

L-Carnitine: a potential treatment for blocking apoptosis and preventing skeletal muscle myopathy in heart failure

GIORGIO VESCOVO,¹ BARBARA RAVARA,² VALERIO GOBBO,² MARCO SANDRI,²
ANNALISA ANGELINI,³ MILA DELLA BARBERA,³ MASSIMO DONA,²
GIANFRANCO PELUSO,⁴ MENOTTI CALVANI,⁵ LUIGI MOSCONI,⁵
AND LUCIANO DALLA LIBERA²

¹Internal Medicine, City Hospital, 45011 Adria; ²Consiglio Nazionale delle Ricerche Institute of Neuroscience, Unit for Muscle Biology and Pathophysiology, Department of Biomedical Sciences, and ³Department of Cardiovascular Pathology, University of Padua, 35100 Padua;

⁴Department of Experimental Oncology, National Cancer Institute, 80072 Naples;

⁵Scientific Department, Sigma Tau, 00040 Rome, Italy

Received 29 January 2002; accepted in final form 17 May 2002

Vescovo, Giorgio, Barbara Ravara, Valerio Gobbo, Marco Sandri, Annalisa Angelini, Mila Della Barbera, Massimo Dona, Gianfranco Peluso, Menotti Calvani, Luigi Mosconi, and Luciano Dalla Libera. L-Carnitine: a potential treatment for blocking apoptosis and preventing skeletal muscle myopathy in heart failure. *Am J Physiol Cell Physiol* 283: C802–C810, 2002. First published May 22, 2002; 10.1152/ajpcell.00046.2002.—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 tumor necrosis factor- α (TNF- α) 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, caspase-3 and -9, were increased, as well as serum levels of TNF- α 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 and to decrease the levels of TNF- α and sphingosine, as well as the number of apoptotic myonuclei. Staurosporine was used in *in vitro* experiments to induce apoptosis in skeletal muscle cells in culture. When L-carnitine was 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 muscle cells and has a role in the treatment of congestive heart failure-associated myopathy.

tumor necrosis factor; sphingosine; caspases; staurosporine

HEART FAILURE IS CHARACTERIZED by a decreased exercise capacity with early appearance of fatigue and dyspnea (15, 18). These symptoms are due in part to a skeletal muscle myopathy with atrophy, which 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 fibers (31, 30, 29), which in turn causes the increased muscle fatigability (29). Apoptosis has been shown to be one of the major determinants of skeletal muscle atrophy (7, 34, 33), and its magnitude correlates with the severity of the syndrome (33). 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 (TNF- α), can induce apoptosis either directly or through secondary messengers, such as the sphingolipid sphingosine (SPH) (10, 13, 22). We have also shown that SPH serum levels are increased in heart failure in parallel with TNF- α and that this molecule is able to induce skeletal myocyte apoptosis both *in vivo* and *in vitro* (6, 25). Although clinical trials aimed to block TNF- α with specific antibodies such as infliximab or etanercept have failed (17), there is compelling evidence that apoptosis can be inhibited, with favorable consequences on muscle atrophy. This can be achieved for instance by blocking the angiotensin II (ANG II) receptors (4). L-Carnitine is a quaternary amine that is fundamental in skeletal muscle metabolism, in that it promotes fatty acids oxidation (23), and it has been shown effective in producing a selective trophic effect on type I and IIa skeletal muscle fibers (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 represents 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

Address for reprint requests and other correspondence: L. Dalla Libera, Consiglio Nazionale delle Ricerche Institute of Neuroscience, Unit for Muscle Biology and Pathophysiology, Dept. of Biomedical Sciences, Univ. of Padua, Viale G. Colombo 3, 35100 Padua, Italy (E-mail: ldl@civ.bio.unipd.it).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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 study, 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, which we all know is one of the major determinants of exercise capacity (18, 35), and to give a clue for the understanding of the pathophysiology of the heart failure myopathy, which in turn may have future implications in the development of further treatment for heart failure.

MATERIALS AND METHODS

In Vivo Study

Experimental model. Four groups of animals were studied for a total of 56 animals. Thirty-six male (80–100 g) Sprague-Dawley rats had congestive heart failure (CHF) induced by monocrotaline (Sigma, St. Louis, MO). This alkaloid produces severe pulmonary hypertension followed by right ventricular failure (4, 34) without inducing by itself changes in skeletal muscle myosin heavy chain (MHC) composition and apoptosis (28). 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 50 mg·kg⁻¹·die⁻¹ L-carnitine (Sigma Tau, Rome, Italy) given in drinking water (CHFCar); 10 rats therefore formed the CHF group. Rats were kept in single cages, and the water that had been drunk was measured daily. The other 10 additional rats had only L-carnitine in drinking water, and they formed the L-carnitine control group (ConCar). Ten 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 developed, rats were killed and body, heart, and tibialis anterior weights 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, TNF- α , ANG II, aspartate amino transferase (AST), alanine amino transferase (ALT), and L-carnitine measurements.

Experiments were approved by the Biological Ethical Committee of the University of Padua, according to the Italian law.

Assessment of right ventricle hypertrophy and failure. To make sure that the monocrotaline-treated animals developed right ventricle failure, beyond the postmortem signs such as pericardial, pleural, and peritoneal effusions (28), 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 (4).

Electrophoretic separation of MHCs. We used the method described in details by Vescovo et al. (31, 32). Tibialis anterior muscles were homogenized and solubilized in SDS buffer. Analytical SDS-PAGE was performed on 7% polyacrylamide slabs with 37.5% vol/vol glycerol to separate MHCs (MHC IIa and MHC IIb).

Assessment of MHC distribution. The percent distribution of the MHCs was determined by a densitometric scan (Sigma Gel, SPSS, Chicago, IL) after image acquisition of the stained gels (4).

Single fiber cross-sectional area. We used single fiber cross-sectional areas (CSA) as an index of myofiber atrophy (4, 6). On each muscle a cross cryosection was taken for histological examination and stained with hematoxylin and eosin. The fiber CSA was calculated with a computerized interactive method (4, 6). At least 400 fibers per specimen were counted at a magnification of $\times 250$ and then averaged.

Assessment of apoptosis. *IN SITU DNA NICK-END LABELING.* In situ terminal transferase dUTP nick-end labeling (TUNEL) of fragmented DNA was performed on cryosections using the in situ cell death detection kit POD (Boehringer Mannheim). Labeled nuclei were identified from the negative nuclei counterstained by Hoechst 33258 and were counted after being photographed. The total number of positive nuclei was determined by counting (magnification $\times 250$) all of the labeled nuclei present in the whole specimen. The number of positive nuclei was then expressed as the number of TUNEL-positive nuclei per millimeter cubed (4, 6). TUNEL-positive myofibers 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 and TUNEL-positive myonuclei.

TIBIALIS ANTERIOR WESTERN BLOT FOR ACTIVATED CASPASE-3 AND -9 AND BCL-2. Western blot was performed on 12.5 polyacrylamide gels as previously described (7). Anti-Bcl-2 (29 kDa) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used with anti-rabbit alkaline-phosphatase (Sigma). Anti-cleaved caspase-3 (17 kDa) and anti-cleaved caspase-9 (37 kDa, Cell Signaling Technology, Beverly, MA) were used with anti-rabbit peroxidase-conjugated antibody and revealed by chemiluminescent substrate (SuperSignal West Pico, Pierce, IL).

CONFOCAL MICROSCOPY IMMUNOFLUORESCENCE. Frozen sections were incubated for 1 h at room temperature with anti-dystrophin antibody (Novocastra, Newcastle Upon Tyne, UK) diluted 1:300 in 1% BSA, which stains sarcolemma. After three washings with PBS, sections were incubated with anti-mouse IgG FITC-conjugated (Sigma) diluted 1:500 at 37°C for 1 h. The sections were then washed three times with PBS and fixed with 4% paraformaldehyde and incubated with anti-caspase-3 antibody (Cell Signaling Technology) diluted 1:10 overnight at 4°C. Slices were then incubated with anti-rabbit Cy3-conjugated antibody for 1 h at room temperature and analyzed by Bio-Rad (Hercules, CA) confocal microscopy.

ELISA FOR APOPTOSIS. DNA ladder assay was performed according to Yasuhara et al. (36). In brief, 10 muscle cryosections, 20 μ m thick, were solubilized in 200 μ l of lysis buffer [0.1% Triton X-100, 5 mM Tris·HCl (pH 8.0), 20 mM EGTA, 20 mM EDTA]. 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 by the method of Bradford and adjusted to 0.01 μ g/ μ l. Cell death ELISA analysis was performed according to manufacturer's instructions (Boehringer Mannheim). After incubation, the plates were analyzed with a multiwell ELISA reader.

TNF- α determination. TNF- α was measured with a solid-phase sandwich ELISA, using a monoclonal antibody specific for rat TNF- α (Euroclone).

ANG II assay. ANG II was measured on serum, using an enzyme-immunometric assay kit from SPI-BIO (Massy Cedex, France).

SPH determination. For the extraction of sphingolipids, 100- to 300- μ l samples of serum were deproteinized by adding warmed butanol (70°C, 800 μ l), vortexing, and incubating at 70°C while rocking. The mixture was then placed in a sonicating water bath for 10 min. Denatured protein and aqueous phase were separated from the butanol layer by centrifugation at 15,300 g . The upper butanol layer was transferred into a new extraction tube and saponified by the addition of 0.5 M KOH (200 μ l). After vortexing, samples were incubated at 70°C while rocking for 1 h with intermittent vortexing and sonicating. HPLC-grade water (400 μ l) was added to each sample, and the sample was returned to the incubator for 10 min. After sonicating for 1 min, the layers were separated by centrifuging at 15,300 g for 3 min. 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 μ l) and agitated in a bath sonicator for 2 min.

The extracts were then derivatized with *o*-phthalaldehyde (Molecular Probes, Eugene, OR) as previously described (6). The derivatized samples (50- μ l injection) were separated on a Bio-Rad Hi-Pore reversed phase column RP-318 (4.6 mm ID \times 250 mm) column with a 1.5 cm Perkin-Elmer (Norwalk, CT) NewGuard RP-18 guard column. Samples were run on a Bio-Rad HPLC system with a Perkin-Elmer fluorescence detector (LS-1) (excitation 340 nm, emission 455 nm). The solvent system was methanol, glacial acetic acid, 1 M tetrabutylammonium dihydrogen phosphate, and HPLC-grade water (82.9:1.5:0.9:14.7, vol/vol) run at 1 ml/min.

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 vol of 10 mM phosphate buffer (pH 3.5). After centrifugation at 20,000 g for 5 min, 400 μ l of water were added to 100 μ l 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-Aminoanthracene was dissolved in acetone (16 mg/ml) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in 10 mM phosphate buffer (pH 3.5). A 20- μ l volume of 1 M HCl, 100 μ l of the 1-aminoanthracene solution, and 100 μ l of the 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide solution (in 20- μ l aliquots) were sequentially added to the sample with continuous vortex mixing. The mixture was incubated at 25°C for 25 min, and the excess reagent was removed by washing the mixture with 5 ml of diethylether. After the organic phase was discarded, a 300- μ l aliquot of the aqueous phase was then transferred in a plastic test tube, 700 μ l 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 μ l 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 \times 4.6 mm ID) column (Eka Chemicals AB, Bohus, Sweden). Samples were run on a Bio-Rad HPLC system with a Perkin-Elmer fluorescence detector (LS-1). The excitation and emission wavelengths were 310 and 420 nm, respectively.

Preparation of membranes from skeletal muscles and GLUT-4 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 20 mM HEPES, 250 mM sucrose, 1 mM EDTA, 5 mM benzamide, 1 μ M aprotinin A, 1 μ M pepstatin, 1 μ M leupeptin, and 1 mM 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 GLUT-4 by Western blotting. All centrifugations were performed at 4°C. Western blot was performed on 10% polyacrylamide gel (7). Anti-GLUT-4 polyclonal antibody (Santa Cruz Biotechnology) diluted 1:500 was used with anti-rabbit peroxidase conjugated antibody (Dako) diluted 1:3,000 and revealed by chemiluminescent substrate (Super Signal West Pico).

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

In Vitro Study

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 \sim 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 myotube formation, which was completed after 7 days.

Cultured myotubes were treated with 1 μ M staurosporine (Sigma; 1-mM stock in dimethyl sulfoxide) in fresh serum-containing DMEM and harvested for 18 h. Treatment of cultures with different concentrations of L-carnitine (2, 5, 10 mM) was initiated 1 h before staurosporine treatment and was maintained throughout the subsequent incubation. Control experiments were performed by adding only the vehicle each time (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 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 multiwell ELISA reader. Data were expressed as percentage of living cells.

Statistical analysis. Means \pm SD are reported. Student's *t*-test for unpaired data was used. $P \leq 0.05$ was considered statistically significant. ANOVA was used when appropriate.

RESULTS

Occurrence of Heart Failure in the Monocrotaline-Treated Animals

After 4 wk, all the CHF rats showed the presence of pericardial, pleural, and peritoneal effusions at post-mortem examination (Table 1). RVM/LVM was $0.26 \pm$

Table 1. Cardiac and serum features in all rats

	RVM/RVV	RVM/LVM	ANG II, pg/ml
Control	6.0 ± 1.4 ^{a,b}	0.26 ± 0.09 ^{d,e}	30 ± 10 ^{g,h}
Carnitine	5.8 ± 1.7	0.24 ± 0.1	27 ± 9
CHF	2.7 ± 0.7 ^{a,c}	0.45 ± 0.09 ^{d,f}	88 ± 2 ^{g,i}
CHF + carnitine	3.4 ± 1.0 ^{b,c}	0.43 ± 0.13 ^{e,f}	52 ± 23 ^{h,i}

Values are means ± SE. CHF, congestive heart failure; RVM, right ventricle mass; RVV, right ventricle volume; LVM, left ventricle mass; ANG II, angiotensin II. RVM/RVV: ^a*P* < 0.0006; ^b*P* < 0.001; ^c*P* = not significant (NS). RVM/LVM: ^d*P* < 0.00006; ^e*P* < 0.00005. ^f*P* < NS. ANG II: ^g*P* < 0.008; ^h*P* < 0.02; ⁱ*P* < 0.04.

0.09 in control, 0.45 ± 0.09 in CHF (*P* < 0.0006), and 0.43 ± 0.13 in CHFCar [*P* = nonsignificant (NS) vs. CHF and *P* < 0.0001 vs. control]. 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 ± 1.4 in the control group (*P* < 0.0006) and 3.4 ± 1 in the CHFCar group (*P* = NS vs. CHF and *P* < 0.001 vs. control).

ANG II Levels

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

MHC Pattern

We found a shift from the fast oxidative (MHC IIa) to the fast glycolytic isoform in the CHF animals (MHC IIb), which was 25 ± 2.7 and 76 ± 2.7% in the control vs. 19 ± 3.6 (*P* < 0.04) and 81 ± 3.6% in the CHF animals, respectively (Table 2). L-Carnitine produced a reshift toward the MHC IIa (23 ± 3.2; *P* < 0.04), while MHC IIb was 77 ± 3.2%.

Tibialis Anterior CSA

The degree of muscle atrophy as measured by the tibialis anterior muscle CSA was 1,969 ± 257 μm² in the control vs. 1,281 ± 335 in the CHF rats (*P* < 0.01; Table 2). The L-carnitine treatment produced an increase in CSA (1,558 ± 331), which did not reach statistical significance (*P* = NS) compared with CHF

Table 2. Skeletal muscle features in all rats

	MHC IIa (% of total)	MHC IIb (% of total)	CSA, μm ²
Control	25 ± 2.7 ^{a,b}	76 ± 2.7	1,969 ± 257 ^{d,e}
Carnitine	26 ± 1.7	74 ± 1.7	2,140 ± 340
CHF	19 ± 3.6 ^{a,c}	81 ± 3.6	1,281 ± 335 ^{d,f}
CHF + carnitine	23 ± 3.2 ^{b,c}	77 ± 3.2	1,558 ± 331 ^{e,f}

Values are means ± SE. MHC, myosin heavy chain; CSA, cross-sectional area MHC IIa: ^a*P* < 0.04; ^b*P* = NS; ^c*P* < 0.04. CSA: ^d*P* < 0.01; ^e*P* < 0.004; ^f*P* = NS.

rats. Despite this effect, there was still a significantly lower (*P* < 0.004) CSA vs. control rats.

Count of In Situ DNA TUNEL-Positive Nuclei

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

Activated Caspase-3 and -9, Bcl-2, and Confocal Microscopy for Caspase-3

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

Bcl-2, that is an antiapoptotic factor, behaved in the opposite manner. It was decreased in CHF (Fig. 1B, lanes c and d) compared with control rats (lanes a and b). The CHFCar animals showed an expression of Bcl-2 similar to that of control animals (lanes e-i).

ELISA for Apoptosis

Evidence for apoptosis was also confirmed by detection of fragmented DNA by ELISA, where we found an increased optical density in the CHF rats compared with control. The CHFCar animals showed intermediate values (Fig. 2).

Table 3. Apoptotic cascade features in skeletal muscle and serum in all rats

	TUNEL+, nuclei/mm ³	TNF-α, pg/ml	SPH, pmol/ml
Control	1 ± 1 ^{a,b}	106 ± 59 ^{d,e}	912 ± 145 ^{g,h}
Carnitine	1 ± 1	100 ± 35	843 ± 148
CHF	189 ± 27 ^{a,c}	240 ± 86 ^{d,f}	2250 ± 777 ^{g,i}
CHF + carnitine	56 ± 62 ^{b,c}	150 ± 41 ^{e,f}	1538 ± 409 ^{h,i}

Values are means ± SE. TUNEL, in situ terminal transferase dUTP nick-end labeling; TNF-α, tumor necrosis factor; SPH, sphingosine. TUNEL+: ^a*P* < 0.0003; ^b*P* < 0.02; ^c*P* < 0.001. TNF-α: ^d*P* < 0.01; ^e*P* < 0.08; ^f*P* < 0.01. SPH: ^g*P* < 0.03; ^h*P* < 0.0005; ⁱ*P* < 0.05.

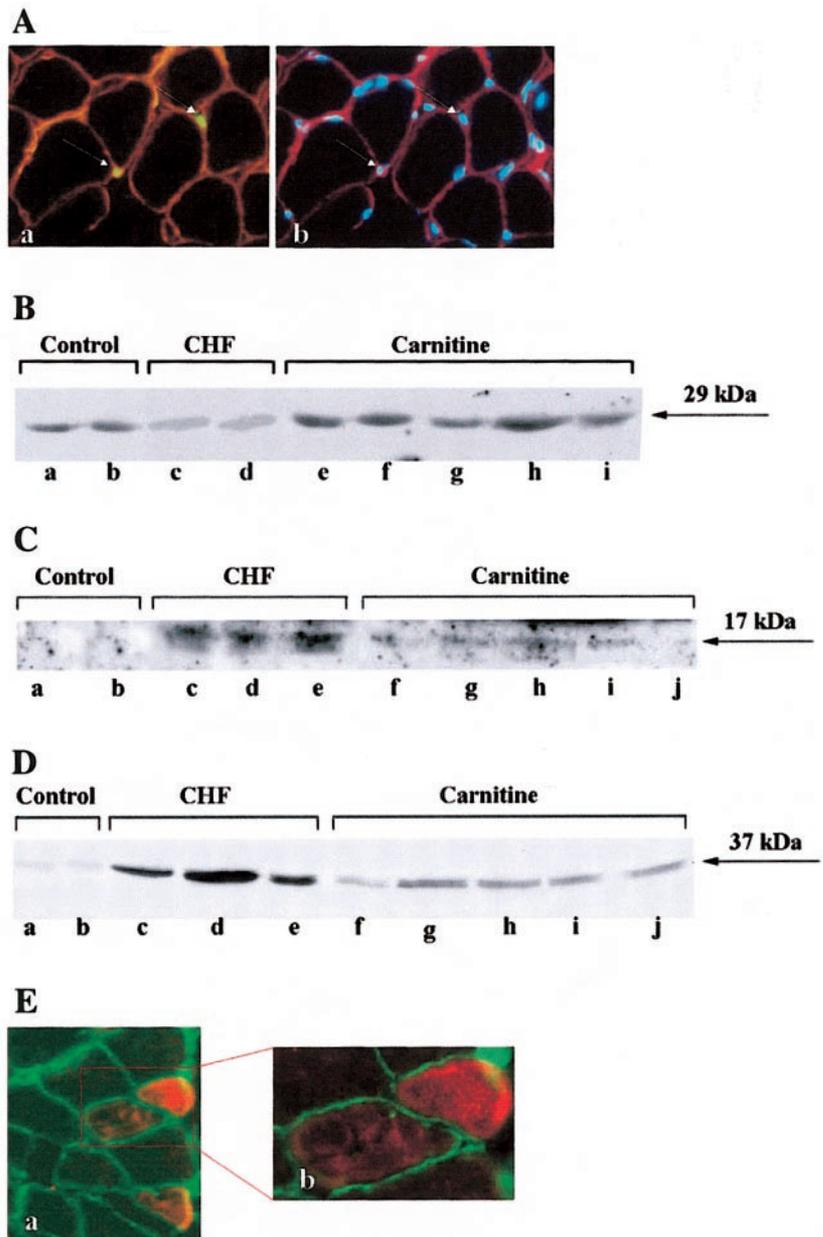


Fig. 1. Apoptotic features of congestive heart failure (CHF) skeletal muscles. **A:** terminal transferase dUTP nick-end labeling (TUNEL)-positive nuclei in tibialis anterior of CHF rat. *a:* Double labeling for TUNEL and laminin; *b:* double exposure for laminin and Hoechst of a serial section. Arrows, apoptotic nuclei (magnification $\times 400$; bar = $50 \mu\text{m}$). **B:** Western blot for Bcl-2. *Lanes a and b,* controls; *lanes c and d,* untreated CHF rats; *lanes e-i,* L-carnitine-treated rats. **C:** Western blot for activated caspase-3. *Lanes a and b,* controls; *lanes c-e,* untreated CHF rats; *lanes f-j,* L-carnitine-treated rats. **D:** Western blot for activated caspase-9; key is same as that for **C**. **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:* $\times 200$; *b:* detail $\times 400$.

L-Carnitine Determination

In the serum of CHF animals, L-carnitine increased from 41.2 ± 9.9 to 67.5 ± 17 nm/ml compared with 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 ± 22.2 and 119.2 ± 12.7 ; Tables 4 and 5).

In the tibialis anterior muscle, CHF produced a dramatic decrease of L-carnitine, acetyl-carnitine, and propionyl-carnitine content compared with controls. L-Carnitine treatment restored almost normal values of the three carnitines in the tibialis anterior muscle (see Table 5).

TNF- α

There was, as expected, a rise in plasma levels of TNF- α in the CHF animals compared with control rats

(240 ± 86 vs. 106 ± 59 pg/ml; $P < 0.01$; Table 3). L-Carnitine treatment produced a slight, though significant, decrease in TNF- α serum levels compared with CHF animals ($P < 0.01$); these values were not significantly different from those of control rats ($P = 0.08$).

SPH

SPH increased in CHF rats ($2,250 \pm 777$ vs. 912 ± 145 pmol/ml in controls; $P < 0.03$; Table 3). L-Carnitine produced a small decrease in SPH serum levels ($1,538 \pm 409$), which were significantly different from both CHF and control rats ($P < 0.05$ and $P < 0.0005$, respectively).

Effect of L-Carnitine on Staurosporine-Induced Apoptosis

Control myotubes showed 100% survival after 18 h in DMEM (Fig. 3). Staurosporine alone caused a drop

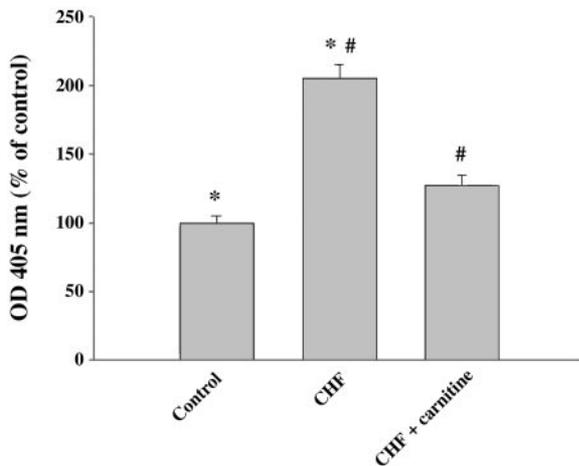


Fig. 2. Evidence of apoptosis by ELISA assay in tibialis anterior muscle. Extracted DNA was diluted, and ELISA was used for detection of fragmented DNA. Data indicate spectrophotometric absorption at 405-nm wavelength, expressed as percentage of control. ELISA demonstrates significant increase of DNA fragmentation in muscles from CHF animals compared with control and L-carnitine-treated animals. OD, optical density. * $P < 0.01$; # $P < 0.05$.

in skeletal myotubes survival, which reached $79 \pm 6\%$ 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 an 18-h incubation in staurosporine plus L-carnitine, the percentage of surviving cells was 86 ± 7 , 94 ± 4 , $100 \pm 8\%$ at the concentration of 2, 5, and 10 mM L-carnitine, respectively ($P = \text{NS}$ for 2 mM L-carnitine vs. staurosporine and $P < 0.01$ for 5 and 10 mM, ANOVA).

Effect of L-Carnitine on GLUT-4 Expression

Previous studies (12, 37) have demonstrated, by Western blot analysis and enzymatic assay, that the pellet obtained from the supernatant of homogenized muscles after high-speed centrifugation in a continuous 10–30% sucrose gradient contains virtually all plasma membrane marker proteins, such as $\beta 1$ -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.

Figure 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, and e). This increase was present

Table 4. Serum carnitine levels in all rats

	Carnitine, nmol/ml
Control	$41.2 \pm 9.9^*$
Carnitine	182.5 ± 22.2
CHF	$67.5 \pm 17.0^*$
CHF + carnitine	119.2 ± 12.7

Values are means \pm SE; * $P < 0.006$.

Table 5. Muscle carnitine levels in all rats

	LC	ALC	PLC
Control	$716 \pm 132^{a,c}$	$234 \pm 63^{d,e}$	$9 \pm 2^{g,h}$
Carnitine	809 ± 60	335 ± 32	8 ± 2
CHF	$334 \pm 65^{a,b}$	$150 \pm 45^{d,f}$	$1 \pm 0.5^{g,i}$
CHF + carnitine	$493 \pm 163^{b,c}$	$321 \pm 133^{e,f}$	$15 \pm 4^{h,i}$

Values are means \pm SE in nmol/gram wet tissue. LC: L-carnitine; ALC: acetyl-L-carnitine; PLC: propionyl-L-carnitine. LC: ^a $P < 0.0001$; ^b $P < 0.0001$; ^c $P < 0.01$, ALC: ^d $P < 0.06$; ^e $P = \text{NS}$; ^f $P < 0.04$, PLC: ^g $P < 0.004$; ^h $P = \text{NS}$; ⁱ $P < 0.003$.

roughly to the same extent in the muscle plasma membrane fractions from rats treated with monocrotaline plus L-carnitine (Fig. 4, lanes f, g, and h).

Indexes of Liver Function

We did not find any alteration in liver function enzymes in the monocrotaline-treated animals, compared with controls (AST 25.0 ± 2.3 , 21.4 ± 4.1 , ALT 73.8 ± 7.2 vs. 52.0 ± 17.4 , $P = \text{NS}$).

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 (7, 28, 34). The presence of CHF is supported, beyond the postmortem findings of pleural, pericardial, and peritoneal effusions, by the right ventricle dilatation, as indicated by the decreased RVM/RVV index (7, 28, 34) and by the elevated plasma levels of ANG II (4, 6).

The occurrence of apoptosis in our study was confirmed by different techniques, including immunoblotting, immunohistochemistry, confocal microscopy, and ELISA of DNA ladder. Activated caspase-3 and -9 were also present; this ensured that the caspase cascade,

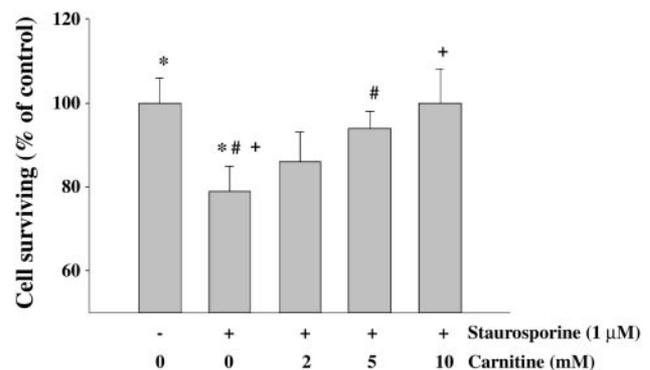


Fig. 3. Effect of L-carnitine on staurosporine-triggered apoptosis in C2C12 mouse myotubes. C2C12 myotubes were preincubated in the absence or presence (2, 5, 10 mM) of L-carnitine for 1 h, followed by 1-h incubation with or without $1 \mu\text{M}$ staurosporine. Culture medium was removed, and myotubes were further incubated for 12 h. Cell viability was assessed by the colorimetric XTT assay. Results are means \pm SE of 3 independent experiments (*, #, + $P < 0.01$). Data are number of cells surviving as percentage of controls.

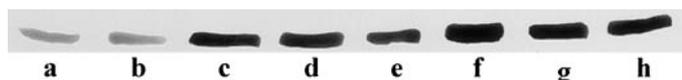


Fig. 4. GLUT-4 expression on skeletal muscle surface membranes. Pellet fractions from homogenized muscles, isolated as described in MATERIALS AND METHODS, were analyzed by western blotting with anti-GLUT-4 polyclonal antibodies. Lanes a and b, control; lanes c, d, and e, monocrotaline rats; lanes f, g, and h: carnitine rats.

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 oxidation (23), and it has been shown effective in producing a selective trophic effect on type I and IIa skeletal muscle fibers (11). In this study, L-carnitine muscle levels are decreased in CHF rats. Plasma levels of carnitine in the CHF animals are even higher than 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 favorable changes in the tibialis anterior muscle. These consisted in a decreased degree of muscle atrophy, as demonstrated by the CSA of tibialis anterior fibers. This was in fact significantly higher than that of CHF rats, although it was not brought to the control 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 overexpression, 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, whereas 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 physiological concentrations of carnitine mimic 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 MHC composition after L-carnitine treatment can be due as well to the well-known trophic metabolic effect of L-carnitine on type IIa skeletal muscle fibers (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: 1) by blocking TNF- α and sphingolipids activation cascade as previously shown in the heart (2), and 2) by inhibiting the cleavage of caspases substrates at mitochondrial level, making it a general caspase inhibitor (21). The partially reduced levels of TNF- α and SPH found in this study may have blunted the phospholipid-induced apoptosis.

In this study, the mitochondrial pathway is certainly involved, in that activated caspase-3 and -9 are inhibited and Bcl-2 is increased. In vitro inhibition of staurosporine-induced apoptosis, which in C2C12 cells acts via the mitochondrial cascade (20, 26), strengthens this hypothesis. It is not surprising that L-carnitine may be active at the mitochondrial level in that it accumulates at this site. It has been recently suggested by Andrieu-Abadie et al. (2) that ceramide generation is linked to mitochondrial metabolism. If that were the case, we would not be dealing with two so independent pathways. It is hard to split the beneficial effects of L-carnitine between its action on myocyte metabolism and its role in modulating apoptosis. We cannot also exclude indirect effects on TNF- α , SPH, and ANG II 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 not only apoptosis but also TNF- α (4) 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 and improve exercise capacity and maybe prognosis in the CHF patients.

The clinical relevance of these findings may be speculated. 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 humans 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 trophism. 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 were there 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 important to understand the mechanisms by which L-carnitine is acting so that they may shed some light on the pathophysiology of the CHF myopathy and possibly lead to the prevention of atrophy, the preservation of exercise capacity, and the design of clinical trials targeted on particular populations, which look not only at clinical but even at biological end points.

REFERENCES

- Anand I, Chandrashekhan Y, De Giuli F, Pasini E, Mazzolletti A, Confortini R, and Ferrari R. Acute and chronic effects of propionyl-L-carnitine on the hemodynamics, exercise capacity, and hormones in patients with congestive heart failure. *Cardio-vasc Drugs Ther* 12: 291–299, 1998.
- Andrieu-Abadie N, Jaffrezou JP, Hatem S, Laurent G, Levade T, and Mercadier JJ. L-Carnitine prevents doxorubicin-induced apoptosis of cardiac myocytes: role of inhibition of ceramide generation. *FASEB J* 13: 1501–1510, 1999.
- Anker SD and Coats AJ. Cachexia in heart failure is bad for you. *Eur Heart J* 19: 191–193, 1998.
- Dalla Libera L, Ravara B, Angelini A, Rossini K, Sandri M, Thiene G, Ambrosio GB, and Vescovo G. Beneficial effects on skeletal muscle of the angiotensin II type 1 receptor blocker irbesartan in experimental heart failure. *Circulation* 103: 2195–2200, 2001.
- Dalla Libera L, Ravara B, Betto R, Sabbadini R, and Vescovo G. The role of sphingosine in inducing skeletal muscle apoptosis and atrophy in heart failure (Abstract). *Eur J Heart Fail* 2: 31a, 2000.
- Dalla Libera L, Sabbadini R, Renken C, Ravara B, Sandri M, Betto R, Angelini A, and Vescovo G. Apoptosis in the skeletal muscle of rats with heart failure is associated with increased serum levels of TNF α and sphingosine. *J Mol Cell Cardiol* 33: 1871–1878, 2001.
- Dalla Libera L, Zennaro R, Sandri M, Ambrosio GB, and Vescovo G. Apoptosis and atrophy in rat slow skeletal muscles in chronic heart failure. *Am J Physiol Cell Physiol* 277: C982–C986, 1999.
- El-Aroussy W, Rizk A, Mayhoub G, Aleem SA, El-Tobgy S, and Mokhtar MS. Plasma carnitine levels as a marker of impaired left ventricular functions. *Mol Cell Biochem* 213: 37–71, 2000.
- Ferrari R and De Giuli F. The propionyl-L-carnitine hypothesis: an alternative approach to treating heart failure. *J Card Fail* 3: 217–241, 1997.
- Ghosh S, Strum JC, and Bell RM. Lipid biochemistry: functions of glycerolipids and sphingolipids in cellular signaling. *FASEB J* 11: 45–50, 1997.
- Giovenali P, Fenocchio D, Montanari G, Cancellotti C, D'Iddio S, Buoncristiani U, Pelagaggia M, and Ribacchi R. Selective trophic effect of L-carnitine in type I and IIa skeletal muscle fibers. *Kidney Int* 46: 1616–1619, 1994.
- Kandror KV, Conderre L, Pushkin AV, and Pilch PF. Comparison of glucose transporter-containing vesicles from rat fat and muscle cells — evidence for a unique endosomal compartment. *Biochem J* 307: 383–390, 1995.
- Krown KA, Page MT, Nguyen C, Zechner D, Gutierrez V, Comstock K, Glembotski CC, Quintana PJE, and Sabbadini R. Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. *J Clin Invest* 98: 2854–2865, 1996.
- Levine B, Kalman J, Mayer L, Fillit H, and Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med* 323: 236–241, 1990.
- Lipkin D, Jones D, Round J, and Poole-Wilson P. Abnormalities of skeletal muscle in patients with chronic heart failure. *Int J Cardiol* 18: 187–195, 1988.
- Longo A, Bruno G, Curti S, Mancinelli A, and Miotto G. Determination of L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine in human plasma by high-performance liquid chromatography after precolumn derivatization with 1-aminoanthracene. *J Chromatogr B Biomed Appl* 686: 129–139, 1996.
- Louis A, Cleland JC, Crabbe S, Ford S, Thackray S, Houghton T, and Clark A. Clinical trials update: CAPRICORN, COPERNICUS, MIRACLE, STAF, RITZ-2, and REIN-AISSANCE and cachexia and cholesterol in heart failure. Highlights of the Scientific Sessions of the American College of Cardiology, 2001. *Eur J Heart Fail* 3: 381–387, 2001.
- Mancini DM, Reichel N, Chance B, Lenkinski R, Mullen J, and Wilson JR. Contribution of skeletal muscle atrophy to exercise intolerance and altered muscle metabolism in heart failure. *Circulation* 85: 1364–1373, 1992.
- Martin MA, Gomez MA, Guillen F, Bornstein B, Campos Y, Rubio JC, de la Calzada CS, and Arenas J. Myocardial carnitine and palmitoyltransferase deficiencies in patient with severe heart failure. *Biochim Biophys Acta* 1502: 330–336, 2000.
- McArdle A, Maglara A, Appleton P, Watson AJM, Grierson I, and Jackson M. Apoptosis in multinucleated skeletal muscle myotubes. *Lab Invest* 79: 1069–1076, 1999.
- Mutomba MC, Yuan H, Konyavko M, Adachi S, Yokoyama CB, Esser V, McGarry JD, Babio BM, and Gottlieb RA. Regulation of the activity of caspases by L-carnitine and palmitoylcarnitine. *FEBS Lett* 478: 19–25, 2000.
- Oral H, Dorn GW, and Mann DL. SPH mediates the immediate negative inotropic effects of tumor necrosis factor- α in the adult mammalian cardiac myocyte. *J Biol Chem* 272: 4836–4842, 1997.
- Peluso G, Barbarisi A, Savica V, Reda E, Nicolai R, Benfatti P, and Calvani M. Carnitine: an osmolyte that plays a metabolic role. *J Cell Biochem* 80: 1–10, 2001.
- Rodgers RL, Christie ME, Tremblay GC, Babson JR, and Daniels T. Insulin-like effects of a physiologic concentration of carnitine on cardiac metabolism. *Mol Cell Biochem* 226: 97–105, 2001.

25. **Sabbadini RA, Danieli-Betto D, and Betto R.** The role of sphingolipids in the control of skeletal muscle function: a review. *Ital J Neurol Sci* 20: 423–430, 1999.
26. **Sandri M and Carraro U.** Apoptosis of skeletal muscle during development and disease. *Int J Biochem Cell Biol* 31: 1373–1390, 1999.
27. **Tsao T, Li J, Chang KS, Stenbit AE, Galuska D, Anderson JE, Zierath JR, McCarter RJ, and Charron MJ.** Metabolic adaptations in skeletal muscle overexpressing GLUT-4: effects on muscle and physical activity. *FASEB J* 15: 958–969, 2001.
28. **Vescovo G, Ceconi C, Bernocchi P, Ferrari R, Carraro U, Ambrosio GB, and Dalla Libera L.** Skeletal muscle myosin heavy chain expression in rats with monocrotaline-induced cardiac hypertrophy and failure. Relation to blood flow and degree of muscle atrophy. *Cardiovasc Res* 39: 233–241, 1998.
29. **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: correlation with changes in skeletal muscle myosin heavy chain composition. *Circulation* 98: 1742–1749, 1998.
30. **Vescovo G, Serafini F, Dalla Libera L, Leprotti C, Facchin L, Tenderini P, and Ambrosio GB.** Skeletal muscle myosin heavy chains in heart failure. Correlation between magnitude of the isozyme shift, exercise capacity and gas exchange measurements. *Am Heart J* 135: 130–137, 1998.
31. **Vescovo G, Serafini F, Facchin L, Tenderini P, Carraro U, Dalla Libera L, Catani C, and Ambrosio GB.** Specific changes in skeletal muscle myosin heavy chain composition in cardiac failure: differences compared with disuse atrophy as assessed on microbiopsies by high resolution electrophoresis. *Heart* 76: 337–343, 1996.
32. **Vescovo G, Serafini F, Facchin L, Tenderini P, Dalla Libera L, Leprotti C, Catani C, and Ambrosio GB.** A new electrophoretic micromethod for assessing myosin heavy chain composition of skeletal muscle in chronic heart failure. *Basic Appl Myol* 5: 365–370, 1995.
33. **Vescovo G, Volterrani M, Zennaro R, Sandri M, Ceconi C, Lorusso R, Ferrari R, Ambrosio GB, and Dalla Libera L.** Apoptosis in the skeletal muscle of patients with heart failure: investigation of clinical and biochemical changes. *Heart* 84: 431–437, 2000.
34. **Vescovo G, Zennaro R, Sandri M, Carraro U, Leprotti C, Ceconi C, Ambrosio GB, and Dalla Libera L.** Apoptosis of skeletal muscle myofibers and interstitial cells in experimental heart failure. *J Mol Cell Cardiol* 30: 2449–2459, 1998.
35. **Volterrani M, Clark AL, Ludman PF, Swan JW, Adamopoulos S, Piepoli M, and Coats AJS.** Predictors of exercise capacity in chronic heart failure. *Eur Heart J* 15: 801–809, 1994.
36. **Yasuhara S, Perez ME, Kanakubo E, Yasuhara Y, Shin YS, Kaneki M, Fujita T, and Martyn JA.** A skeletal muscle apoptosis after burns is associated with activation of proapoptotic signals. *Am J Physiol Endocrinol Metab* 279: E1114–E1121, 2000.
37. **Zhou M, Sevilla L, Vallega G, Chen P, Palacin M, Zorzano A, Pilch PF, and Kandror KV.** Insulin-dependent protein trafficking in skeletal muscle cells. *Am J Physiol Endocrinol Metab* 275: E187–E196, 1998.

