L-CARNITINE: A POTENTIAL TREATMENT FOR BLOCKING APOPTOSIS AND PREVENTING SKELETAL MUSCLE MYOPATHY IN HEART FAILURE.

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ABSTRACT

Skeletal muscle in congestive heart failure is responsible for increased fatigability and decreased exercise capacity. A specific myopathy with increased expression of fast-type myosins, myocyte atrophy, secondary to myocyte apoptosis triggered by high levels of circulating TNFα has been described. In an animal model of heart failure, the monocrotaline-treated rat, we have observed an increase of apoptotic skeletal muscle nuclei. Proapoptotic agents, caspases 3 and 9, were increased, as well as serum levels of TNFα and its second messenger sphingosine. Treatment of rats with L-carnitine, known for its protective effect on muscle metabolism injuries, was found to inhibit caspases, to decrease the levels of TNFα and sphingosine as well the number of apoptotic myonuclei. Staurosporine was used in in vitro experiments, to induce apoptosis in skeletal muscle cells in culture. When L-carnitine were applied to skeletal muscle cells, before staurosporine treatment, we observed a reduction in apoptosis. These findings show that L-carnitine can prevent apoptosis of skeletal muscles cells and have a role in the treatment of congestive heart failure-associated myopathy.

Key words: Tumor necrosis factor; sphingosine; caspases; staurosporine
INTRODUCTION

Heart Failure is characterized by a decreased exercise capacity with early appearance of fatigue and dyspnea (15,18). These symptoms are in part due to a skeletal muscle myopathy with atrophy, that is responsible for the decreased muscle strength and endurance (18,35), and shift from the “slow, fatigue resistant” Type I, to the “fast, fatigable” Type II fibres (29,31,33), that in turn causes the increased muscle fatigability (33). Apoptosis has been shown to be one of the major determinants of skeletal muscle atrophy (4,32,34), and its magnitude correlates with the severity of the syndrome (34). Myocyte death is triggered by cytokine activation which is almost always present in Heart Failure as a sign of a chronic inflammatory state (13,14). Cytokines, such as Tumor Necrosis Factor α (TNFα), can induce apoptosis either directly or through secondary messengers, such as the sphingolipoid sphingosine (SPH) (10,13,22). We have also shown that sphingosine serum levels are increased in Heart Failure in parallel with TNFα and that this molecule is able to induce skeletal myocyte apoptosis both in vivo and in vitro (7,25).

Although clinical trials aimed to block TNFα with specific antibodies such as Infliximab or Etanercept have failed (17), there is a compelling evidence that apoptosis can be inhibited, with favourable consequences on muscle atrophy. This can be for instance achieved by blocking the Angiotensin II (AngII) receptors (6). L-carnitine is a quaternary amine that is fundamental in skeletal muscle metabolism, in that promotes fatty acids oxidation (23), and that has been shown effective in producing a selective trophic effect on Type I and IIa skeletal muscle fibres (11). However there are recent observations that L-carnitine, beyond the well known metabolic effect, possesses some more complex activities in regulating gene expression and activity of caspases, the activation of which represent the compulsory step for cell death execution (2,21). Moreover it has been shown that L-carnitine prevents doxorubicin-induced apoptosis in cardiac myocytes, by inhibiting the doxorubicin-induced sphingomyelin hydrolysis and ceramide generation (2). It seems therefore likely that sphingolipids play a determinant role in inducing apoptosis in the
skeletal muscle and it could be argued that programmed cell death may be stopped by blocking the sphingolipid activation cascade. In this paper we tried to block sphingolipid formation and prevent apoptosis in a well known model of heart failure, the monocrotaline-treated rat, by giving L-carnitine. The ultimate aim of this approach was to prevent the development of muscle atrophy, that we all know is one of the major determinant of exercise capacity (18,35), and to give a clue for the understanding of the pathophysiology of the heart failure myopathy, which may in turn have future reflections in the development of further treatment for heart failure.

MATERIALS AND METHODS

1) IN VIVO STUDY

a) Experimental model

Four groups of animals were studied for a total of 56 animals. 36 males 80 to 100 gr Sprague Dawley rats with congestive heart failure (CHF) induced by monocrotaline (Sigma, St. Louis, Mo., USA). This alkaloid produces severe pulmonary hypertension followed by right ventricular (RV) failure (6,32) without inducing by itself changes in skeletal muscle myosin heavy chains (MHCs) composition and apoptosis (30). Monocrotaline was injected intraperitoneally in the rats at the dose of 30 mg/kg. Starting at the same day, 26 out of 36 rats were treated with L-carnitine (Sigma Tau, Roma, Italy) 50 mg/kg/die given in drinking water (CHFCarn). 10 rats therefore formed the CHF group. Rats were kept in single cages and the drunk water was measured daily. Other 10 additional rats had only L-carnitine in drinking water and they formed the L-carnitine control group (ConCar). 10 age and diet matched rats were injected with saline and served as controls. After 28 days, when in the monocrotaline treated animals, overt heart failure has developed, rats were killed and body, heart and tibialis anterior weight were measured. Muscles were immediately frozen in liquid nitrogen and stored at -80°C. Hearts were stored in 10%
formaldehyde solution. Blood was drawn for SPH, TNFα, AngII AST (Aspartate amino transferase), ALT (Alanine amino transferase) and L-carnitine measurements. Experiments were approved by the Biological Ethical Committee of the University of Padua, according to the Italian law.

**b) Assessment of Right Ventricle hypertrophy and failure.**

To make sure that the monocrotaline treated animals developed right ventricle failure, beyond the post-mortem signs such as pericardial, pleural and peritoneal effusions (30), the following measurements were taken: Right Ventricle Mass/Left Ventricle Mass (RVM/LVM) and Right Ventricular Mass/Right Ventricular Volume index (RVM/RVV) calculated with a computerized planimeter on photographic pictures of formalin fixed transverse sections of the heart taken in the middle portion of the interventricular septum (6).

c) **Electrophoretic separation of Myosin Heavy Chains (MHCs)**

We used the method described in details by Vescovo *et al*. (28,29). TA muscles were homogenized and solubilized in sodium dodecyl sulfate (SDS) buffer. Analytical SDS Page was performed on 7% polyacrylamide slabs with 37.5% vol/vol glycerol to separate MHCs (MHC2a and MHC2b).

d) **Assessment of MHCs distribution**

The percent distribution of the MHCs was determined by a densitometric scan (Sigma Gel, SPSS, Chicago, IL, USA) after image acquisition of the stained gels (6).

e) **Single fibers cross sectional area**

We used single fibers cross sectional areas (CSA) as an index of myofibers atrophy (6,7). On each muscle a cross cryo-section was taken for histological examination and stained with
Hematoxylin-Eosin. The fibers CSA was calculated with a computerized interactive method (6,7). At least 400 fibers per specimen were counted at magnification 250X and then averaged.

\( f \) Assessment of apoptosis

**In situ DNA nick-end labelling (TUNEL)**

In situ nick end labeling of fragmented DNA was performed on cryo-sections using the In Situ Cell Death Detection Kit, POD (Boehringer Mannheim). Labeled nuclei were identified from the negative nuclei counter-stained by Hoechst 33258 and counted after being photographed. The total number of positive nuclei was determined by counting (magnification 250X) all the labeled nuclei present in the whole specimen. The number of positive nuclei was then expressed as number of TUNEL positive nuclei/mm\(^3\) (6,7). TUNEL positive myofibres and interstitial nuclei were distinguished on the basis of their location on sections stained with laminin, which selectively reacts with the basal lamina. TUNEL positive nuclei within the basal lamina were taken as myonuclei (4,6,7). Separate calculations were made for total TUNEL positive nuclei, TUNEL positive myonuclei.

**TA Western blot for activated Caspases 3 and 9, and Bcl-2**

Western blot was performed on 12.5 polyacrylamide gels as previously described (4). Anti-Bcl-2 (29kDa) antibodies (Santa Cruz Biotechnology, St. Cruz, CA, USA) were used with anti rabbit alkaline-phosphatase (Sigma Chemical Co., St. Luis, MO). Anti-cleaved Caspase-3 (17 kDa) and anti-cleaved Caspase-9 (37 kDa) Cell Signaling Tecnology (Beverly, MA, USA) were used with anti-rabbit peroxidase-coniugated antibody and revealed by chemiluninescent substrate (SuperSignal West Pico, Pierce, ILL, USA).
Confocal Microscopy Immunofluorescence

Frozen sections were incubated for 1 hour at room temperature with anti-dystrophin antibody (Novocastra, Newcastle Upon Tyne, UK) diluted 1:300 in 1% BSA, which stains sarcolemma. After 3 washings with PBS sections were incubated with anti mouse IgG FITC-conjugated (Sigma) diluted 1:500 at 37°C for 1 hour. The sections were then washed 3 times with PBS and fixed with 4% PFA and incubated with anti caspase 3 antibody (Cell Signaling Tecnology, Beverly, MA, USA) diluted 1:10 overnight at 4°C. Slices were then incubated with anti-rabbit Cy3-conjugated antibody for 1 hour at room temperature and analysed by a Bio-Rad (Hercules, CA, USA) Confocal Microscopy.

ELISA for apoptosis

DNA ladder assay was performed according to Yasuhara et al. (36) In brief 10 muscle cryosections, 20 μm thick, were solubilized in 200 μl of lysis buffer (Triton X-100 0.1%, Tris-HCl (pH 8.0) 5 mM, EGTA 20 mM, EDTA 20 mM). Then, polyethylene glycol 8000 and NaCl were added to a final concentration of 2.5% and 1 M, respectively. Samples were centrifuged at 16,000 g for 10 min at 4°C. Protein concentration of supernatant was determined with Bradford and adjusted to 0.01 μg/μl. Cell death ELISA analysis was performed according to manufacturer’s instructions (Boehringer Mannheim). After incubation the plates were analyzed with a multi-well ELISA reader.

g) TNFα determination

TNFα was measured with a solid phase sandwich ELISA, using a monoclonal antibody specific for rat TNFα (Euroclone Ltd. UK).

h) Angiotensin II Assay

AngII was measured on serum, using an enzyme-immunometric assay kit, from SPI-BIO (Massy CEDEX FRANCE).

i) SPH determination
For the extraction of sphingolipids, 100-300 µl samples of serum were deproteinized by adding warmed butanol (70°C, 800 µl), vortexing and incubating at 70°C while rocking. The mixture was then placed in a sonicating water bath for 10 minutes. Denatured protein and aqueous phase were separated from the butanol layer by centrifugation at 15,300 x g. The upper butanol layer was transferred into a new extraction tube and saponified by the addition 0.5 M KOH (200 µl). After vortexing, samples were incubated at 70°C while rocking for 1 hour with intermittent vortexing and sonicating. HPLC-grade water (400 µl) was added to each sample and returned to the incubator for 10 minutes. After sonicating for 1 minute, the layers were separated by centrifuging at 15,300 x g for 3 minutes. The butanol layer was transferred to a new tube and dried down using a Savant (Holbrook, NY) SpeedVac Plus. Dried samples were completely resuspended in methanol (375 µl) and agitated in a bath sonicator for 2 minutes.

The extracts were then derivatized with O-phthalaldehyde (Molecular Probes, Eugene, OR) as previously described (7). The derivatized samples (50 µl injection) were separated on a Bio-Rad (Hercules, CA) Hi-Pore Reversed Phase Column RP-318 (4.6 mm I.D. x 250 mm) column with a 1.5 cm Perkin Elmer (Norwalk, CT) NewGuard RP-18 guard column. Samples were run on a Bio-Rad (Hercules, CA) HPLC system with a Perkin Elmer (Norwalk, CT) fluorescence detector (LS-1)(excitation 340 nm, emission 455 nm). The solvent system was methanol, glacial acetic acid, 1 M tetrabutlyammonium dihydrogen phosphate, HPLC-grade water (82.9:1.5:0.9:14.7, v/v) run at 1.0 ml/minute.

j) Carnitine assay

Free carnitine, acylcarnitine and propionylcarnitine were measured in serum and in muscle homogenate as described by Longo et al. (16) In brief, muscles were homogenized in 4 volumes of 10 mM phosphate buffer (pH 3.5). After centrifugation at 20,000 g for 5 minutes, 400 µl of water were added to 100 µl of supernatant or serum and loaded onto a SAX cartridge.
(SUPELCO, Bellefonte, PA), previously conditioned with 0.5 ml of methanol and 1 ml of water. After elution, the cartridges were washed with 0.5 ml of 10 mM phosphate buffer (pH 3.5) and the eluates were collected. The so-obtained samples (1 ml) were used for the derivatization reaction. 1-aminoantracene (1AA) was dissolved in acetone (16 mg/ml) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) in 10 mM phosphate buffer (pH 3.5). A 20-µl volume of 1 M HCl, 100 µl of the 1AA solution and 100 µl of the EDC solution (in 20 µl aliquots) were sequentially added to the sample with continuous vortex-mixing. The mixture was incubated at 25°C for 25 minutes and the excess reagent was removed by washing with 5 ml of diethylether. After discarding the organic phase, a 300-µl aliquot of the aqueous phase was then transferred in plastic test tube, 700 µl of 10 mM phosphate buffer (pH 9.1) was added and the mixture was washed with 5 ml of chloroform. After centrifugation 0.5 ml of upper aqueous phase was transferred in a new plastic tube containing 0.5 ml of HPLC-grade water. 50-100 µl were analyzed by HPLC. The HPLC mobile phase was prepared by mixing 700 ml of 0.1 M ammonium acetate (pH 3.5) with 300 ml of acetonitrile; the solution was then filtered and degassed. Chromatographic separation was performed at a flow-rate of 0.6 ml/min using a Kromasil 100-5C18 (250 x 4.6 mm I.D) column (Eka Chemicals AB, Bohus, Sweden). Samples were run on a Bio-Rad (Hercules, CA) HPLC system with a Perkin Elmer (Norwalk, CT) fluorescence detector (LS-1). The excitation and emission wavelength were 310 and 420, respectively.

k) Preparation of membranes from skeletal muscles and GLUT4 determination

Muscles from rat hindlimb were removed and trimmed of connective tissue, fat, and nerves. The muscles were then minced and homogenized on ice three times using a Polytron homogenizer set at 13,500 rpm in a buffer containing 20mM HEPES, 250mM sucrose, 1mM EDTA, 5mM benzamidine, 1µM aprotinin A, 1µM pepstatin, 1µM leupeptin, and 1mM phenylmethylsulfonyl
fluoride, pH 7.4. The homogenate was centrifuged at 2,000 g for 10 min. The pellet, which contained mainly unhomogenized pieces of tissue, was discarded, and the supernatant was centrifuged at 9,000 g for 20 min. The 9,000 g pellet (P1) was resuspended in PBS with the standard cocktail of protease inhibitors listed above. The supernatant was centrifuged at 180,000 g for 90 min. The 180,000 g pellet was resuspended in PBS with protease inhibitors, loaded on a 10-30% (wt/wt) continuous sucrose gradient (3-4 mg protein/5 ml gradient), and centrifuged at 48,000 rpm for 55 min in a SW-50.1 rotor. The pellet of the sucrose-gradient centrifugation (P2) was resuspended in PBS and analyzed for the presence of GLUT4 by western blotting. All centrifugations were performed at 4° C. Western blot was performed on 10% polyacrylamide gel (4). Anti-GLUT4 polyclonal antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:500 was used with anti-rabbit peroxidase conjugated antibody (Dako) diluted 1:3000 and revealed by chemiluminescent substrate (Super Signal West Pico, Pierce, ILL, USA).

1) Indices of liver function

We used AST and ALT as indices of liver function. They were measured on plasma with immunoenzymatic method and red with a spectrophotometer at 340 nm.

2) IN VITRO STUDY

a) Skeletal muscle cultures

Mouse myogenic C2C12 cell line was cultured in DMEM proliferating medium supplied with 10 % fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (complete medium) on flasks and on collagen-coated glass coverslips at the density of about 50,000 cells/cm². After 3 days of cell culture, fetal calf serum was substituted with 2 % horse serum to induce myoblasts fusion and the myotubes formation, which was completed after 7 days. Cultured myotubes were treated with 1 µM staurosporine (Sigma, St. Louis, Mo, USA) (1 mM stock in dimethyl sulfoxide) in fresh serum-containing DMEM and harvested for 18 hours.
Treatment of cultures with different concentrations of L-carnitine (2, 5, 10 mM) was initiated 1 hour before staurosporine treatment and was maintained throughout the subsequent incubation. Control experiments were performed by adding each time only the vehicle (20,26).

Cell viability was analyzed by the colorimetric XTT assay (Cell Proliferation Kit II XTT, Roche). For this assay, equal numbers of C2C12 myogenic cells were plated on 96-well plates and maintained in the growing media as above described in order to obtain myotubes. Differentiated myotubes were then incubated with staurosporine (added as a DMSO solution) either with or without L-carnitine. XTT reagents were eventually added to each well and incubated at 37° C for 4 h. After incubation the plates were analyzed with a multi-well ELISA reader. Data were expressed as percent of living cells.

3) Statistical analysis

Mean ± SD is reported. Student t test for unpaired data was used. P≤0.05 was considered statistically significant. Analysis of variance (ANOVA) was used when appropriate.

RESULTS

a) Occurrence of heart failure in the monocrotaline-treated animals (Tab. 1)

After 4 weeks all the CHF rats showed at post-mortem examination the presence of pericardial, pleural and peritoneal effusions. RVM/LVM was 0.26±0.09 in control, 0.45±0.09 in CHF (p<0.0006) and 0.43±0.13 in the CHFCar (p=NS vs CHF and p<0.0001 vs Con). The right ventricular cavity was markedly dilated in all the CHF rats as reflected by the RVM/RVV index that was 2.7±0.7 in the CHF rats vs 6.0±1.4 in the control group (p<0.0006), and 3.4±1.0 in the CHFCar group (p=NS vs CHF and p<0.001 vs control).

b) AngII levels (Tab. 1)
Ang II levels were 30±10 pg/ml in the control rats, which were the same of the ConCar rats (27±9 pg/ml). The CHF rats showed a significant (p<0.008) increase in Ang II (88±2), while L-carnitine produced in the monocrotaline-treated rats a significant decrease in the Ang II levels (52±23, p<0.04 vs CHF and p<0.02 vs control).

c) MHCs pattern (Tab. 2)
We found in the CHF animals a shift from the fast oxidative (MHC2a) to the fast glycolytic isoform (MHC2b) which were 25±2.7 % and 76±2.7 % in the Con vs 19±3.6 % (p<0.04) and 81±3.6 % in the CHF respectively. L-carnitine produce a reshift toward the MHC2a (23±3.2; p<0.04), while MHC2b was 77±3.2 %.

d) Tibialis anterior CSA (Tab. 2)
The degree of muscle atrophy as measured by the tibialis anterior muscle CSA was 1969±257 µm² in the control vs 1281±335 in the CHF rats (p<0.01). The L-carnitine treatment produced an increase in CSA (1558±331) which did not reach statistical significance (p=NS) when compared to CHF. Despite this effect, there was still a significantly lower (p<0.004) CSA versus control.

e) Count of in situ DNA nick-end labeling (TUNEL) positive nuclei (Tab. 3 and Fig. 1A) The CHF rats showed in the tibialis anterior muscle an enormous increase in the number of TUNEL positive nuclei which was 189±27 nuclei/mm³, as opposed to 1±1 of the control and ConCar animals (p<0.0003). The treatment with L-carnitine produced in the monocrotaline treated animals a significant reduction in the number of TUNEL positive nuclei (56±62; p<0.001 vs CHF and p<0.02 vs control).

f) Activated Caspases 3 and 9, Bcl2 (Fig. 1B, C, D) and confocal microscopy for Caspase 3(Fig. 1E)
We confirmed the occurrence of apoptosis in animals with high number of TUNEL-positive nuclei by testing for the presence of activated Caspases 9 and 3 (the mitochondrial regulator and the executioner, respectively). Caspase 3 is detectable during the execution phase of apoptosis,
when the caspase cascade leads inevitably to programmed cell death. The presence of activated Caspases 3 (panel C) and 9 (panel D) was shown with immunoblotting, and that of activated Caspase 3 was shown with immunofluorescence and confocal microscopy (panel E). Higher levels of Caspase 3 and 9 were present at immunoblotting (lanes c,d,e in panels C and D) in CHF animals, when compared to controls (lanes a,b). The CHFCar rats (lanes f-j) showed a reduction on the expression of these two pro-apoptotic molecules, when compared to CHF, although they did not reach the level of the control animals.

Bcl-2, that is an anti-apoptotic factor, behaved in the opposite manner. It was decreased in CHF (panel B, lanes c,d) in comparison to control (lanes a,b). The CHFCar animals showed an expression of Bcl-2 similar to that of control. (lanes e-i).

g) **ELISA for Apoptosis (Fig. 2)**

Evidence for apoptosis was also confirmed by detection of fragmented DNA by ELISA, where we found an increased optic density in the CHF rats as compared to control. The CHFCar animals showed intermediate values.

h) **L-carnitine determination (Tab. 4 and 5)**

In the serum of CHF animals L-carnitine increased from $41.2 \pm 9.9$ to $67.5 \pm 17$ nm/ml in comparison to controls ($p<0.006$). L-carnitine treatment sorted out a further increase in L-carnitine serum levels both in normal and monocrotaline rats ($182.5 \pm 22.2$ and $119.2 \pm 12.7$).

In the tibialis anterior muscle CHF produced a dramatic decrease of L-carnitine, Acetyl-carnitine and Propionyl-carnitine content when compared to controls. L-carnitine treatment restored almost normal values of the three carnitines in the Tibialis anterior muscle (see Table 5).

i) **TNFα (Tab. 3)**

There was as expected a rise in plasma levels of TNFα in the CHF animals as compared to control ($240 \pm 86$ vs $106 \pm 59$ pg/ml; $p<0.01$). L-carnitine treatment produced a slight, though
significant decrease in TNFα serum levels when compared to CHF (p<0.01): These values were not significantly different from those of control (p=0.08).

**j) SPH (Tab 3)**

SPH increased in CHF (2250±777 vs 912±145 pmol/ml in control; p<0.03). L-carnitine produced a small decrease in SPH serum levels (1538±409) which were significantly different from both CHF and control rats (p<0.05 and p<0.0005 respectively).

**k) Effect of L-carnitine on staurosporine-induced apoptosis (Fig. 3)**

Control myotubes showed a 100% survival after 18 hours in DMEM. Staurosporine alone caused a drop in skeletal myotubes survival, that reached 79±6% after the same incubation time. L-carnitine treatment was able to improve in a dose-dependent fashion the survival rate of the cultured myotubes. In fact after 18 hours incubation in staurosporine plus L-carnitine the percentage of surviving cells was 86±7, 94±4, 100±8% at the concentration of 2, 5 and 10 mM L-carnitine respectively (p=NS for 2mM L-carnitine vs staurosporine and p<0.01 for 5 and 10 mM, ANOVA).

**l) Effect of L-carnitine on GLUT-4 expression (Fig.4)**

Previous studies (12,37) have demonstrated, by western blot analysis and enzymatic assay, that the pellet obtained from supernatant of homogenized muscles after high-speed centrifugation in a continuous 10-30% sucrose gradient, contains virtually all plasma membrane marker proteins, such as β1-integrin, none of which could be detected in the other gradient fractions. Thus, the experimental conditions we used allowed us to separate the surface membranes from intracellular vesicles, and to detect the insulin-dependent GLUT-4 translocation.

Fig.4 shows the western blot analysis of GLUT-4 in muscle from control and treated animals. Monocrotaline injection induced a significant increase in GLUT-4 expression in the pellet fractions of the gradient (Fig.4, Lanes c, d, e). This increase was present to roughly the same
extent in the muscle plasma membrane fractions from rats treated with monocrotaline plus L-carnitine (Fig.4, Lane f, g, h).

**m) Indices of liver function**

We did not find any alteration in liver function enzymes in the monocrotaline-treated animals, when compared to controls (AST 25.0±2.3, 21.4±4.1, ALT 73.8±7.2 vs 52.0±17.4 p=ns).

**DISCUSSION**

In the rat, monocrotaline induces right ventricle hypertrophy followed by right ventricle dilatation and failure. In these animals we found skeletal muscle atrophy secondary to myocyte nuclei apoptosis and shift of MHCs toward the fast glycolytic isoforms, confirming previously published data (4,30,32). The presence of CHF is supported, beyond the post-mortem findings of pleural, pericardial and peritoneal effusions, by the right ventricle dilatation, as indicated by the decreased RVM/RVV index (4,30,32) and by the elevated plasma levels of Ang II (6,7).

The occurrence of apoptosis in our study was confirmed by different techniques, including immunoblotting, immunohistochemistry, confocal microscopy and ELISA of DNA ladder. Activated caspases 3 and 9 were also present; this ensured that the caspase cascade, which inevitably leads to programmed cell death, was initiated.

L-carnitine is a quaternary amine that is fundamental in skeletal muscle metabolism, in that promotes fatty acids oxydation (23), and that has been shown effective in producing a selective trophic effect on Type I and IIa skeletal muscle fibres (11). In this study L-carnitine muscle levels are decreased in CHF rats. Plasma levels of carnitine in the CHF animals are even higher that those of controls, indicating that liver biosynthesis is intact (the absence of liver damage is also proved by the normal transaminases). The low levels of muscle carnitine detected in the CHF rats are therefore probably due to low muscle uptake. There are recent observations that L-
carnitine, beyond the well known metabolic effect, possesses some more complex activities in regulating gene expression and activity of caspases (21). In our study L-carnitine was unable to prevent the development of heart failure, but, despite that, we could detect favourable changes in the tibialis anterior muscle. These consisted in a decreased degree of muscle atrophy, as demonstrated by the CSA of tibialis anterior fibres. This was in fact significantly higher than that of CHF rats, although it wasn’t brought to the Con levels. It may be suggested that the reduction in muscle atrophy may be due to correction of metabolic impairment and to the lower levels of apoptosis observed in the CHFCar animals.

**Metabolic Impairment:** In the CHF rats we have shown a profoundly altered glucose metabolism, in fact, after monocrotaline treatment, skeletal muscle GLUT-4 expression dramatically increases. The contemporary administration of L-carnitine does not counterbalance the monocrotaline-induced GLUT-4 over-expression, therefore, suggesting that L-carnitine does not play a role in modulating insulin responsiveness of skeletal muscles in monocrotaline-treated rats.

In a mouse model of skeletal muscle GLUT-4 overexpression it was demonstrated that the increased glycolysis in muscle was associated both with increased serum lactate levels and with increased flux of this metabolite through the Cori-cycle (27).

The glycolysis rates were increased in these animals while their glucose oxidation rates seemed to be considerably lower than of glycolysis (probably secondary to inhibition of pyruvate dehydrogenase complex by acetyl-CoA derived from increased substrate oxidation). By a parallelism between our model and the transgenic model, it is possible to postulate that L-carnitine could have an important role in regulating glucose metabolism in skeletal muscle of monocrotaline treated rats. This has been also shown in the rat perfused heart, where physiologic concentrations of carnitine mimics insulin-like metabolic effects by increasing glucose oxidation (24).
By increasing muscle L-carnitine levels in the CHF rats may stimulate glucose oxidation by the action of L-carnitine on inner mitochondrial L-carnitine acetyltransferase, which enhances conversion of mitochondrial acetyl-CoA to cytoplasmic acetylcarnitine, resulting in a decrease in the inner mitochondrial acetyl-CoA ratio. A decrease in this ratio will stimulate the pyruvate dehydrogenase complex, the enzyme that converts pyruvate to acetyl-CoA, and is the rate limiting step of glucose oxidation. As a result of an increase of L-carnitine in muscle overexpressing GLUT-4, a greater proportion of the pyruvate derived from glycolysis as well as pyruvate derived from lactate can be oxidized.

The reported changes in MHCs composition after L-carnitine treatment can be as well due to the well known trophic metabolic effect of L-carnitine on type 2a skeletal muscle fibres (11).

**Apoptosis**: in the L-carnitine treated CHF rats we found a substantially lower degree of TUNEL positive nuclei and DNA break strands (ELISA ladder), which were accompanied by a lower expression of caspases and by an increased expression of Bcl-2. We can speculate on the mechanisms by which L-carnitine may have prevented apoptosis:

a) by blocking TNFα and sphingolipids activation cascade as previously shown in the heart (2);

b) by inhibiting the cleavage of caspases substrates at mitochondrial level, making it a general caspases inhibitor (21).

The partially reduced levels of TNFα and SPH found in this paper may have blunted the phospholipid-induced apoptosis.

In this study the mitochondrial pathway is certainly involved, in that activated caspases 3 and 9 are inhibited and Bcl-2 is increased. In vitro inhibition of staurosporine-induced apoptosis, that in C2C12 cells acts via the mitochondrial cascade, (20,26) strengthen this hypothesis. It is not surprising that L-carnitine may be active at mitochondrial level in that it accumulates at this site. It has been recently suggested by Mercadier and coworkers (2) that ceramide generation is linked
to mitochondrial metabolism. If that was the case we would not be dealing with two so independent pathways. It is hard to split the beneficial effects of L-carnitine among its action on myocyte metabolism and its role in modulating apoptosis. We cannot also exclude indirect effects on TNFα, SPH and AngII downregulation. The complexity of the interplay between these molecules has been previously shown by our group in the same animal model: Ang II receptor blockade was in fact able to decrease apoptosis, but also TNFα (6) and SPH (5).

We know that skeletal muscle bulk is one of the major determinants of exercise capacity, in that muscle strength is related to bulk (18,35). Muscle waste is also linked to prognosis: patients with cardiac cachexia, in whom muscle waste is extreme, have a very poor prognosis (3). It is therefore clear how blocking apoptosis and improving oxidative metabolism may prevent skeletal muscle bulk loss, improve exercise capacity and maybe prognosis in the CHF patients.

It may be speculated on the clinical relevance of these findings. In agreement with previous studies carried out in patients with CHF, muscle levels of L-Carnitine are decreased (19) and plasma levels are increased (8), confirming a muscle deficiency and a reduced uptake of L-carnitine both in man and in our animal model of CHF. It is clear that interventions trying to restore normal levels of carnitine at muscle level have the potential for improving skeletal muscle metabolism and also throphism. Although a large randomized trial with L-carnitine on exercise capacity (9) has been published, it has shown neutral results on the long term outcome. Only in a subgroup of patients with preserved ejection fraction, and in another small study there were partially positive effects of the active treatment on peak oxygen consumption (1). We think that a conclusive interpretation cannot be drawn yet. Our data show only a partial improvement in preventing apoptosis, atrophy, cytokine release and SPH formation. In overt heart failure, if production of cytokines and sphingolipids is overwhelming, as it is in sickest or cachectic patients, the consequent apoptosis may be only partially modulated. It is therefore possible that
intervention may be effective only in well defined subgroups of patients. However, it is of importance the understanding of the mechanisms by which L-carnitine is acting in that they may contribute to shed some light on the pathophysiology of the CHF myopathy, leading to prevention of atrophy, preservation of exercise capacity and to design clinical trials targeted on particular populations looking not only at clinical but even at biological end-points.
REFERENCES


33. Vescovo G, Dalla Libera L, Serafini F, Leprotti C, Facchin M, Volterrani M, Ceconi C, and Ambrosio GB. Improved exercise tolerance after losartan and enalapril in heart failure:


Figure 1. Apoptotic features of CHF skeletal muscles.

Panel A: TUNEL-positive nuclei in the TA of CHF rat. (a): double labelling for TUNEL and laminin. (b): double exposure for laminin and Hoechst of a serial section. Arrows indicate apoptotic nuclei (magnification x400; bar = 50 µm).

Panel B: Western blot for Bcl-2. Lanes (a) and (b): controls. Lanes (c) and (d): untreated CHF rats. Lanes (e) through (i): L-carnitine-treated rats.

Panel C: Western blot for activated Caspase 3. Lanes (a) and (b): controls. Lanes (c) to (e): untreated CHF rats. Lanes (f) through (j): L-carnitine-treated rats.

Panel D: Western blot for activated Caspase 9. The key is the same as that for panel C.

Panel E: Immunofluorescence and confocal microscopy for activated Caspase 3 and dystrophin from a CHF animal. Thin, granulated pattern indicates diffuse cytosolic distribution of Caspase 3. Positive fiber (with red dots) is surrounded by negative fibers (black). Sarcolemma is stained in green. (a) x200; (b) detail x400.
Figure 2. Evidence of apoptosis by ELISA assay in TA muscle.

Extracted DNA was diluted, and ELISA was used for detection of fragmented DNA. Data indicate spectrophotometric absorption at 405 nm wavelength, expressed as percent of the control. ELISA demonstrates significant increase of DNA fragmentation in muscles from CHF animals compared with control and L-carnitine-treated animals. * p<0.01; # p<0.05.
C-2C12 myotubes were preincubated in the absence or presence (2, 5, 10 mM) L-carnitine for 1 hour, followed by 1 hour incubation with or without 1 µM staurosporine. Culture medium was removed and myotubes were further incubated for 12 hour. Cell viability was assessed by the colorimetric XTT assay. Results are means ± SE of three independent experiments (*,#,+ p<0.01). Data are expressed as number of cells surviving as percent of controls.
Figure 4. GLUT-4 expression on skeletal muscle surface membranes. Pellet fractions from homogenized muscles, isolated as described in Materials and Methods section, were analysed by western blotting with anti-GLUT-4 polyclonal antibodies. Lanes (a) and (b): control; Lanes (c), (d) and (e): monocrotaline rats; Lanes (f), (g) and (h): carnitine rats.
### TABLE 1
Cardiac and serum features in all rats

<table>
<thead>
<tr>
<th></th>
<th>RVM/RVV</th>
<th>RVM/LVM</th>
<th>AngII (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.0 ± 1.4 * †</td>
<td>0.26 ± 0.09 # **</td>
<td>30 ± 10 §</td>
</tr>
<tr>
<td>Carnitine</td>
<td>5.8 ± 1.7</td>
<td>0.24 ± 0.1</td>
<td>27 ± 9</td>
</tr>
<tr>
<td>CHF</td>
<td>2.7 ± 0.7 * ‡</td>
<td>0.45 ± 0.09 # ††</td>
<td>88 ± 2 § ¶</td>
</tr>
<tr>
<td>CHF+Carnitine</td>
<td>3.4 ± 1.0 † ‡</td>
<td>0.43 ± 0.13 ** ††</td>
<td>52 ± 23</td>
</tr>
</tbody>
</table>

RVM/RVV : * p< 0.0006 ; † p<0.001 ; ‡ p= N.S.
RVM/LVM: # p<0.00006; ** p<0.00005; ††p=NS
AngII: § p<0.008 ; || p<0.02 ; ¶ p<0.04
Key : CHF, congestive heart failure; RVM, right ventricle mass; RVV, right ventricle volume; LVM, left ventricle mass; AngII, Angiotensin II

### TABLE 2
Skeletal muscle features in all rats

<table>
<thead>
<tr>
<th></th>
<th>MHC 2A (% of total)</th>
<th>MHC 2B (% of total)</th>
<th>CSA (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25 ± 2.7 * †</td>
<td>76 ± 2.7</td>
<td>1969 ± 257 §</td>
</tr>
<tr>
<td>Carnitine</td>
<td>26 ± 1.7</td>
<td>74 ± 1.7</td>
<td>2140 ± 340</td>
</tr>
<tr>
<td>CHF</td>
<td>19 ± 3.6 * ‡</td>
<td>81 ± 3.6</td>
<td>1281 ± 335 § ¶</td>
</tr>
<tr>
<td>CHF+Carnitine</td>
<td>23 ± 3.2 † ‡</td>
<td>77 ± 3.2</td>
<td>1558 ± 331</td>
</tr>
</tbody>
</table>

MHC 2A: * p< 0.04; † p= N.S.; ‡ p<0.04
CSA : § p<0.01 ; || p<0.004 ; ¶ p= N.S.
Key: CHF, congestive heart failure; MHC, myosin heavy chain; CSA, cross sectional area.
TABLE 3
Apoptotic cascade features in skeletal muscle and serum in all rats

<table>
<thead>
<tr>
<th></th>
<th>TUNEL+ (nuclei/mm³)</th>
<th>TNFα (pg/ml)</th>
<th>SPH (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1 ± 1 * †</td>
<td>106 ± 59 §</td>
<td>912 ± 145 # **</td>
</tr>
<tr>
<td>Carnitine</td>
<td>1 ± 1</td>
<td>100 ± 35</td>
<td>843 ± 148</td>
</tr>
<tr>
<td>CHF</td>
<td>189 ± 27 * †‡</td>
<td>240 ± 86 ¶</td>
<td>2250 ± 777 # ††</td>
</tr>
<tr>
<td>CHF+Carnitine</td>
<td>56 ± 62 † ‡</td>
<td>150 ± 41 ¶</td>
<td>1538 ± 409 ** ††</td>
</tr>
</tbody>
</table>

TUNEL+: * p<0.0003; † p<0.02; ‡ p<0.001
TNFα: § p<0.01; ¶ p<0.08; ¶ p<0.01
SPH: # p<0.03; ** p<0.0005; †† p<0.05

Key: TUNEL, in situ DNA nick-end labeling; TNFα, tumor necrosis factor; SPH, sphingosine; CHF, congestive heart failure.

TABLE 4
Serum carnitine levels in all rats

<table>
<thead>
<tr>
<th></th>
<th>Carnitine (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.2 ± 9.9 *</td>
</tr>
<tr>
<td>Carnitine</td>
<td>182.5 ± 22.2</td>
</tr>
<tr>
<td>CHF</td>
<td>67.5 ± 17.0 *</td>
</tr>
<tr>
<td>CHF+Carnitine</td>
<td>119.2 ± 12.7</td>
</tr>
</tbody>
</table>

* p< 0.006

TABLE 5
Muscle carnitine levels in all rats

<table>
<thead>
<tr>
<th></th>
<th>LC</th>
<th>ALC</th>
<th>PLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>716 ± 132 * ‡</td>
<td>234 ± 63 §</td>
<td>9 ± 2 # †</td>
</tr>
<tr>
<td>Carnitine</td>
<td>809 ± 60</td>
<td>335 ± 32</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>CHF</td>
<td>334 ± 65 * †</td>
<td>150 ± 45 ¶</td>
<td>1 ± 0.5 ^</td>
</tr>
<tr>
<td>CHF+Carnitine</td>
<td>493 ± 163 † ‡</td>
<td>321 ± 133 ¶</td>
<td>15 ± 4 # ^</td>
</tr>
</tbody>
</table>

Values are expressed as nmol/gram wet tissue.
LC: L-carnitine; ALC: acetyl L-carnitine; PLC: propionyl L-carnitine.
LC: * p< 0.0001; ‡ p< 0.0001; † p< 0.01
ALC: § p< 0.06; ¶ p= NS; ¶ p< 0.04
PLC: ^ p< 0.004; # p= NS; ^ p< 0.003