# Therapeutical Treatments for Blocking Apoptosis and Preventing Skeletal Muscle Myopathy in Heart Failure

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**Abstract**

Heart failure is a syndrome leading to a skeletal muscle myopathy with atrophy and shift toward fast contracting fibres type. It has been shown that the major casue of atrophy is muscle waste due to skeletal muscle myonuclei apoptosis. Apoptosis is triggered by circulating citokines and their second messengers, in particular sphingolipids. Several attempts to block apoptosis have been tried: TNFα has been blocked with Embrel, Infliximab and thalidomide without success and this experimental avenue has been diverted. There are recent observation showing that apoptosis and atrophy can be prevented by blocking the agiotensin II receptors. Another possible approach is to block the apoptotic cascade involving sphyngomyelinase and caspases. This can be done *in vivo* with natural occurring substances such as carnitine that have been shown able to block both sphyngomyelinase and mitochondrial caspases, or *in vitro* with other substances like n-oleilethanol-amine. Aim of our future studies is to shed some light on the pathophysiology of the heart failure myopathy, but at the same time to see whether apoptosis can be inhibited with subsequent prevention of the myopathy (atrophy and MHCs shift) and improvement of exercise capacity and symptoms. We have decided to test this hypothesis both *in vivo*, in a well known and accepted animal model of heart failure (the monocrotaline treated rat, that is a model of right ventricle failure secondary to pulmonary hypertension) and *in vitro* (on cultured myotubes where apoptosis can be induced either with staurosporine or doxorubicine). In case it was possible to block the apoptotic cascade and prevent muscle waste and atrophy, these research could be extended to heart failure in humans and to other myopathies in which it has been shown that apoptosis plays a determinant role in producing myocyte loss.

**Key words**: angiotensin II, apoptosis, carnitine, chronic heart failure, myosin heavy chains, skeletal muscle, sphingosine, TNFα.

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Despite data showing major benefits of medical therapy in terms of reduced mortality in chronic heart failure (CHF), little progress has been made in improving the quality of life in these patients. Quality of life for the majority of patients is limited by two major symptoms: fatigue and shortness of breath. Fatigue is due to skeletal muscle dysfunction rather than to reduced skeletal muscle blood flow [36]: changes in histology [13], mitochondria [7], oxidative enzyme activity [25] and high energy phosphate handling [16]. In patients with CHF there is a reduction of high energy substrates [3]. Minotti [18] showed that there is no reduction in mean force per unit area, implying that myofibril force production was normal, but that loss of skeletal muscle mass is an important determinant of muscle strength. We have shown that in the gastrocnemius of patients with CHF there is a shift from the slow to the fast myosin isoforms [28]. The magnitude of this shift correlates with the severity of CHF. Similar observations were made by Sullivan [26]. It is possible that a high percentage of glycolitic fibres reduces exercise capacity because of the early appearance of anaerobic metabolism, due to the prevalence of fast fatigueable fibres with low anaerobic threshold. Muscle atrophy by itself is a determinant of exercise capacity [15] and the debate on its possible role in CHF is still open. We have found in the leg muscles of rats with experimental CHF [32] an increased number of apoptotic nuclei and a decreased

muscle fibres cross sectional area [4]. The pro-apoptotic factor caspase-3 was significantly increased, while Bcl-2, that is anti-apoptotic, dropped significantly [32]. Apoptosis in the skeletal muscle is paralleled by increased levels of circulating TNFα. This cytokine is known to produce muscle waste either by triggering apoptosis or by activating ubiquitin [11]. In our model ubiquitin seems not to be involved [34]. We think that muscle atrophy is apoptosis-dependent and a similar picture was found in biopsies of the vastus lateralis of patients with severe heart failure where muscle atrophy and apoptosis [1,33] were present. Similarly apoptosis has been detected in the failing human heart [21]. There is a strong inverse correlation between the number of apoptotic myonuclei, the degree of muscle atrophy [1] and peak $VO_2$ [31]. The degree of fibres atrophy correlates with muscle endurance, and exercise capacity, suggesting that muscle strength depends on mass and is in turn a determinant of exercise capacity. Apoptosis is involved in myocyte loss both in CHF [20] and in cardiomyopathies [10]. In the heart apoptosis can be triggered by activation of cytokines, such as TNF-α [11,12] or by hormones, such as AngII [10]. We have shown that apoptosis is accompanied by increased levels of circulating sphingosine, a phospholipid which is the second messenger of TNFα [6,24], and that is able to induce skeletal myocyte apoptosis both in vivo and in vitro [6]. Although clinical trials aimed to block TNFα with specific antibodies [14] or thalidomide [34] have failed, 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 receptors [5].

L-carnitine is a quaternary amine that is fundamental in skeletal muscle metabolism, in that promotes fatty acids oxydation [23], and that produces a selective trophic effect on skeletal muscle fibres [8]. However L-carnitine regulates also gene expression and caspases activity, the activation of which represent the compulsory step for cell death execution [2, 19]. Moreover it has been shown that L-carnitine prevents doxorubicin-induced apoptosis in cardiac myocytes, by inhibiting sphingomyelin hydrolysis and ceramide generation [19]. It seems therefore likely that sphingolipids play a determinant role in inducing apoptosis in the skeletal muscle and it may be argued that programmed cell death may be stopped by blocking the sphingolipid activation cascade.

## Materials and Methods

*In vivo study*

Experimental model

Experiments are approved by the Biological Ethical Committee of the University of Padua, according to the Italian law. Four groups of animals have been studied. Males 80 to 100 gr Sprague Dawley rats with congestive heart failure (CHF) induced by monocrotaline. This alkaloid produces severe pulmonary hypertension followed by right ventricular (RV) failure [4, 29, 32] without inducing by itself changes in skeletal muscle myosin heavy chains (MHCs) composition and apoptosis [29]. Monocrotaline has been injected intraperitoneally in the rats at the dose of 30 mg/kg. Starting at the same day some rats have been treated with substances that could prevent skeletal muscle myopathy. The remaining rats therefore formed the CHF group (CHF). Other age and diet matched rats have been injected with saline and served as controls (Con). After 28 days, when in the monocrotaline treated animals, overt heart failure has developed, rats have been killed and body, heart and tibialis anterior (TA) weight measured. Muscles have been immediately frozen in liquid nitrogen and stored at -80°C. Hearts are stored in 10% formaldehyde solution and blood drawn for SPH, TNFα and AngII measurements.

Assessment of Right Ventricle hypertrophy and failure

To make sure that the monocrotaline treated animals developed RV failure, beyond the post-mortem signs such as pericardial, pleural and peritoneal effusions [29], the following measurements have been 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 formaline fixed transverse sections of the heart taken in the middle portion of the interventricular septum [32].

Electrophoretic separation of Myosin Heavy Chains (MHCs)

We used the method described in details by Vescovo *et al*. [27, 28]. TA muscles are homogenized and solubilized in sodium dodecyl sulfate (SDS) buffer. Analytical SDS page is performed on 7% polyacrylamide slabs with 37.5% vol/vol glycerol to separate MHCs (MHC2a and MHC2b).

Assessment of MHCs distribution

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

Single fibers cross sectional area

We use single fibers cross sectional areas (CSA) as an index of myofibers atrophy [5]. On each muscle a cross cryo-section is taken for histological examination and stained with Hematoxylin-Eosin. The fibers CSA is calculated with a computerized interactive method [5]. At least 400 fibers per specimen are counted at magnification 250X and then averaged.

Assessment of apoptosis

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

In situ nick end labeling of fragmented DNA is performed on cryo-sections using the In Situ Cell Death

Detection Kit, POD. Labeled nuclei are identified from the negative nuclei counter-stained by Hoechst 33258 and counted after being photographed. The total number of positive nuclei is determined by counting (magnification 250X) all the labeled nuclei present in the whole specimen. The number of positive nuclei is then expressed as number of TUNEL positive nuclei/$mm^3$ [4]. TUNEL positive myofibres and interstitial nuclei are 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 are taken as myonuclei [4, 32]. Separate calculations are made for total TUNEL positive nuclei, TUNEL positive myonuclei.

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

Western blot is performed on 12.5 polyacrylamide gels. Anti-Bcl-2 (29kDa) antibodies are used with anti rabbit alkaline-phosphatase. Anti-cleaved Caspase-3 (17 kDa) and anti-cleaved Caspase-9 (37 kDa) are used with anti-rabbit peroxidase-coniugated antibody and revealed by chemiluminescent substrate.

c) Confocal Microscopy immunofluorescence

Frozen sections are incubated for 1 hour at room temperature with anti-dystrophin antibody diluted 1:300 in 1% BSA, which stains sarcolemma. After 3 washings with PBS sections are incubated with anti mouse IgG FITC-conjugated diluted 1:500 at 37°C for 1 hour. The sections are then washed 3 times with PBS and fixed with 4% PFA and incubated with anti caspase 3 antibody diluted 1:10 overnight at 4°C. Slices are then incubated with anti-rabbit Cy3-conjugated antibody for 1 hour at room temperature and analysed by a Bio-Rad Confocal Microscopy.

d) ELISA for apoptosis

DNA ladder assay is performed as follows. 10 muscle cryosections, 20 µm thick, are 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 are added to a final concentration of 2.5% and 1 M, respectively. Samples are centrifuged at 16,000 g for 10 min at 4°C. Protein concentration of supernatant is determined with Bradford and adjusted to 0.01 µg/µl. Cell death ELISA analysis is performed according to manufacturer's instructions (Boehringer Mannheim). After incubation the plates are analyzed with a multi-well ELISA reader.

TNFα determination

TNFα is measured with a solid phase sandwich ELISA, using a monoclonal antibody specific for rat TNFα.

Angiotensin II Assay

AngII is measured on serum, using an enzyme-immunometric assay kit, from SPI-BIO.

SPH determination

For the extraction of sphingolipids, 100-300 µl samples of serum are deproteinized by adding warmed butanol (70°C, 800 µl), vortexing and incubating at 70°C while rocking. The mixture is then placed in a sonicating water bath for 10 minutes. Denatured protein and aqueous phase are separated from the butanol layer by centrifugation at 15,300 x g. The upper butanol layer is transferred into a new extraction tube and saponified by the addition 0.5 M KOH (200 µl). After vortexing, samples are 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 are separated by centrifuging at 15,300 x g for 3 minutes. The butanol layer is transferred to a new tube and dried down using a Savant SpeedVac Plus. Dried samples are completely resuspended in methanol (375 µl) and agitated in a bath sonicator for 2 minutes.

The extracts are then derivatized with O-phthalaldehyde. The derivatized samples (50 µl injection) are separated on a Bio-Rad Hi-Pore Reversed Phase Column RP-318 (4.6 mm I.D. x 250 mm) column with a 1.5 cm Perkin Elmer NewGuard RP-18 guard column. Samples are run on a Bio-Rad HPLC system with a Perkin Elmer fluorescence detector (LS-1)(excitation 340 nm, emission 455 nm). The solvent system is 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.

*In vitro study*

Skeletal muscle cultures

Mouse myogenic C2C12 cell line is cultured in DMEM proliferating medium supplied with 10% fetal calf serum (FCS), 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/$cm^2$. After 3 days of cell culture, FCS is substituted with 2% horse serum (HS) to induce myoblasts fusion and the myotubes formation, which is completed after 7 days.

Cultured myotubes are treated with 1 µM staurosporine or 0.5 µM doxorubicin in fresh serum-containing DMEM to induce apoptosis and harvested for 18 hours [17]. Treatment of cultures with different concentrations of apoptosis inhibitors is initiated 1 hour before staurosporine treatment and is maintained throughout the subsequent incubation. Control experiments are performed by adding each time only the vehicle.

Cell viability is analyzed by the colorimetric XTT assay. For this assay, equal numbers of C2C12 myogenic cells are plated on 96-well plates and maintained in the growing media as above described in order to obtain myotubes. Differentiated myotubes are then incubated with staurosporine (added as a DMSO solution) either with or without apoptosis inhibitors. XTT reagents are eventually added to each well and incu-

bated at 37°C for 4 h. After incubation the plates are analyzed with a multi-well ELISA reader.

The apoptotic features have been analyzed as described for the *in vivo* experiments.

*Statistical analysis*

Student t test for unpaired data will be used. $P \leq 0.05$ will be considered statistically significant. Analysis of variance (ANOVA) will be used when appropriate.

## Results

In the recent years our group has gained a vast experience in the field of the skeletal muscle myopathy in CHF. In fact we have significantly contributed to the understanding of this syndrome [4-6, 28-31, 33]. All the rats with monocrotaline induced CHF showed the presence of right ventricle dilatation, pericardial, pleural and peritoneal effusions at post-mortem examination (Figure 1).

Congestive heart failure is a clinical syndrome characterized by decreased exercise capacity due to early appearance of fatigue and dyspnoea. It has been hypothesized that skeletal muscle in CHF undergoes profound changes leading to the development of a Specific Skeletal Muscle Myopathy that may explain the origin of symptoms: "The muscle hypothesis". Dyspnoea can be caused by an hyperventilatory response to exercise due to exaggerated ergoreflex activity (afferents sensitive to skeletal muscle work). Fatigue is associated with early anaerobic metabolism and lactate accumulation [30, 31]. In other words, patients with CHF show a reduced muscle endurance that is the results of changes in fibres type, with a shift from slow, fatigue resistant oxidative, to fast more fatigable, glycolytic fibres. This shift in fibres type is accompanied by a similar shift of Myosin Heavy Chains (MHC) expression from MHC1 (slow oxidative) to MHC2a (fast oxidative) and MHC2b fast glycolytic [28, 33] (Figure 2). Fibres with fast MHCs possess high speed of shortening and high ATP consumption that make them reach anaerobic threshold early: it is therefore understandable why muscles with higher expression of MHC2a and 2b are more prone to develop fatigue. Measures of muscle fatigability correlate with peak VO2 [30, 31]. Another independent determinant of exercise capacity is muscle atrophy [4, 32]. Measures of muscle bulk and functions are the best predictors of exercise capacity. Triggers for change in fibres type and their relation to muscle atrophy are still poorly understood. It is possible that the impaired oxygen delivery to skeletal muscle due to reduced blood flow and capillary endothelial dysfunction or damage may cause a relative ischaemia which the muscle fibre may adapt to by inducing the preferential synthesis of anaerobic myosin isoforms. Fibres atrophy may be however explained by myocytes apoptosis as we have demonstrated both in man [33] and in an animal model of CHF [4, 5, 29, 32] (Figure 3). Apoptosis is triggered by elevated circulating levels of TNFα and its second messenger sphingosine (SPH) [6] (Table I).

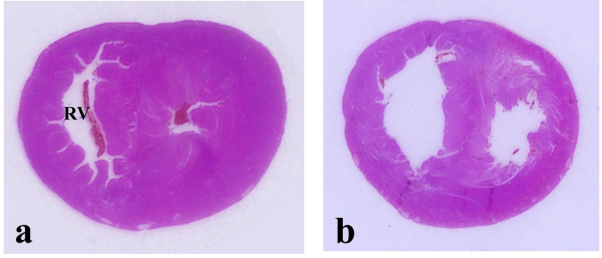


*Figure 1. Transverse sections of heart at level of mid-interventricular septum from control (a) and CHF rat (Heidenheim stain). RV: right ventricle.*

There is strong relationship between the limitation in exercise capacity (VO2) and the degree of skeletal muscle apoptosis, which in turn correlates with myocyte atrophy [33].

*In vitro* experiments have confirmed the link between TNFα, SPH and apoptosis [6]. The shift in MHCs is reversible: six month treatment with ACE inhibitors or Angiotensin II antagonist may improve peak VO2 in man with CHF and is paralleled by restoration of MHCs pattern (Figure 4).

Similarly in an animal model of CHF atrophy and apoptosis can be prevented by blocking the Angiotensin II receptor AT1 [5]. Circulating levels of TNFα and SPH can also be reduced by this treatment (Table II).

## Perspectives

Heart Failure is characterized by a decreased exercise capacity with early appearance of fatigue and dyspnea [13, 35]. These symptoms are in part due to a skeletal muscle myopathy with atrophy, that is responsible for the decreased muscle strength and endurance [31, 35], and shift from the "slow, fatigue resistant" Type I, to the "fast, fatigable" Type II fibres [4, 28, 29], that in turn causes the increased muscle fatigability. Apoptosis has been shown to be one of the major determinants of skeletal muscle atrophy [1, 5, 32], and its magnitude correlates with the severity of the syndrome [1]. Myocyte death is triggered by cytokine activation which is almost always present in Heart Failure as a sign of a chronic inflammatory state [22]. Cytokines, such as TNFα, can induce apoptosis either directly or through secondary messengers, such as the sphingolipid sphingosine (SPH) [11,

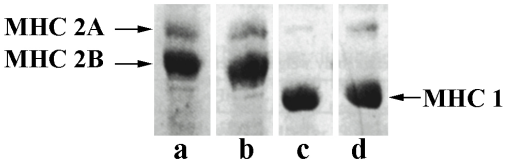


*Figure 2. Electrophoretic separation on polyacrylamide SDS slab-gel of myosin heavy chains (MHC) of rat muscles. Arrows indicate the three isoforms: MHC1 "slow type"; MHC2a "fast oxidative" and MHC2b "fast glycolytic". Key: (a) Extensor digitorum longus (EDL) from control rat. (b) EDL from CHF rat. (c) Soleus from control rat. (d) Soleus from CHF rat.*

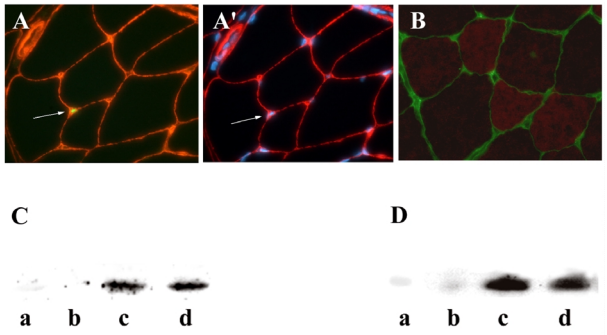


*Figure 3. Evidence of apoptosis in skeletal muscle of CHF rats. (A) TUNEL-laminin staining. (A')TUNEL-Hoechst-laminin staining in a serial section of (A). Arrows in (A) and (A') indicate apoptotic nuclei. (B) Caspase-3 immunostaining. Thin, granulated pattern indicates diffuse cytosolic distribution of caspase-3. (C) Immunoblotting for activated caspase-3. (a) and (b): control. (c) and (d): CHF. (D) Immunoblotting for activated caspase-9. (a) and (b): control. (c) and (d): CHF.*

29]. 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* [5, 6]. Although clinical trials aimed to block TNFα with specific antibodies such as Infliximab or Etanercept have failed [14], 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 [5].

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 [8]. 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]. 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. We will try

*Table I*

| | Apoptotic nuclei (number/mm$^3$) | TNFα (percent of control) | Sphingosine (nmoles/ml) |
|---|---|---|---|
| Control | 1.2 ± 2.8 | 100 | 0.74 ± 0.14 |
| CHF | 39.6 ± 42.7 | 135 | 1.16 ± 0.45 |

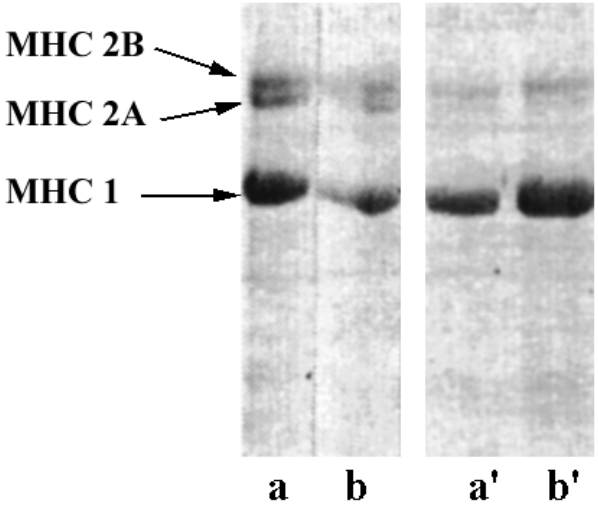


*Figure 4. Electrophoretic separation on polyacrylamide SDS slab-gel of myosin heavy chains (MHC) of human muscles. Arrows indicate the three isoforms: MHC1 "slow type"; MHC2a "fast oxidative" and MHC2b "fast glycolytic". Key: (a) and (b): baseline patients. (c) and (d): after ACE inhibitor treatment.*

to block sphingolipid formation and prevent apoptosis in a well known model of heart failure, the monocrotaline-treated rat, by giving L-carnitine. Parallel experiments will be performed *in vitro* utilizing the C2C12 murine cell line to see if L-carnitine is able to block the staurosporine-induced apoptosis. 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 [31, 35], and to give a clue for the understanding of the pathophysiology of the heart failure myopathy.

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*Table II*

| | TNFα (percent of control) | Sphingosine (nmoles/ml) |
|---|---|---|
| Control | 98 ± 7 | 0.75 ± 0.12 |
| CHF | 145 ± 10 | 1.21 ± 0.40 |
| Irbesartan | 102 ± 9 | 0.72 ± 0.22 |

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