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Research Article

Myocardial function and energy metabolism in carnitine-deficient rats

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Abstract. Carnitine is essential for mitochondrial metabolism of long-chain fatty acids and thus for myocardial energy production. Accordingly, carnitine deficiency can be associated with cardiomyopathy. To better understand this disease, we determined myocardial function and energy metabolism in a rat model of carnitine deficiency. Carnitine deficiency was induced by a 3- or 6-week diet containing N-trimethyl-hydrazine-3-propionate, reducing cardiac and plasma carnitine by 70–85%. Myocardial function was investigated in isolated isovolumic heart preparations. Carnitine-deficient hearts showed left ventricular systolic dysfunction, reduced contractile reserve, and a blunted frequency-force relationship independently of the substrate used (glucose or palmitate). After glycogen depletion, palmitate could not sustain myocardial function. Histology and activities of carnitine palmitoyl transferase, citrate synthase, and cytochrome c oxidase were unaltered. Thus, as little as 3–6 weeks of systemic carnitine deficiency can lead to abnormalities in myocardial function. These abnormalities are masked by endogenous glycogen and are not accompanied by structural alterations of the myocardium or by altered activities of important mitochondrial enzymes.

Key words. Cardiomyopathy; contractile function; oxidative phosphorylation; mitochondria; systemic carnitine deficiency; fatty acid; glycogen; N-trimethyl-hydrazine-3-propionate.

Carnitine is essential for mitochondrial transport and metabolism of long-chain fatty acids and, thus, for myocardial energy metabolism. Specifically, because the inner mitochondrial membrane is impermeable to long-chain fatty acyl-CoAs, carnitine is required to transport these substrates across this membrane [1]. Furthermore, carnitine is important for carbohydrate metabolism by stimulating glucose oxidation via reversing the inhibition of pyruvate dehydrogenase [2, 3]. Moreover, carnitine shifts the ratio of acyl-CoA to CoA-SH in the direction of CoA-SH and is involved in trapping acyl residues from peroxisomes and mitochondria [1, 4, 5].

As a consequence of these important functions, deficiency of carnitine manifests mainly as a dysfunction of skeletal muscles and myocardium, where fatty acids constitute an essential energy substrate [4]. Due to decreased metabolism of fatty acids, carnitine deficiency results in accumulation of lipids within skeletal muscle, myocardium, and liver. Ultrastructurally, myofibrils are disrupted and aggregates of mitochondria and lipid deposits accumulate within the skeletal muscle and my-

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ocardium [6]. For these reasons, carnitine deficiency can cause hypertrophic or dilated cardiomyopathy in children [7], which is associated with a mortality of over 50% [8]. Moreover, myocardial carnitine levels are reduced in the hearts of patients with dilated cardiomyopathy, but the significance of this observation is currently not known [7].

The results of clinical and animal studies suggest that a short period (2 weeks) of moderate carnitine deficiency (50% reduction) does not have a major effect on cardiac contractile function [9]. However, after longer periods (>20 weeks) of carnitine deficiency, alterations occur in the heart that may result in impaired contractile performance, particularly at high workloads [9]. At this point, however, the precise mechanisms responsible for this cardiac depression are uncertain. To better understand the pathophysiology of carnitine deficiency, some of us have recently developed and characterized a rat model of carnitine deficiency [10]. In this model, rats developed systemic carnitine deficiency after 3 weeks on a diet containing N-trimethyl-hydrazine-3-propionate (THP; also known as mildronate or MET-88) [11] which inhibits carnitine biosynthesis and increases renal carnitine excretion [10].

In the present study, we sought to investigate in this model whether as little as 3 or 6 weeks of *severe* systemic carnitine deficiency can lead to abnormalities of myocardial function. Additionally, we sought to investigate whether this carnitine deficiency would lead to structural alterations of the myocardium or to altered activities of important mitochondrial enzymes involved in fatty acid and energy metabolism including carnitine palmitoyl transferase, citrate synthase, and cytochrome c oxidase. For this purpose, we studied isolated isovolumically contracting hearts of control and THP-treated rats. Perfusing glucose (carnitine independent) or palmitate (carnitine dependent) as substrate, we measured various hemodynamic variables including left ventricular (LV) pressure, indices of contractility and relaxation, as well as coronary flow. Additionally, we measured both the contractile reserve (assessed as postextrasystolic potentiation) and the frequency-force relationship (staircase), as these myocardial characteristics are often altered in cardiomyopathy. Finally, as myocardial glycogen may compensate for an insufficient energy supply during perfusion with palmitate in carnitine-deficient hearts, we depleted glycogen in some hearts using substrate-free perfusion before LV pressure analysis.

Materials and methods

Animals and induction of carnitine deficiency

Male Sprague-Dawley rats (Süddeutsche Versuchstierfarm, Tuttlingen, Germany and RCC, Füllinsdorf, Switzerland) weighing 290–490 g were used in this study. The animal use protocol was approved by the veterinary department of Basel (Switzerland) and was in line with the 'Ethical Principles and Guidelines for Scientific Experiments on Animals' of the Swiss Academy of Medical Sciences. Carnitine deficiency was induced as previously described [10] using vegetarian food poor in carnitine (Kliba Futter 2435, Basel, Switzerland) and THP (20 mg/100 g per day) for 3 or 6 weeks (n = 6 rats each). Control rats were kept for the same periods of time with vegetarian rat chow ad libitum (n = 7 and 6 rats, respectively). All rats weighed approximately 200 g at the beginning of the diet/THP treatment and received tap water ad libitum throughout the study.

Perfused heart model

After anesthesia (50 mg/kg sodium pentobarbital intraperitoneally; Nembutal; Abbott Laboratories, Chicago, Ill.), rat hearts were excised rapidly and perfused according to a modified Langendorff method as previously described [12]. All hearts were perfused at a perfusion pressure of 110 cm H_2O at 36 °C with a filtered (pore size 0.65 μm) nonrecirculating modified Krebs-Henseleit solution containing 117.0 mM NaCl, 4.3 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM Mg Cl_2, 25.0 mM $NaHCO_3$, 1% albumin, and 11.0 mM glucose or 0.4 mM Na palmitate as substrate. The perfusate was saturated with a gas mixture of 95% O2 and 5% CO_2. Ventricular pacing was carried out using a pair of platinum wire electrodes connected to PowerLab/8e using Chart and Scope software (ADInstruments, Castle Hill, Australia) on a G3 Macintosh computer (Ingeno, Basel, Switzerland). The pacing electrodes were implanted superficially in the right ventricular free wall. To record a bipolar epicardial electrocardiogram (ECG), a pair of electrodes was placed on the right atrial appendage and the right ventricular base.

Experimental protocol

These experiments were designed to investigate the physiological contractile and energetic state of the hearts with a normal myocardial glycogen content. Therefore, rats were studied in the fed state, one control and one THP-treated rat per day in randomized order. Twenty minutes after isolation and perfusion of the hearts, stable baseline values of LV pressure and coronary flow were recorded with one of the substrates at a pacing rate of 200 beats per minute (bpm). The sequence of the substrates (glucose or palmitate) was randomized to eliminate confounding effects of varying perfusion time or glycogen content. To determine the LV contractile reserve, all hearts underwent the postextrasystolic potentiation protocol (see below). Subsequently, to determine the frequency-force relationship, we studied the effects of increasing the heart rate on LV pressure in the staircase protocol (see below). Then, the substrate was switched and after 20 min, of perfusion with the new substrate, baseline recordings and the two

protocols (staircase and postextrasystolic potentiation) were repeated. At the end of the experiments, hearts were immediately cut transversely. The base was stored in 10% formalin for histological analysis and the apex was frozen in liquid nitrogen for measurement of cardiac carnitine concentrations, mitochondrial enzymes (carnitine palmitoyl transferase, citrate synthase, and cytochrome c oxidase), and of the high-energy phosphates, adenosine triphosphate (ATP) and phosphocreatine.

In the fed state, endogenous myocardial glycogen may compensate for insufficient energy supply during perfusion with palmitate in carnitine-deficient hearts. This may mask contractile differences between control and carnitine-deficient animals. Therefore, we depleted glycogen in some hearts using substrate-free perfusion (n = 4 hearts per group) as described previously [13]. Pilot experiments showed that 60 min of substrate-free perfusion was sufficient to deplete the glycogen stores in rat harts (see fig. 1). Glycogen was isolated and analyzed by a spectrophotometric method described previously [14]. After 60 min of substrate-free perfusion, LV-developed pressure began to decrease. Subsequently, the hearts were first perfused with Na palmitate and then with glucose (to prevent restoring of glycogen). In these hearts, we analyzed LV pressure but could not perform further protocols due to the continuing deterioration of LV pressure particularly during palmitate perfusion of hearts from carnitine-deficient rats.

For baseline recordings as well as during the protocols for staircase and postextrasystolic potentiation, simultaneous measurements of LV pressure, coronary flow, and the ECG were performed on PowerLab/8e at a sampling rate of 1000 Hz. Further dependent variables included peak positive first-order time derivative of the LV pressure decline (dP/dt_{max}) as an index of contractility as well as two relaxation indices τ and β (computed off-line using the differential or curve fit extensions of Chart).

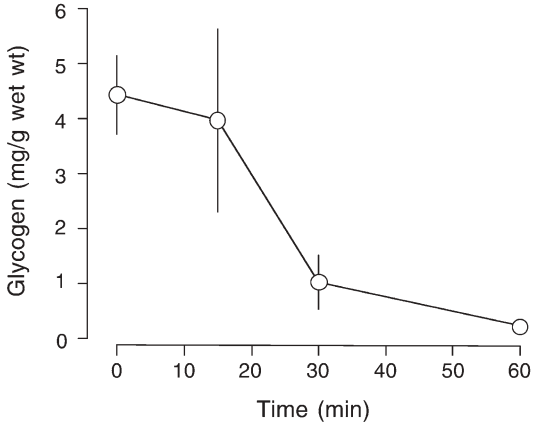

Figure 1. Effect of substrate-free perfusion (0, 15, 30, or 60 min) on the glycogen concentration in the left ventricle of isolated hearts from control rats. Values are mean ± SD; n = 6, 5, 6, or 7 hearts, respectively.

Measurement of carnitine concentrations in the heart and plasma

Carnitine content in the heart and plasma (collected after sternotomy) was determined using a radioenzymatic method as described previously [15]. Heart and plasma samples were prepared for analysis in 3% perchloric acid (v : v). The perchloric acid-soluble fraction was assayed directly for free carnitine, and after alkaline hydrolysis for total acid-soluble carnitine (representing the sum of free carnitine and short-chain acylcarnitine). The short-chain acylcarnitine content (acyl group chain length less than ten carbons) was obtained by subtraction (total acid-soluble minus free carnitine content). Long-chain acylcarnitine contents (acyl group chain length ten or more carbons) were determined in the perchloric acid pellets. Total carnitine content refers to the sum of long-chain acylcarnitine content and total acid-soluble carnitine content.

Measurements of LV pressure and coronary flow

A 60-µl thin latex balloon (size 4; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) was inserted into the left ventricle through the left atrium. LV pressure was measured by a plastic tube located inside the balloon, sutured at the proximal end of the balloon, and connected to an Isotec pressure transducer (Hugo Sachs Elektronik-Harvard Apparatus) that was at the same height as the heart. The volume of the saline-filled balloon was adjusted using a microsyringe to establish a constant physiological end-diastolic pressure in the range of 5–10 mm Hg [12]. Thus, the left ventricle contracted isovolumically throughout the experiment. LV developed pressure was defined as the difference between systolic and diastolic values of LV pressure. Coronary flow was measured within the aortic canula using an in-line flow-probe (Transonic 2N) connected to a transit time flowmeter (Transonic TTFM-SA type 700; Hugo Sachs Elektronik-Harvard Apparatus).

Analysis of LV relaxation

LV relaxation was analyzed on LV pressure recordings using two different relaxation indices. These indices were based on mono- and double-exponential function curve fitting to digital LV pressure data at baseline and a consistent heart rate of 200 bpm. The first index was the widely used time constant τ of monoexponential LV pressure decline according to equation 1:

$$P(t) = (P_0 - P_\infty)\, e^{-\frac{t}{\tau}} + P_\infty \qquad \text{(Eq. 1)}$$

where P(t) denotes LV pressure as a function of time t, P_0 is LV pressure at minimal dP/dt (inflection point), and P_∞ relates to lower asymptotic LV pressure [16]. However, τ considers only the final LV pressure decline, and contains no information about the initial decline before the inflection point of LV pressure. Therefore, a second relaxation

index was applied using parameter β of an asymmetric double-exponential function according to equation 2:

$$P(t) = \gamma_0 - \gamma e^{-\alpha \cdot e^{-\beta \cdot t}} \quad \text{(Eq. 2)}$$

where P(t) denotes LV pressure as a function of time t, and γ_0 and γ relate to upper asymptote and amplitude of LV pressure. Parameter α relates to the phasic delay of decline onset, whereas β principally relates to the rate of the entire LV pressure decline [17].

Measurement of contractile reserve and interval-force relationship

To determine the contractile reserve, three different postextrasystolic potentiation protocols were introduced using PowerLab 8/e and Scope software. Specifically, after eight regular beats at 250-ms intervals (240 bpm), one premature beat was introduced at different intervals (75, 130, and 145 ms) followed by a pause (215, 335, and 265 ms, respectively). The intervals of the premature beats and the pauses were chosen to maximize postextrasystolic potentiation of the following beat [12]. The contractile reserve was estimated as the difference of LV developed pressure between the potentiated beats and the previous regular beat (averaged from three to five repetitions of each of the three potentiation protocols). Because of perturbing effects of arrhythmias on LV pressure, only arrhythmia-free sequences were used for this analysis.

To determine the interval-force relationship, we assessed the effects of increasing heart rates on LV developed pressure. For this purpose, the heart rate was increased stepwise over a physiological range in a preprogrammed staircase pacing protocol at 240, 300, 360, 420, and 480 bpm generated by PowerLab 8/e using the stimulator function of Chart. Each step lasted for 6 s allowing LV pressure to reach a new steady state. Again, only arrhythmia-free sequences were used for this analysis. It should be noted that, in contrast to human hearts, rat hearts demonstrate a negative staircase at physiological heart rates [18].

Histological analysis

Histological examination of the hearts (carnitine-deficient diet for 3 or 6 weeks and controls, n = 6 rats each) was performed on 10% formalin-fixed and paraffin-embedded tissue as well as on fresh frozen tissue. Paraffin-embedded tissue sections were stained with hematoxylin-eosin (H & E) and elastica van Gieson while frozen sections where stained with H & E, periodic acid-Schiff (with and without diastase treatment), and modified Gomori trichrome. The histochemical analyses included myofibrillar ATPase (preincubated at pH 4.3 and pH 4.6), nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH) and cytochrome oxidase. Oil red O was used as a stain for neutral lipids.

Measurement of carnitine palmitoyl transferase, citrate synthase, and cytochrome c oxidase

Activities of the mitochondrial enzymes carnitine palmitoyl transferase, citrate synthase, and cytochrome c oxidase were determined using routine methods [19–21]. Briefly, carnitine palmitoyl transferase activity was measured after incubation of the heart tissue homogenate with palmitoyl CoA and radioactively labeled carnitine to produce labeled palmitoylcarnitine. Palmitoylcarnitine was extracted with butanol and counted in a Packard 1900 TR β-counter (Canberra, Groningen, The Netherlands) [21]. Citrate synthase activity was measured spectrophotometrically at 412 nm using 5,5′-dithio-bis-2-nitrobenzoic acid reacting with CoA-SH that is produced in the reaction from acetyl-CoA and oxaloacetate to citrate [19]. Finally, cytochrome c oxidase activity was measured spectrophotometrically at 550 nm after oxygen-induced oxidation of ferrocytochrome c (produced freshly reducing ferricytochrome c with dithionite) [20]. The net activity was obtained by subtracting the rate obtained in the presence from the rate in the absence of the specific inhibitor sodium azide [20].

Measurement of ATP, phosphocreatine, and lactate

Concentrations of ATP and phosphocreatine in frozen heart samples were measured spectrophotometrically at 340 nm using enzymatic assays as described previously (Sigma-Aldrich Diagnostics ATP assay, no. 366 and custom-made assay, respectively) [22, 23]. The plasma lactate concentrations were determined fluorimetrically as described by Olsen [24].

Data evaluation and statistical analysis

LV pressure values, dP/dt_{max}, and the relaxation indices τ and β were averaged from five cardiac cycles digitally recorded at a heart rate of 200 bpm. To eliminate confounding effects of varying LV developed pressure on the contractility index, dP/dt_{max} was normalized for LV-developed pressure. Furthermore, to compare frequency-force relationships among groups, the sums of the rate-pressure products were computed over the entire heart rate range for each experiment (240–480 bpm). Moreover, out of 57 hearts, 3 were excluded from analysis due to unstable perfusion conditions (1 from the 3-week control group and 2 before glycogen depletion).

All results are expressed as mean ± SD except for carnitine data that were not normally distributed and were therefore expressed as median with interquartile range (25th–75th percentile). Comparisons of normally distributed interval variables among groups (carnitine-deficient vs control rats and 3-week vs 6-week treatment) were performed by one-way ANOVA followed by Bonferroni's test for selected multiple comparisons. Comparisons of carnitine data among groups (carnitine-deficient vs control rats and 3-week vs 6-week treatment) were performed by corre-

sponding nonparametric tests (Kruskal-Wallis test) followed by Dunn's test for selected multiple comparisons. Comparisons of variables within groups (glucose vs palmitate) were performed by repeated-measures ANOVA followed by Bonferroni's test for selected multiple comparisons.

Statistical computations were done using Prism software (version 3.0a; GraphPad, San Diego, Calif.). For all statistical analyses, the null hypothesis was rejected at the 95% level, considering a two-tailed $p < 0.05$ significant. In an approximation of sample size determination for this study, six rats in each group had 90% power to detect differences of 20% in most variables assuming a SD of 10% and a 0.05 two-sided significance level [25].

Results

Carnitine concentrations in heart and plasma

After both 3 and 6 weeks of diet and THP treatment, carnitine concentrations in the heart and plasma were significantly reduced (table 1). Specifically, hearts and plasma of THP-treated rats had 15–30% of the total carnitine concentrations of control rats. Furthermore, all THP-treated rats showed signs of systemic carnitine deficiency including steatosis and enlargement of the liver. Despite these signs, THP-treated rats had similar body and heart weights in comparison to control rats (table 1). Additionally, after 6 weeks of diet and THP treatment, total carnitine concentrations in the heart and plasma were not significantly lower than after 3 weeks. However, after 6 weeks, control rats had higher total carnitine concentrations than after 3 weeks.

The plasma lactate concentration was 2.4 ± 0.5 mmol/l in control rats, and 2.1 ± 0.5 or 1.7 ± 0.1 mmol/l in rats treated with THP for 3 or 6 weeks (each group $n = 6$, $p < 0.05$ for rats treated with THP for 6 weeks vs control rats).

Hemodynamics, contractility, and relaxation

After 3 weeks of diet and THP treatment, no significant differences could be detected among the groups regarding LV pressure, dP/dt_{max}, or the relaxation indices τ and β (table 2). Specifically, hearts of carnitine-deficient rats did not differ from hearts of control rats in any hemodynamic variable independently of the substrate used (11 mM glucose or 0.4 mM Na palmitate).

However, after 6 weeks, hearts of carnitine-deficient rats showed significantly lower LV systolic pressure than control hearts during both glucose and palmitate perfusion (table 2). Consequently, hearts of carnitine-deficient rats also showed lower LV developed pressure than hearts of control rats (as LV end-diastolic pressure was set at a physiological range of 5–10 mm Hg). However, hearts of carnitine-deficient rats showed a similar dP/dt_{max} as control hearts indicating unaltered contractility. Similarly, hearts of carnitine-deficient rats showed similar relaxation indices τ and β as control hearts. Finally, hearts of carnitine-deficient rats had a similar coronary flow as control hearts independently of the substrate throughout all experiments (overall mean 16.3 ml/min).

Contractile reserve and frequency-force relationship

After 3 and 6 weeks of carnitine deficiency, the contractile reserve was significantly reduced independently of the substrate used (fig. 2). Specifically, hearts of carnitine-deficient rats showed a reduced postextrasystolic potentiation of LV developed pressure relative to control hearts. This reduction was similar after 3 and 6 weeks of

Table 1. Characterization of the animals and carnitine concentrations in the heart and plasma.

	3 weeks		6 weeks	
	control	THP-treated	control	THP-treated
Body weight (g)	334 ± 16	319 ± 28	418 ± 46**	414 ± 46**
Heart wet weight (g)	1.16 ± 0.11	1.20 ± 0.08	1.17 ± 0.07	1.23 ± 0.18
Heart				
Free carnitine (nmol/mg muscle)	0.53 (0.46–0.61)	0.19 (0.19–0.45)	0.30 (0.22–0.34)	0.09 (0.06–0.11)
SCA carnitine (nmol/mg muscle)	0.44 (0.28–0.52)	0.00 (0.00–0.01)*	0.43 (0.42–0.73)	0.09 (0.08–0.11)*
LCA carnitine (nmol/mg muscle)	0.04 (0.03–0.05)	0.01 (0.01–0.02)*	0.04 (0.03–0.06)	0.01 (0.01–0.01)*
Total carnitine (nmol/mg muscle)	1.05 (1.01–1.07)	0.24 (0.21–0.48)*	0.78 (0.66–1.17)	0.21 (0.17–0.22)*
Plasma				
Free carnitine (µmol/l)	40.7 (30.7–43.3)	10.0 (9.4–10.8)*	67.4 (64.3–71.5)	8.1 (7.0–8.7)*
SCA carnitine (µmol/l)	6.2 (5.3–7.3)	1.9 (1.1–2.5)	14.1 (6.1–36.8)	3.2 (1.6–3.8)*
LCA carnitine (µmol/l)	7.3 (6.0–9.9)	2.0 (1.8–2.1)*	10.4 (6.0–10.7)	1.8 (1.7–2.0)*
Total carnitine (µmol/l)	53.4 (41.7–58.4)	13.9 (12.6–14.9)*	89.0 (84.6–93.0)**	12.8 (12.5–13.4)*

Values are mean ± SD (weights) or median with interquartile range (carnitine) after 3 or 6 weeks diet and treatment with THP ($n = 6$ hearts per group). SCA, short-chain acylcarnitine; LCA, long-chain acylcarnitine: * $p < 0.05$ vs control at corresponding age; ** $p < 0.05$ vs corresponding group after 3 weeks.

Table 2. Effect of carnitine deficiency on LV pressure, contractility, and relaxation indices in isolated rat hearts.

	3 weeks				6 weeks			
	glucose		palmitate		glucose		palmitate	
	control	carnitine-deficient	control	carnitine-deficient	control	carnitine-deficient	control	carnitine-deficient
LVSP (mm Hg)	75 ± 6	70 ± 10	74 ± 4	70 ± 13	84 ± 14	65 ± 6*	83 ± 12	66 ± 11*
LVEDP (mm Hg)	10 ± 2	8 ± 2	8 ± 3	8 ± 3	9 ± 4	8 ± 2*	10 ± 3	10 ± 3
LVDP (mm Hg)	65 ± 5	62 ± 9	65 ± 5	61 ± 12	75 ± 15	57 ± 6*	73 ± 15	55 ± 11*
dP/dt_{max} (mm Hg/s)	1151 ± 161	1159 ± 99	1157 ± 161	1167 ± 117	1123 ± 131	1136 ± 124	1139 ± 66	1144 ± 126
τ (ms)	52 ± 9	50 ± 8	45 ± 9	50 ± 15	50 ± 6	57 ± 13	49 ± 14	59 ± 19
β	24 ± 4	25 ± 4	27 ± 5	25 ± 6	22 ± 6	23 ± 4	25 ± 6	24 ± 6

Values are mean ± SD after 3 or 6 weeks diet and treatment with THP (n = 6 hearts per group) recorded at 200 bpm. LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure; LVDP, LV developed pressure; dP/dt_{max} was normalized for LVDP. * $p < 0.05$ vs control during corresponding substrate perfusion.

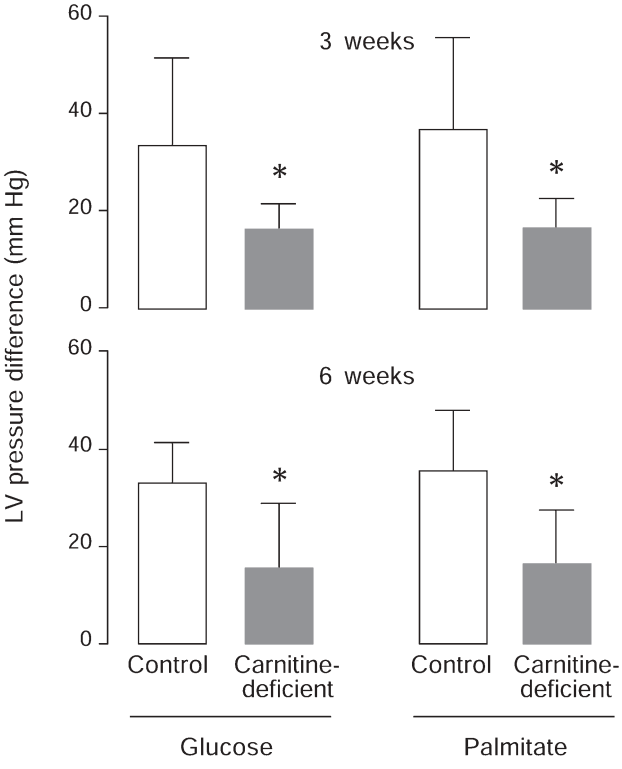

Figure 2. Contractile reserve of isolated hearts from control rats and carnitine-deficient rats during glucose or palmitate perfusion after 3 and 6 weeks diet and THP treatment. The contractile reserve was assessed as the difference of LV developed pressure between postextrasystolic potentiated beats and preceding regular beats. Values are mean ± SD; n = 6 hearts per group. * $p < 0.05$ vs control at corresponding age and perfusion condition.

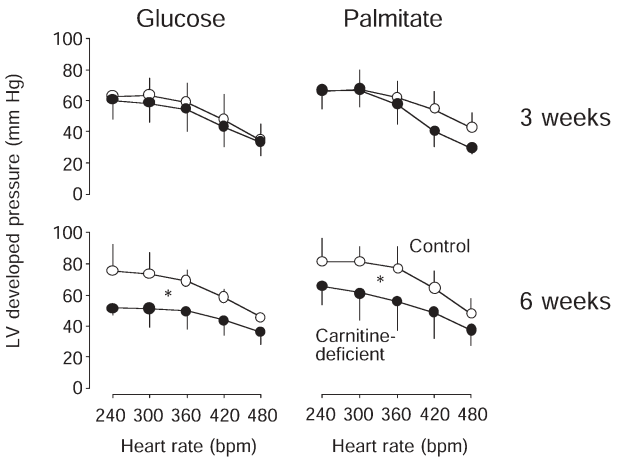

Figure 3. Frequency-force relationship of isolated hearts from control rats and carnitine-deficient rats during glucose or palmitate perfusion after 3 and 6 weeks diet and THP treatment. The frequency-force relationship was assessed as effect of increasing heart rate from 240 to 480 bpm on LV developed pressure. Values are mean ± SD; n = 6 hearts per group. * $p < 0.05$ vs control evaluated as rate-pressure product over the entire heart rate range.

carnitine deficiency and was not affected by the choice of substrate.

Similar to the contractile reserve, the frequency-force relationship was blunted in hearts of carnitine-deficient rats independently of the substrate used (fig. 3). After 3 weeks of carnitine deficiency, the frequency-force relationship was already slightly altered at high heart rates during palmitate perfusion. However, the rate-pressure product over the entire heart rate range was not significantly different. Nevertheless, after 6 weeks, hearts of carnitine-deficient rats developed significantly less LV pressure than control hearts over the entire heart rate range. This difference was largely due to differences in LV systolic pressure between carnitine-deficient and control rats. LV diastolic pressure, in contrast, did not differ among the groups.

Histological findings

Histological examination revealed no structural changes of the myocardium in carnitine-deficient rats. Specifically, we found no vacuolation of muscle fibers and oil red O staining revealed no excess of lipid storage in our rats. In addition, we found no disruption of muscle fibers, and

Table 3. Mitochondrial enzymes.

	3 weeks		6 weeks	
	control	carnitine-deficient	control	carnitine-deficient
Carnitine palmitoyl transferase (mU/mg heart tissue)	1.7 ± 0.4	1.9 ± 0.2	2.3 ± 0.1	2.1 ± 0.4
Citrate synthase (U/mg heart tissue)	104 ± 32	85 ± 12	99 ± 32	103 ± 32
Cytochrome c oxydase (U/mg heart tissue)	5.9 ± 1.6	8.0 ± 3.9	6.9 ± 2.2	6.0 ± 1.6

Values are mean ± SD after 3 or 6 weeks diet and treatment with THP (n = 5–6 hearts per group).

glycogen storage was normal in both carnitine-deficient and control rats. Finally, we found no fibers with excess mitochondria using Gomori trichrome stain, NADH-TR, SDH, or cytochrome oxidase reaction. Thus, histologically and histochemically, the myocardium of carnitine-deficient rats did not differ from that of control rats.

Mitochondrial enzymes, ATP and phosphocreatine

After 3 and 6 weeks of carnitine deficiency, the activities of all three mitochondrial enzymes measured were not significantly different from controls (table 3). Specifically, hearts of carnitine-deficient rats showed similar activities of carnitine palmitoyl transferase, citrate synthase, and cytochrome c oxidase as control hearts, excluding a significant proliferation of mitochondria in hearts of carnitine-deficient rats.

Concentrations of ATP and phosphocreatine did not significantly differ among hearts of carnitine-deficient rats and control rats after 3 or 6 weeks diet at the end the experimental protocol (data not shown).

Effect of glycogen depletion

Pilot studies using hearts of control rats had shown that after 60 min of substrate-free perfusion, glycogen was almost completely depleted (fig. 1) and LV pressure started to decrease. The effect of glycogen depletion on myocardial function was subsequently investigated using hearts of carnitine-deficient and control rats (n = 4 hearts for both groups). When LV pressure began to decrease (usually after 60 min of substrate-free perfusion), these hearts were perfused with Na palmitate and subsequently with glucose. Compared to values obtained using hearts with normal glycogen stores (see table 2), glycogen-depleted hearts of control rats produced normal LV systolic and diastolic pressures during perfusion with both substrates (fig. 4). In comparison, glycogen-depleted hearts of carnitine-deficient rats produced LV systolic pressures which were approximately 10% lower compared to control rats, irrespective of the substrate used. This decrease did not reach statistical significance, however, due to high variations in control rats. Diastolic pressure was significantly higher in hearts from carnitine-deficient rats perfused with palmitate. Importantly, upon subsequent perfusion with glucose, diastolic pressure decreased to values similar to those of control rats. It is noteworthy that arrhythmias were frequent findings in glycogen-depleted hearts, in particular in hearts from carnitine-deficient rats perfused with palmitate.

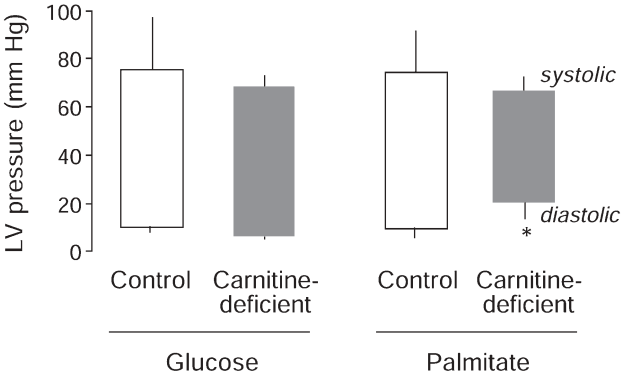

Figure 4. Effect of glycogen depletion on LV systolic and diastolic pressure (upper and lower bar end, respectively) of isolated hearts from control and carnitine-deficient rats during glucose or palmitate perfusion after 6 weeks diet and THP treatment. Values are mean ± SD; n = 3 hearts per group. * $p < 0.05$ vs control at corresponding perfusion condition.

Discussion

In the present study in a rat model of carnitine deficiency, we demonstrated that as little as 3–6 weeks of severe systemic carnitine deficiency can lead to abnormalities in myocardial function including systolic dysfunction, reduced contractile reserve, and a blunted frequency-force relationship. These abnormalities may be masked by endogenous glycogen and are not accompanied by structural alterations of the myocardium or by altered activities of important mitochondrial enzymes involved in fatty acid and energy metabolism.

Specifically, we found that both 3 and 6 weeks of vegetarian food poor in carnitine plus THP treatment (20 mg/100 g per day) led to severe systemic carnitine deficiency in rats (table 1). After 3 weeks of carnitine deficiency, we found a significant reduction in the contractile reserve assessed as postextrasystolic potentiation of isolated isovolumically contracting heart preparations using glucose or palmitate as substrate (fig. 2). After 6 weeks of carnitine deficiency, we found further abnormalities in myocardial function including LV systolic dysfunction (table 2), reduced contractile reserve, and a blunted frequency-force relationship (fig. 3).

At a mechanistic level, our findings suggest that as long as myocardial workloads are low, ATP production may still be sufficient in carnitine-deficient hearts. Accordingly, ATP and phosphocreatine concentrations as well as relaxation indices were similar in carnitine-deficient and control hearts. However, at high workloads, such as during high heart rates, functional abnormalities occurred, suggesting that carnitine deficiency-induced alterations in substrate metabolism result in transiently inadequate ATP production as proposed previously [26]. Furthermore, reduced postextrasystolic potentiation after 3 and 6 weeks of carnitine deficiency in our experiments suggests that altered intracellular calcium handling is an early step in the development of functional abnormalities. Specifically, severe carnitine deficiency may reduce the amount of activator calcium and/or interfere with sarcoplasmic reticular calcium uptake and/or transport [12, 27]. In our histological analysis, we found no disruptions of myofibers that could have explained abnormal myocardial function (in particular systolic dysfunction) in carnitine-deficient rats. Systolic dysfunction might arise from accumulating long-chain acyl-CoAs due to impaired activity of carnitine palmitoyltransferase I (CPT I) in the presence of low intracellular carnitine levels, as demonstrated in the liver of rats treated with THP [28]. Long-chain acyl-CoAs can damage biological membranes and impair the function of membrane-associated enzymes and/or transport proteins such as the adenine nucleotide translocase which is critical for mitochondrial energy metabolism [29]. This mechanism explains why in non-glycogen-depleted hearts cardiac dysfunction was observed under both conditions, perfusion with glucose or with palmitate.

The K_m for M-CPT I, the predominating CPT I isoform in adult rats [30], is in the range of 500 µmol/l for carnitine [31]. Since the cardiac concentrations of free carnitine in carnitine-deficient rats were 190 µmol/kg at 3 and 90 µmol/kg at 6 weeks of THP treatment, an energy deficit with an associated impaired cardiac function was expected in hearts perfused with palmitate. However, endogenous myocardial glycogen stores may have at least partially compensated for an insufficient energy supply during perfusion with palmitate in carnitine-deficient hearts. Our histological examination did not reveal differences in myocardial glycogen content between carnitine-deficient and control hearts. Since this examination was not quantitative and did not control for glycogen formation during perfusion, we repeated our studies with hearts after depletion of cardiac glycogen by substrate-free perfusion for 60 min. After depletion of glycogen, LV diastolic pressure was higher during palmitate perfusion in carnitine-deficient hearts as compared to glucose perfusion, possibly due to insufficient energy supply to maintain a normal diastolic intracellular calcium concentration. Based on this finding, we propose that in severe disorders of fatty acid metabolism such as systemic carnitine deficiency, the myocardial energy supply can depend on endogenous glycogen stores. This is particularly the case in the absence of suitable substrates such as glucose, short-chain fatty acids, ketone bodies, or amino acids, and also during periods of increased energy consumption, for example, during high heart rates. If such periods are prolonged, myocardial glycogen stores may be depleted and glycogenolysis may be insufficient to meet the metabolic needs of the carnitine-deficient myocardium.

In patients suffering from mitochondrial diseases, proliferation of mitochondria in the affected organ (e. g., skeletal muscle and/or heart) is a frequent finding [32, 33]. In contrast, in our carnitine-deficient rats, the observed alterations of myocardial function were not accompanied by increased activities of mitochondrial enzymes or histological abnormalities, excluding mitochondrial proliferation. However, after a longer duration of severe systemic carnitine deficiency, mitochondrial proliferation may occur. Interestingly, juvenile visceral steatosis mice, an animal model of inherited severe systemic carnitine deficiency, develop cardiac hypertrophy and mitochondrial proliferation [34].

Importantly, the present study provides the first description of carnitine deficiency-induced abnormalities of myocardial function in rats after as little as 3–6 weeks of systemic carnitine deficiency. In previous studies, 20 weeks of carnitine deficiency (induced by sodium pivalate in drinking water) was considered the minimal duration to induce functional abnormalities in rats [26]. This difference may be due to the lower degree of carnitine deficiency in previous studies (50–60% reduction) than in the present study (70–85% reduction). As carnitine concentrations did not differ between the third to the sixth week in our experiments, one may reasonably speculate that both the severity and duration of carnitine deficiency determine the development of functional abnormalities and ultimately cardiomyopathy.

In conclusion, our study in rats shows that as little as 3–6 weeks of severe systemic carnitine deficiency can lead to abnormalities in myocardial function including systolic dysfunction, reduced contractile reserve, and a blunted frequency-force relationship. These abnormalities are partially masked by endogenous glycogen and are not ac-

companied by structural alterations of the myocardium or altered activities of important mitochondrial enzymes involved in fatty acid and energy metabolism. The functional abnormalities in carnitine-deficient hearts may represent early signs of a metabolic cardiomyopathy.

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1 Bremer J. (1983) Carnitine – metabolism and functions. Physiol. Rev. **63:** 1420–1480
2 Broderick T. L., Quinney H. A. and Lopaschuk G. D. (1992) Carnitine stimulation of glucose oxidation in the fatty acid perfused isolated working rat heart. J. Biol. Chem. **267:** 3758–3763
3 Bremer J. (1969) Pyruvate dehydrogenase, substrate specificity and product inhibition. Eur. J. Biochem. **8:** 535–540
4 Lango R., Smolenski R. T., Narkiewicz M., Suchorzewska J. and Lysiak-Szydlowska W. (2001) Influence of L-carnitine and its derivatives on myocardial metabolism and function in ischemic heart disease and during cardiopulmonary bypass. Cardiovasc. Res. **51:** 21–29
5 Brass E. P. and Hoppel C. L (1980) Relationship between acid-soluble carnitine and coenzyme A pools in vivo. Biochem. J. **190:** 495–504
6 Gilbert E. F (1985) Carnitine deficiency. Pathology **17:** 161–171
7 Wynne J. and Braunwald E (1997) The cardiomyopathies and myocarditides. In: Heart Disease: A Textbook of Cardiovascular Medicine, 5th edn, pp. 1404–1463, Braunwald E. (ed.), Saunders, Philadelphia
8 Winter S., Jue K., Prochazka J., Francis P., Hamilton W., Linn L. et al. (1995) The role of L-carnitine in pediatric cardiomyopathy. J. Child Neurol. 10 (Suppl 2): S45-S51
9 Paulson D. J. (1998) Carnitine deficiency-induced cardiomyopathy. Mol. Cell. Biochem. **180:** 33–41
10 Spaniol M., Brooks H., Auer L., Zimmermann A., Solioz M., Stieger B. et al. (2001) Development and characterization of an animal model of carnitine deficiency. Eur. J. Biochem. **268:** 1876–1887
11 Simkhovich B. Z., Shutenko Z. V., Meirena D. V., Khagi K. B., Mezapuke R. J., Molodchina T. N. et al. (1988) 3-(2,2,2-Trimethylhydrazinium)propionate (THP) – a novel gamma-butyrobetaine hydroxylase inhibitor with cardioprotective properties. Biochem. Pharmacol. **37:** 195–202
12 Zaugg C. E., Kojima S., Wu S. T., Wikman-Coffelt J., Parmley W. W. and Buser P. T. (1995) Intracellular calcium transients underlying interval-force relationship in whole rat hearts: effects of calcium antagonists. Cardiovasc. Res. **30:** 212–221
13 Soares P. R., Albuquerque C. P. de, Chacko V. P., Gerstenblith G. and Weiss R. G. (1997) Role of preischemic glycogen depletion in the improvement of postischemic metabolic and contractile recovery of ischemia-preconditioned rat hearts. Circulation **96:** 975–983
14 Barbosa V., Sievers R. E., Zaugg C. E. and Wolfe C. L. (1996) Preconditioning ischemia time determines the degree of glycogen depletion and infarct size reduction in rat hearts. Am. Heart J. **131:** 224–230
15 Brass E. P. and Hoppel C. L. (1978) Carnitine metabolism in the fasting rat. J. Biol. Chem. **253:** 2688–2693
16 Gilbert J. C. and Glantz S. A. (1989) Determinants of left ventricular filling and of the diastolic pressure-volume relation. Circ. Res. **64:** 827–852
17 Tamiya K., Beppu T. and Ishihara K. (1995) Double-exponential curve fitting of isometric relaxation: a new measure for myocardial lusitropism. Am. J. Physiol. **269:** H393–H406
18 Field M. L., Azzawi A., Unitt J. F., Seymour A. M., Henderson C. and Radda G. K. (1996) Intracellular [Ca^{2+}] staircase in the isovolumic pressure-frequency relationship of Langendorff-perfused rat heart. J. Mol. Cell. Cardiol. **28:** 65–77
19 Srere P. A. (1969) Citrate synthase. Methods Enzymol. **13:** 3–11
20 Krähenbühl S., Chang M., Brass E. P. and Hoppel C. L. (1991) Decreased activities of ubiquinol:ferricytochrome c oxidoreductase (complex III) and ferrocytochrome c:oxygen oxidoreductase (complex IV) in liver mitochondria from rats with hydroxycobalamin[c-lactam]-induced methylmalonic aciduria. J. Biol. Chem. **266:** 20998–21003
21 Bremer J. (1981) The effect of fasting on the activity of liver carnitine palmitoyltransferase and its inhibition by malonyl-CoA. Biochim. Biophys. Acta **665:** 628–631
22 Adams H. (1963) Adenosin-5′-triphosphate, determination with phosphoglycerate kinase. In: Methods of Enzymatic analysis, pp. 539–543, Bergmayer H. U. (ed.) Academic Press, New York
23 Willer B., Stucki G., Hoppeler H., Bruhlmann P. and Krahenbuhl S. (2000) Effects of creatine supplementation on muscle weakness in patients with rheumatoid arthritis. Rheumatology **39:** 293–298
24 Olsen C. (1971) An enzymatic fluorimetric micromethod for the determination of acetoacetate, beta-hydroxybutyrate, pyruvate and lactate. Clin. Chim. Acta **33:** 293–300
25 Dawson-Saunders B. and Trapp R. G. (1990) Basic and Clinical Biostatistics, Appleton & Lange, San Mateo, Calif.
26 Paulson D. J. (1998) Secondary L-carnitine deficiency-induced cardiomyopathy. Cardiologia **43** (Suppl 2)**:** 677–683
27 Wood E. H., Hepner R. L. and Weidmann S. (1969) Inotropic effects of electrical currents. Circ. Res. **24:** 409–445
28 Spaniol M., Kaufmann P., Beier K., Wuthrich J., Torok M., Scharnagl H. et al. (2003) Mechanisms of liver steatosis in rats with systemic carnitine deficiency due to treatment with trimethylhydraziniumpropionate. J. Lipid Res. **44:** 144–153
29 Faergeman N. J. and Knudsen J. (1997) Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. Biochem. J. **323:** 1–12
30 Brown N. F., Weis B. C., Husti J. E., Foster D. W. and McGarry J. D. (1995) Mitochondrial carnitine palmitoyltransferase I isoform switching in the developing rat heart. J. Biol. Chem. **270:** 8952–8957
31 McGarry J. D., Mills S. E., Long C. S. and Foster D. W. (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
32 Haller R. G., Lewis S. F., Estabrook R. W., DiMauro S., Servidei S. and Foster D. W. (1989) Exercise intolerance, lactic acidosis, and abnormal cardiopulmonary regulation in exercise associated with adult skeletal muscle cytochrome c oxidase deficiency. J. Clin. Invest. **84:** 155–161
33 Hoppel C. L., Kerr D. S., Dahms B. and Roessmann U. (1987) Deficiency of the reduced nicotinamide adenine dinucleotide dehydrogenase component of complex I of mitochondrial electron transport: fatal infantile lactic acidosis and hypermetabolism with skeletal-cardiac myopathy and encephalopathy. J. Clin. Invest. **80:** 71–77
34 Horiuchi M., Yoshida H., Kobayashi K., Kuriwaki K., Yoshimine K., Tomomura M. et al. (1993) Cardiac hypertrophy in juvenile visceral steatosis (jvs) mice with systemic carnitine deficiency. FEBS Lett. **326:** 267–271