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ORIGINAL ARTICLE

A Moderate Carnitine Deficiency Exacerbates Isoproterenol-Induced Myocardial Injury in Rats

Pietro Lo Giudice[1] · **Mario Bonomini**[2] · **Arduino Arduini**[3]

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Abstract
Purpose The myocardium is largely dependent upon oxidation of fatty acids for the production of ATP. Cardiac contractile abnormalities and failure have been reported after acute emotional stress and there is evidence that catecholamines are responsible for acute stress-induced heart injury. We hypothesized that carnitine deficiency increases the risk of stress-induced heart injury.
Methods Carnitine deficiency was induced in Wistar rats by adding 20 mmol/L of sodium pivalate to drinking water (P). Controls (C) received equimolar sodium bicarbonate and a third group (P + Cn) received pivalate along with 40 mmol/L carnitine. After 15 days, 6 rats/group were used to evaluate function of isolated hearts under infusion of 0.1 μM isoproterenol and 20 rats/group were submitted to a single subcutaneous administration of 50 mg/kg isoproterenol.
Results Isoproterenol infusion in C markedly increased the heart rate, left ventricular (LV) systolic pressure and coronary flow rate. In P rats, isoproterenol increased the heart rate and LV systolic pressure but these increases were not paralleled by a rise in the coronary flow rate and LV diastolic pressure progressively increased. Subcutaneous isoproterenol induced 15 % mortality rate in C and 50 % in P ($p < 0.05$). Hearts of surviving P rats examined 15 days later appeared clearly dilated, presented a marked impairment of LV function and a greater increase in tumor necrosis factor α (TNFα) levels. All these detrimental effects were negligible in P + Cn rats.
Conclusions Our study suggests that carnitine deficiency exposes the heart to a greater risk of injury when sympathetic nerve activity is greatly stimulated, for example during emotional, mental or physical stress.

Keywords Sympathetic activation · Isoprotenerol · Carnitine · Pivalic acid · Primary carnitine deficiency · Heart injury

Introduction

Carnitine is essential to transport long-chain fatty acids into the mitochondrial matrix where they undergo β-oxidation and generate energy. The myocardium, which is largely dependent upon oxidation of fatty acids for the production of ATP, is entirely dependent on carnitine uptake from the blood. Transport of carnitine toward the myocardium occurs against a concentration gradient, leading tissue carnitine concentrations to be higher than those in plasma. The carnitine requirement depends on factors such as age, diet and metabolic conditions (stress, dietary state, level of exercise) [1]. The condition in which carnitine concentrations in plasma or tissues are below the levels required for normal functioning of the organism is defined as carnitine deficiency [2]. Primary carnitine deficiency is well recognized as a cause of impaired lipid metabolism, which frequently results in pediatric cardiomyopathy [3, 4]. Secondary carnitine deficiency syndromes can be caused by acquired pathological conditions or from iatrogenic factors such as dialysis treatment in ESRD (end stage renal

✉ Arduino Arduini
a.arduini@corequest.ch

[1] Research and Development, Sigma Tau Pharmaceuticals, Via Pontina Km 30.400, 00071 Pomezia, Italy

[2] Institute of Nephrology, Department of Medicine, G. d'Annunzio University, Via dei Vestini, 66013 Chieti, Italy

[3] Department of Research & Development, CoreQuest Sagl, Tecnopolo, Stabile Suglio, Via Cantonale 18, 6928 Manno, Switzerland

disease) patients and pivalate use in pharmaceutical and cosmetic industries [5–8].

One well-established animal model of carnitine deficiency consists in administration of sodium pivalate to rats [9]. Pivalic acid forms an ester with carnitine, which is excreted as pivaloylcarnitine; sustained loss of carnitine in the form of this ester induces a state of carnitine deficiency. This model is useful as a way of studying the cardiac consequences of carnitine deficiency syndromes [10]. The treatment of patients suffering from primary carnitine deficiency with antibiotics containing pivalic acid as a prodrug has caused several deaths [11].

Cardiac contractile abnormalities and heart failure have been reported after acute emotional stress [12–15]. For instance, stress-induced cardiomyopathy is commonly present in postmenopausal women and may be precipitated by emotional stress, and numerous other stress states. Symptoms may be indistinguishable from acute coronary syndrome or only detectable via electrocardiogram alterations, and differential diagnosis may be challenging. Although most patients show complete recovery, there is a high risk of complications at the initial presentation, requiring intense support. The underlying etiology is most likely related to release of catecholamines, both locally in the myocardium and in the circulation. Catecholamine administration induces necrotic lesions similar to those induced by stress [16–20], whereas adrenergic blockers reduce the extent of stress-induced heart injury [21]. We hypothesized that if stress-induced heart injury occurs in an asymptomatic carnitine deficiency patient, a more severe clinical outcome of such cardiomyopathy may be expected. To test this hypothesis we investigated the consequences of β-adrenergic stimulation on the myocardium of carnitine-deficient rats. As a β-adrenergic agonist, we used isoproterenol administration, which in animals causes structural alterations at the myocardial level just as occurs after acute sympathetic activation due to psychosocial stress [18, 21].

Materials and Methods

Animals

All experiments were conducted according to international guidelines and approved by the Italian Ministry of Health. Animals were carefully monitored by a veterinarian.

Male Wistar rats (200–225 g, Charles River, Calco, Lecco, Italy) were used. All animals were allowed access to food (GLP-4RF-21, Mucedola S.r.l., Settimo Milanese, Italy) and water ad libitum and maintained under controlled environmental conditions (temperature 22 ± 2 °C, relative humidity 55 ± 10 %, 12-h light/dark cycle).

Induction of Carnitine Deficiency

Carnitine deficiency was induced by adding 20 mmol/L of sodium pivalate (Sigma Chemical Co, St Louis, MO, USA) to drinking water (P group) [22]. Control rats (C group) received an equimolar concentration of sodium bicarbonate. A third group of rats were treated with sodium pivalate and at the same time, in order to counteract carnitine depletion, with 40 mmol/L L-carnitine (Sigma Tau SpA, Pomezia, Italy) in drinking water (P + Cn group). An overview of the experimental protocol is shown in Fig. 1.

Assessment of Carnitine Deficiency and Biochemical Parameters

At the 15th day of sodium pivalate treatment 5 rats/group were anaesthetized [sodium pentobarbital (Sigma Chemical Co, St Louis, MO, USA) 60 mg/kg i.p.] followed by blood collection from the abdominal aorta and excision of the heart. Hearts were rinsed in saline solution and left ventricular samples (about 100 mg) were frozen in liquid nitrogen and stored at −80 °C. Plasma glucose, free fatty acids (FFAs), and triglycerides (TGDs) were determined using standard procedures [23]. Plasma carnitine was analyzed using a radiochemical assay [24]. To determine left ventricular carnitine, muscle samples were sonicated in 1 ml of 0.5 KOH and subjected to alkaline hydrolysis for 60 min at 50 °C. After hydrolysis, the solution was neutralized and centrifuged. The supernatant was analyzed for carnitine as above [24]. The carnitine measured included the free (non-esterified) carnitine originally present within the sample and free carnitine resulting from the hydrolysis of all esterified forms of carnitine.

Isoproterenol Infusion in Isolated Hearts

On the 15th day of sodium pivalate treatment six rats/group were anaesthetized (pentobarbital 60 mg/kg i.p.); their hearts were quickly excised and arrested in ice-cold heparin-containing Krebs–Henseleit buffer. Ascending aortas were cannulated for retrograde Langendorff-perfusion at a constant temperature of 37 °C and a constant pressure of 100 mmHg. Hearts were perfused with modified Krebs–Henseleit buffer (pH 7.4) containing (in mM) NaCl 117, KCl 4.7, $CaCl_2$ 2.7, $MgSO_4$ $7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and 11 glucose, and continuously gassed with a mixture of 95 % O_2 and 5 % CO_2.

A polyethylene PE50 catheter was inserted directly into the left ventricular cavity and connected to a pressure transducer (Stathman P23XL). After 30 min of equilibration, isoproterenol 0.1 μM (Sigma Chemical Co, St Louis, MO, USA) was infused into the perfusion solution for a further 30 min. The heart rate and left ventricular systolic and diastolic pressures were recorded on a polygraph (bm614 amplifier, bm613

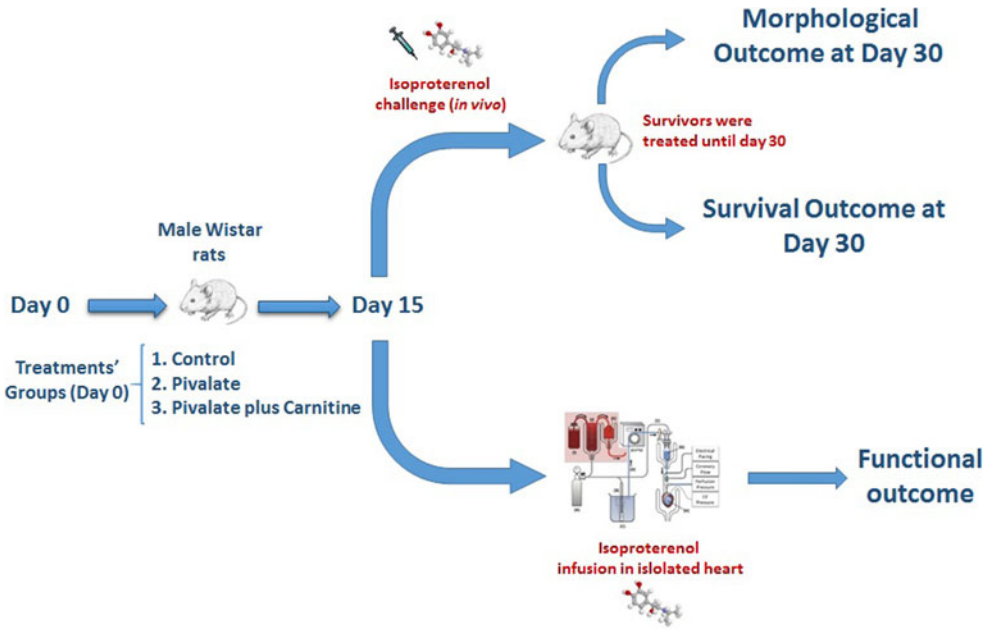

Fig. 1 Experimental protocol for evaluating the effect of isoproterenol challenge in carnitine deficient animals

E.C.G. amplifier, and BM IDAS Programmable Acquisition System; Biomedica Mangoni, Pisa, Italy). The coronary flow was measured by collecting the effluent.

β-Adrenergic Challenge-Induced Myocardial Injury

Twenty rats from each group were submitted, on day 15, to a single subcutaneous administration of 50 mg/kg of isoproterenol, a dose that was reported to induce myocardial necrosis with a low mortality rate [25].

Ventricular Function, Carnitine and TNFα Levels in Rats Surviving After Isoproterenol Challenge

Fifteen days later (during which period administration of sodium pivalate and carnitine continued) half of the rats from each group that survived isoproterenol and 5 rats from the group that received no isoproterenol (sham) were utilized to measure cardiac function. The second half of the survivors and a further 5 sham group rats were used to measure ventricular carnitine and TNFα content.

Cardiac function was studied in isolated hearts according to the Langendorff method above. Functional parameters were recorded after 30 min for equilibration. To measure ventricular carnitine and TNFα, the rats were anaesthetized (pentobarbital 60 mg/kg i.p.), their hearts excised, rinsed in normal saline, macroscopically examined and left ventricular samples frozen in liquid nitrogen. Ventricular carnitine was determined as above. TNF-α levels were measured in samples from the left ventricle by an ELISA kit (Rat TNFα ELISA, Endogen Inc., Woburn, MO, USA) according to the manufacturer's instructions.

Statistical Analysis

Data are expressed as means ± standard error (SEM); differences between groups were tested for significance by one-way or two-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Mortality rate data are expressed as a percentage and analyzed using the Fisher exact test. In all tests, a p value of 0.05 or lower was considered to be statistically significant.

Results

Assessment of Carnitine Deficiency and Biochemical Parameters

Fifteen days of pivalate treatment induced reduction of carnitine levels by about 80 % and 40 % in plasma and in ventricular tissue, respectively (Table 1). These reductions were completely reversed by concomitant treatment with carnitine. Indeed P + Cn rats showed higher levels of carnitine in plasma and ventricles even when compared with normal rats. As shown in Table 1, although pivalate treatment caused a severe reduction in plasma carnitine, only a moderate reduction of ventricular carnitine content (60 % of controls) was observed. Be it noted, in primary carnitine deficiency, a more severe form of carnitine deficiency that may not be compatible with life if not treated with a generous dose of carnitine [2–4], the muscle carnitine content is less than 6 % of healthy individuals [26]. In addition, our carnitine deficient rats did not show any pathological phenotype before the isoproterenol challenge. No significant differences were seen among the groups for plasma glucose, FFAs and TGDs (Table 1).

Table 1 Concentrations of total glucose, TGDs, FFAs, and total carnitine in C, P, and P + Cn groups

	C (5)	P (5)	P + Cn (5)	ANOVA	
Plasma Glucose (mg/dl)	119 ± 4	122 ± 5	128 ± 1	$F_{(2,12)} = 1.67$,	n.s
Plasma TGDs (mg/dl)	171 ± 34	172 ± 20	162 ± 31	$F_{(2,12)} = 0.04$,	n.s
Plasma FFAs (μM)	705 ± 103	411 ± 72	558 ± 47	$F_{(2,12)} = 3.61$,	n.s
Total carnitine					
Plasma (μM)	42.6 ± 3.4	9.6 ± 1.4##	83.1 ± 8.6## **	$F_{(2,12)} = 232.38$,	$p < 0.0001$
Left Ventricle carnitine (nmol/mg tissue)	177 ± 10	109 ± 3#	291 ± 26## **	$F_{(2,12)} = 32.29$,	$p < 0.0001$

Values are means ± SEM; in brackets the numbers of animals; Tuckey post hoc test: # = $p < 0.05$ and ## = $p < 0.01$ vs C; ** = $p < 0.01$ vs P

Effect of Isoproterenol Infusion in Isolated Hearts

Baseline parameters of hearts isolated from C, P, and P + Cn rats were similar (Fig. 2a–d). Isoproterenol infusion markedly increased heart rate (Fig. 2a) and left ventricular systolic pressure (Fig. 2b) of C hearts. These increases were paralleled by a rise in the coronary flow rate (Fig. 2d) while left ventricular diastolic pressure did not change (Fig. 2c). A similar increase in heart rate and left ventricular systolic pressure was also recorded in hearts of P rats. However, in these hearts the increased cardiac work was not followed by any rise in coronary flow rate, while under isoproterenol infusion left ventricular diastolic pressure progressively rose (Fig. 2c). Hearts from P + Cn rats behaved similarly to C hearts: heart rate, left ventricular systolic pressure and coronary flow rate increased, whereas the left diastolic pressure remained unchanged (Fig. 2b–d).

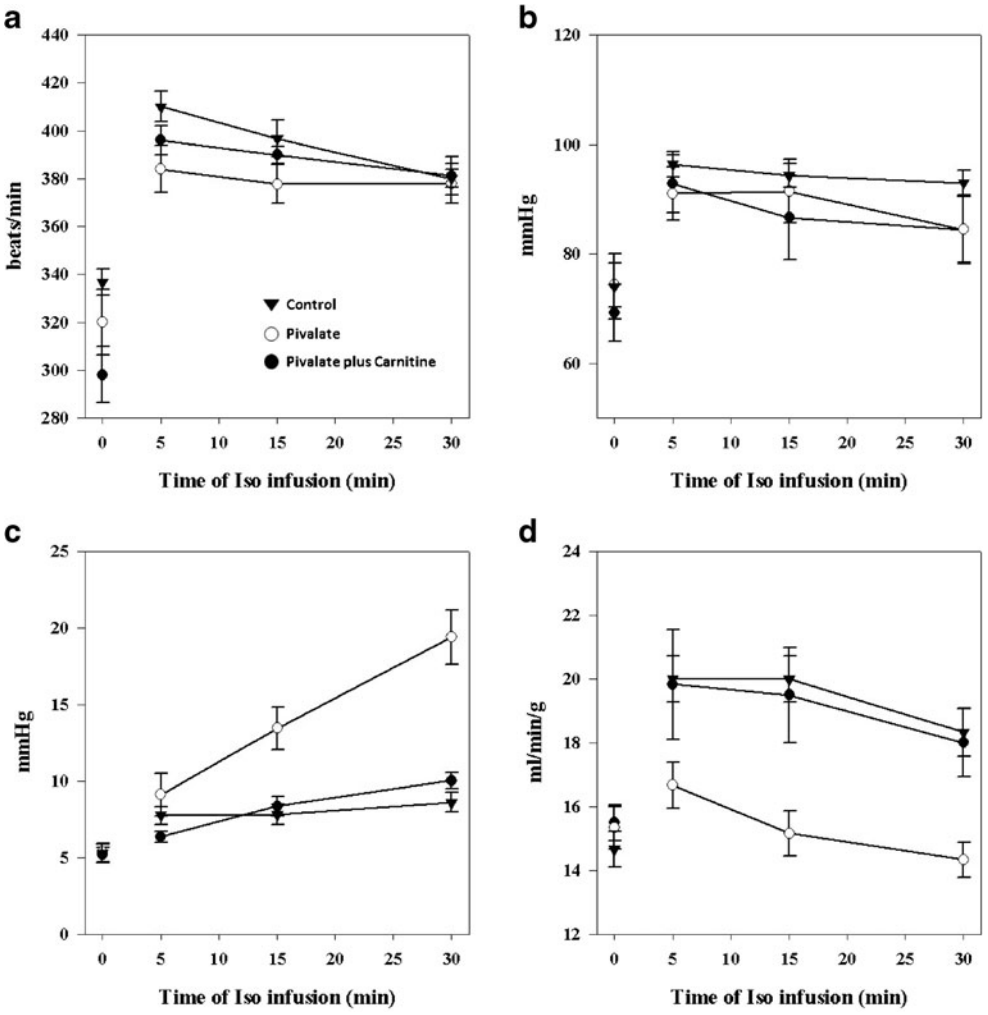

Fig. 2 Effects of isoproterenol (Iso) infusion on function of isolated hearts from rats depleted of carnitine by 15 days' treatment with pivalate (*open circle*), from pivalate plus carnitine-treated rats (*filled circle*) and from sodium bicarbonate treated rats (*filled triangle*). Baseline values were recorded at time 0. Two way ANOVA for repeated measures. Heart rate (Fig. 1a); group: $F_{(2,15)} = 1.62$, not significant; time: $F_{(2,30)} = 11.07$, $p < 0.001$, group x time: $F_{(4,30)} = 2.03$, not significant. Left ventricular systolic pressure (Fig. 1b); group: $F_{(2,15)} = 0.90$, not significant; time: $F_{(2,30)} = 1.55$, not significant, group x time: $F_{(4,30)} = 0.25$, not significant. Left ventricular end diastolic pressure (Fig. 1c); group: $F_{(2,15)} = 14.97$, $p < 0.001$; time: $F_{(2,30)} = 50.18$, $p < 0.0001$, group x time: $F_{(4,30)} = 16.18$, $p < 0.001$. Coronary flow rate (Fig. 1d); group: $F_{(2,15)} = 6.31$, $p < 0.01$; time: $F_{(2,30)} = 9.07$, $p < 0.001$, group x time: $F_{(4,30)} = 0.49$, not significant

β-Adrenergic Challenge-Induced Myocardial Injury

Within minutes of receiving isoproterenol all animals exhibited gross signs of increased sympathetic nervous system activity. Respiratory secretions increased and respiration became deep and irregular. As shown in Fig. 3, in the first 24 h after injection, the P + Cn group fully recovered and no deaths were observed. As expected, in the control group 3 out 20 died [18, 20]. In the pivalate-treated group, however, we were somewhat surprised to see that 50 % of rats (10 out of 20) died (Fig. 3), since the isoproterenol dose used in our study was even lower than that known to possess a low mortality rate (20). Afterwards, signs of increased sympathetic activity progressively disappeared and surviving rats exhibited normal behavior.

Morphological and Functional Evaluation of Hearts from Rats Surviving Isoproterenol Challenge

Fifteen days after isoproterenol challenge, that is to say 30 days after the initiation of pivalate treatment, the heart/body weight ratio, ventricular macroscopic morphology and cardiac function parameters were similar in the three groups of hearts from sham groups (Table 2). On the contrary, the heart/body weight ratio of the C rats submitted to isoproterenol challenge was significantly increased with respect to sham-C (Table 2). These hearts appeared slightly flabby and a few petechiae in the apical epicardium were detected by gross visual observation in all cases (Fig. 4). The hearts from survivors in the P group appeared more damaged, all of them presenting a large fibrotic area that varied from 30 to 50 % of coverage from the apex. The remaining myocardium appeared markedly dilated (Fig. 4).

The heart/body weight ratio of P + Cn rats submitted to isoproterenol challenge was lower to that of either P or C rats (Table 2) and petechiae on the apex were observed in only 5 (25 %) out of 20 (Fig. 4).

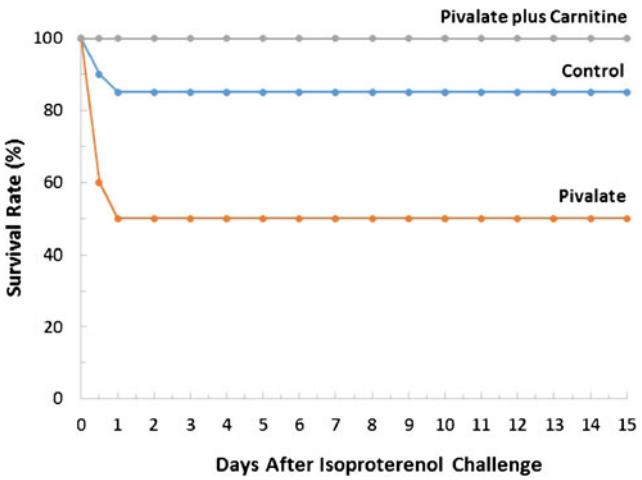

Fig. 3 Survival rate over time after isoproterenol challenge. The different treatment groups are indicated in the figure (n = 20 in each group)

Cardiac function was measured in 9, 5 and 10 hearts respectively for C, P, and P + Cn rats which had survived the β-adrenergic challenge. When compared to C-sham, the hearts of surviving C rats showed a reduction in left ventricular systolic pressure, and increased left-end diastolic pressure (Table 2). Hearts from isoproterenol-challenged P rats presented more pronounced ventricular impairment. They had a marked reduction in heart rate and left ventricular systolic pressure, a marked increase in left diastolic pressure and reduction in the coronary flow rate (Table 2). These adverse alterations were not present in hearts from challenged P + Cn rats, which had functional parameters similar to those of the sham group (Table 2).

Carnitine and TNF-α Levels in Hearts from Rats Surviving Isoproterenol Challenge

Ventricular carnitine was reduced in hearts from both isoproterenol-challenged and sham P rats but increased in hearts of P + Cn rats (Table 3).

Ventricular TNFα levels were similar in the three groups of hearts from sham groups and proved to be increased in all groups after β-adrenergic stimulation. In the latter groups, however, P rats showed the largest TNFα increase, followed by the control group (Table 3). Within the isoproterenol-challenged rats, those treated with carnitine showed the least TNFα elevation (Table 3).

Discussion

The present results indicate that a moderate reduction in cardiac carnitine content makes rats more susceptible to myocardial injury induced by β-adrenergic stimulation along with an unanticipated high mortality rate. Although pivalate treatment led to an 80 % reduction in plasma carnitine, the ventricular carnitine content in these animals was only 40 % less than controls. Hearts isolated from carnitine-deficient rats as well as hearts from controls responded to β-adrenergic stimulation with increased contractility leading to increased nutritional demand. A parallel increase in the coronary flow occurred in controls, whereas in carnitine-deficient hearts the increased coronary flow was lacking as a result of ischemia due to the imbalance between myocardial nutritional needs and coronary supply. This caused ventricular contracture as indicated by the progressive increase in diastolic pressure (Fig. 2c).

It is well known that depressed contractile force, along with significant ATP depletion and derangement of the ion homeostasis, characterize the ischemic myocardium [27]. In addition, the combination of increased rates of fatty acid β-oxidation and higher rates of anaerobic glycolysis along with reduced rates of aerobic glycolysis is thought to play a significant role in ischemia and reperfusion injury. Low carnitine levels further impair the intramitochondrial acetyl-CoA buffering

Table 2 Functional feature of isolated hearts from the different groups

	Sham			Isoproterenol challenged			Two –way ANOVA					
	C	P	P + Cn	C	P	P + Cn	Group		Challenge		Interaction	
	(5)	(5)	(5)	(9)	(5)	(10)	F	P<	F	p<	F	p<
HR (beat/min)	314 ± 10	307 ± 13	312 ± 10	307 ± 11	229 ± 31**, ‡	308 ± 12##	4.6	0.05	5.5	0.05	3.3	0.05
LVSP (mmHg)	70 ± 3	66 ± 4	70 ± 2	59 ± 3†	56 ± 4†	69 ± 3*, #	3.8	0.05	7.7	0.01	1.4	n.s.
LVDP (mmHg)	5.7 ± 0.6	7.6 ± 1.5	8.1 ± 1.5	8.2 ± 0.4†	14.2 ± 0.7**, ‡	6.9 ± 0.3##	12.6	0.001	15.6	0.001	11.3	0.001
CFR (ml/min/g Hw)	10.5 ± 0.5	10.8 ± 0.6	11.3 ± 0.5	8.4 ± 0.5‡	8.1 ± 0.2‡	11.0 ± 0.4**, ##	7.9	0.005	16.6	0.001	2.9	n.s.

Values are means ± SEM; in brackets the numbers of animals; HR, heart rate; LVSP, left ventricular systolic pressure; LVDP, left ventricular diastolic pressure; CFR, coronary flow rate; n.s. = not significant

Tukey Test: † = $p < 0.05$ and ‡ = $p < 0.01$ vs sham; * = $p < 0.05$ and ** = $p < 0.01$ vs C; # = $p < 0.05$ and ## = $p < 0.01$ vs P

capacity of heart cells, leading to activation of pyruvate dehydrogenase kinase (PDHK), which inhibits pyruvate dehydrogenase (PDH) by phosphorylating it [27]. This translates into severe uncoupling between the anaerobic and aerobic span of glycolysis, followed by an increased rate of proton production along with a significant drop in intracellular pH [28–30]. During ischemia much of the rise in cytosolic $[Ca^{2+}]i$ is due to Ca^{2+} entry by reverse mode of the Na^+/Ca^{2+} exchanger secondary to H^+-activated Na^+-influx. Now, since a fall in ATP impairs Na^+/K^+-ATPase, Na^+ that enters the cell on the Na^+/H^+ exchanger is not efficiently pumped out. However, a much greater rise in $[Ca^{2+}]i$ may occur during reperfusion, which contributes to the genesis of ventricular arrhythmia and myocardial stunning [31]. In addition, energy restoration upon reperfusion may activate sarcoplasmic reticulum Ca^{2+} cycling resulting in cytosolic Ca^{2+} oscillations and propagation of Ca^{2+} waves. These SR-driven Ca^{2+} oscillations may lead to myofibrillar hyperactivation and development of hypercontracture. The large burst of oxygen radicals and

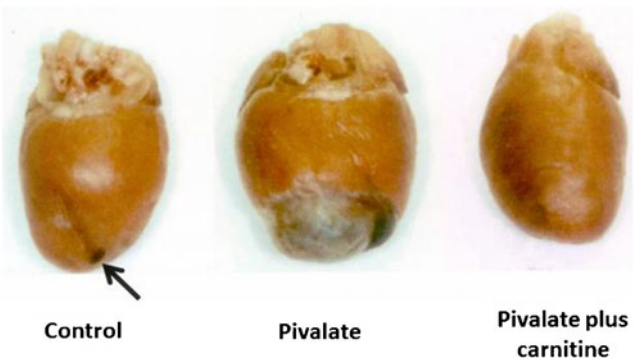

Fig. 4 Representative images of hearts from control, pivalate and pivalate plus carnitine treated groups 15 days after isoproterenol injection (30 days of pivalate treatment). Isoproterenol in hearts from control rats induced petechiae in the apical epicardium (*arrow*). In hearts from pivalate treated rats, isoproterenol injection induced a large fibrotic area in the apex of the myocardium with a marked dilation of the remaining myocardium. Hearts from pivalate plus carnitine treated rats after isoproterenol injection appeared only marginally damaged

$[Ca^{2+}]i$ overload upon reperfusion are also among the major determinants of the mitochondrial permeability transition (MPT) pore [31]. Opening of the MPT pore leads to uncoupling of oxidative phosphorylation, *in vitro* swelling of mitochondria and release of proapoptotic factors such as cytochrome *c*. In addition to Ca^{2+}-overload and the formation of oxygen radicals, however, other mechanisms may be operative in myocardial I/R injury such as the occurrence of a no reflow phenomenon due to cell swelling and impaired vascular relaxation [32, 33].

Our results are in agreement with previous data indicating that hearts isolated from carnitine-deficient rats have baseline functions similar to controls and a marked depression in response to increased metabolic demand [34]. Moreover, it has been reported that carnitine supplementation protects rats from isoproterenol-induced myocardial infarction [35, 36]. Our study, however, provides the first-ever clear evidence that a moderate reduction in heart carnitine content renders the animals much more vulnerable to severe and fatal cardiac dysfunction when submitted to increased sympathetic stimulation *in vivo*. On the other hand, it has been shown that a 60 % reduction of heart carnitine content in pivalate-treated rats leads to a shift in cardiac substrate utilization without compromising cardiac functional capacity under physiological conditions of workload [37]. This seems to suggest that a silent carnitine deficient phenotype may reveal its brittleness when subjected to strong β-adrenergic stress. Our data also suggest that this can be fully prevented as shown in our carnitine treated animals.

Juvenile visceral steatosis (JVS) heterozygous mutants (+/−) from a murine model of carnitine/organic cation transporter *(OCTN2)* mutation, the JVS mouse, showed a moderate reduction in myocardial carnitine content similar to what we observed in our study [38]. These *OCTN2* mutants showed no differences in the heart weight: body weight ratio or mortality rate compared to WT mice, though the homozygous mutants developed pathological cardiac hypertrophy along with a large lipid accumulation [39, 40]. However, if the heterozygous JVS mice were challenged with a surgically induced pressure

Table 3 Feature of isolated hearts from the different groups

	Sham			Isoproterenol challenged			Two –way ANOVA					
	C	P	P + Cn	C	P	P + Cn	Group		Challenge		Interaction	
	(10)	(10)	(10)	(17)	(10)	(20)	F	P<	F	p<	F	p<
BW (g)	408 ± 8	400 ± 9	397 ± 5	391 ± 5 †	361 ± 6‡, **	387 ± 4##	4.9	0.05	20.2	0.001	3.2	0.05
HW (mg)	1349 ± 29	1363 ± 44	1276 ± 18	1477 ± 28‡	1537 ± 36‡	1306 ± 23**, ##	14.7	0.001	19.0	0.001	2.82	n.s
HW/BW (mg/g)	3.31 ± 0.06	3.42 ± 0.14	3.22 ± 0.05	3.77 ± 0.05‡	4.26 ± 0.08‡, **	3.37 ± 0.04**, ##	28.7	0.001	69.2	0.001	11.3	0.001
Ventricular	(5)	(5)	(5)	(8)	(5)	(10)						
Carnitine (nmol/mg tissue)	197 ± 8	110 ± 3**	312 ± 20**, ##	179 ± 6	92 ± 9**	236 ± 12 ‡, **, ##	105.3	0.001	14.8	0.001	4.4	0.05
TNFa (pg/mg prot.)	7.2 ± 1.6	4.8 ± 1.1	5.5 ± 1.0	21.7 ± 2.6‡	33.9 ± 5.0‡, **	12.1 ± 1.1†, **, ##	10.6	0.001	79.7	0.001	11.9	0.001

Values are means ± SEM; in brackets the numbers of animals; BW, body weight; HW, heart weight; n.s. = not significant

Tukey Test: † = $p < 0.05$ and ‡ = $p < 0.01$ vs sham; * = $p < 0.05$ and ** = $p < 0.01$ vs C; # = $p < 0.05$ and ## = $p < 0.01$ vs P

overload, severe cardiomyopathy and heart failure with energy depletion occurred, and this could be prevented by carnitine treatment [38]. These findings lend further support to the concept that a phenotypically silent carnitine deficiency may become dramatically evident if an acute or chronic insult stresses cardiac bioenergetics. Interestingly enough, it has been estimated that 0.5 to 1 % of the population carries one abnormal allele of the gene coding for the OCTN2, an asymptomatic form of primary carnitine deficiency [41, 42]. Humans who are heterozygous for primary carnitine deficiency show a moderate deficiency of plasma carnitine levels due to increased urinary losses [43] and may develop cardiac hypertrophy [42]. ESRD patients under automatized peritoneal dialysis treatment are exposed to more severe secondary carnitine deficiency than patients dialyzed with other modalities [44]. It is worth mentioning that increased cardiovascular risk and sympathetic overactivity is often present in patients on chronic dialysis [45]. Finally yet importantly, the use of pivalate, an efficient carnitine-depleting prodrug, in the pharmaceutical and cosmetic industries, may pose a significant cardiovascular risk [6–8, 11]. Abrahamsson et al. have found that healthy volunteers treated for 7–8 weeks daily with pivmecillinam (1200 mg), an antibiotic drug containing pivalic acid, developed a statistically significant decrease in both interventricular septum thickness and left ventricular mass [46]. Fifteen months after the end of treatment, these dimensions had only partially returned to pretreatment values. In addition, a more recent study reported that the administration of antibiotics containing pivalic acid to asymptomatic primary carnitine deficient subjects was strongly associated with encephalopathy and lethal cardiac arrhythmias [11].

Increased sympathetic stimulation is one of the main compensatory mechanisms by which the heart can augment its performance during times of stress. The administration of isoproterenol causes dose-related myocardial ischemic necrosis in rats, which closely resembles damage seen in human myocardial infarction [27, 47].

Inflammation has been recognized as one of the main driving forces in the ischemic process, and increasing evidence has shown that enhanced levels of inflammatory markers are related to ischemia [48, 49]. The proinflammatory cytokine TNFα is a small secreted protein that mediates and regulates inflammation. The inflammatory stresses induced by isoproterenol were reflected by TNFα elevation in both control and pivalate treated groups, though the latter group showed the higher elevation. In the pivalate-carnitine treated group, TNFα levels were significantly lower even than those measured in the control group, suggesting that, because of correcting carnitine deficiency, part of the cardioprotective effects might also be associated with anti-inflammatory action. Interestingly enough, a recent randomized study showed that L-carnitine treatment significantly reduced the levels of TNF-α and other inflammation markers in coronary artery disease patients [50]. Although the mechanisms by which L-carnitine ameliorates inflammation and

circulating pro-inflammatory markers are not definitely established, they may include direct antiapoptotic action [51], regulation of CPT I-dependent PPARγ signaling [52], and/or suppression of inflammatory responses via the Transient Potential Vanilloid type 1 (TRPV1) pathway [53].

Our findings on the adverse effect of a moderate carnitine deficiency in the heart also confirm the worse remodeling process and pronounced ventricular impairment observed in pivalate-treated rats 15 days after isoproterenol challenge. Nevertheless, from the present data it is not possible to conclude whether the worsening of cardiac function and remodeling in such rats is due to the enhanced initial myocardial injury or to the reduction in carnitine levels during the post infarction period. More likely, both of these mechanisms are involved. Carnitine supplementation fully counteracted the alterations caused by pivalate, suggesting that the increased susceptibility to β-adrenergic stimulation seen is not due to a direct effect by pivalate itself. Indeed, several pharmacological agents capable of affecting mitochondria heart energy substrate selection have been shown to exert a favorable therapeutic action in animal and human studies on acute ischemia-reperfusion [54, 55]. Despite the different mode of action, these agents have in common the capacity to promote aerobic glycolysis, attenuating the severe uncoupling between the two arms of myocardial glucose metabolism. The beneficial action of carnitine treatment is therefore expected to lower the intramitochondrial acetyl-CoA "pressure" and relieve PDH inhibition by PDHK, allowing the tricarboxylic acid (TCA) cycle to flow again [27, 56].

Conclusion

Carnitine deficiency in rats is associated with a high mortality rate, enhanced myocardial injury and a worse remodeling process after β-adrenergic agonist challenge. This suggests that a moderate reduction in carnitine levels may expose the heart to a greater risk of myocardial infarction when sympathetic nerve activity is greatly stimulated, for example during emotional, mental or physical stress [12–15].

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Compliance with Ethical Standards

Conflict of Interest There are no known conflicts of interest associated with this publication for Prof. Mario Bonomini. Dr. Pietro Lo Giudice and Dr. Arduino Arduini confirm that they are employees of Sigma Tau Pharmaceuticals and CoreQuest Sagl, respectively.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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