# IS MYOSIN UBIQUITINISATION A MECHANISM OF SKELETAL MUSCLE ATROPHY IN HEART FAILURE?

Luciano Dalla Libera D Chem [a], Giorgio Vescovo MD PhD FESC [b]

[a] *National Reserch Council, Department of Neurosciences, University of Padova, 35100 Padua, Italy.* [b] *Internal Medicine 1, S. Bortolo Hospital 36100 Vicenza Italy*

**Abstract**

Purpose of this paper is to enlighten the mechanisms of muscle wastage in experimental heart failure with particular attention to myosin ubiquitinisation, which is linked to pro-inflammatory cytokines that are also responsible for triggering apoptosis.

In fact the mechanisms leading to muscle wastage in CHF include cytokine-triggered skeletal muscle apoptosis, but also ubiquitin-proteasome and non-ubiquitin-dependent pathways.

The imbalance between protein synthesis and degradation is another important pathway as well. Protein degradation can also occur through ubiquitin dependent and non-ubiquitin dependent pathways. Very recently systems controlling the ubiquitin-proteasome activation have been described: the FOXO-ubiquitin ligase, the NFkB ubiquitin ligase. These are triggered by TNFα and GH-IGF1.

However an important role is played by apoptosis. Both in man and in experimental models of heart failure programmed cell death has been found in skeletal muscle and interstitial cells. Apoptosis is triggered by TNFα and in vitro experiments have shown that it can be induced by its second messenger SPH. Apoptosis correlates with the severity of the heart failure syndrome. It involves caspases 3 and caspases 9 activation and mitochondrial citochrome C release. We have demonstrated in pereviuos papers that apoptosis is involved in muscle atrophy in heart failure, and in this series of experiments we tested the hypothesis that the increased levels of pro-inflammatory cytokines, such as TNFα, can produce myosin ubiquitinisation and selective destruction of contractile proteins with further worsening of muscle atrophy and function.

**Key Words***:* Experimental Heart Failure, Skeletal Muscle, Ubiquitin-proteasome.

*Basic Appl Myol, 15 (5&6): 177-182, 2005*

## INTRODUCTION

It is well known that skeletal muscle can influence exercise capacity and symptoms in heart failure (7, 13). Changes in skeletal muscle composition occurring in heart failure comprise qualitative changes with shift from the slow, aerobic, fatigue resistant Myosin Heavy Chain (MHC)1 toward the fast, glycolytic, MHC2a and MHC2b (27,28,30), decreased mitochondrial synthesis and a blunted oxidative metabolism. Together with these changes muscle bulk, which plays a pivotal role in determining strength, force and endurance, which are in turn linked to exercise capacity as demonstrated by Volterrani (3) and Mancini (16), is significantly reduced. Cardiac cachexia represents the extreme spectrum of the muscle wastage process (2).

The cause of muscle bulk loss has to be searched not only into an imbalance between mechanisms regulating cell hypertrophy and atrophy, where the hypertrophy pathway is repressed and the atrophic one is enhanced (23, 26), but also into the imbalance between intra-cellular protein synthesis and degradation, with reduced synthesis an excessive degradation mediated by the ubiquitin-proteasome pathway. These processes are paralleled by the activation of apoptosis, the cell suicide programme, which leads to a progressive loss of muscle fibre nuclei, which further contributes to the development of muscle atrophy (31,32).

The crucial role of the ubiquitin-proteasome pathway has been demonstrated in several models of muscle atrophy that include starvation, uremia, denervation, sepsis and diabetes mellitus (see 25).

A recent review on cancer cachexia has shown that the increased levels of IL6, TNFα and IFNγ can interfere with the translocation of MyoD which in turns produces an increased expression of E3 ubiquitin ligase.

Ubiquitin binds to myosin and activates the proteasome with myosin degradation (6). The present study was therefore undertaken to investigate whether activation of the ubiquitin-proteasome pathway contributes to muscle atrophy in the syndrome of chronic heart failure as well.

## MATERIALS AND METHODS

### *Experimental model*

Two groups of male 90 to 100 gr Sprague Dawley rats were studied: 5 controls and 10 with CHF induced by monocrotaline. This alkaloid produces severe right ventricular failure that mimics the haemodynamic and neurohormonal pattern of CHF in humans (28). Monocrotaline was injected intraperitoneally at the dose of 30 mg/kg. After 28 days, when the monocrotaline animals developed CHF, rats were killed. Leg muscles were immediately excised and frozen in liquid nitrogen.

Experiments were approved by the University of Padua Biological Ethical Committee.

### *TNFα and SPH determination*

TNFα was measured in serum with a specific solid phase sandwich enzyme-linked immunoabsorbent assay (ELISA) (Euroclone Ltd, UK). Sphingosine was measured in serum as described in (11).

### *Muscle homogenates*

Tibialis Anterior muscles (0.1 g) were homogenized at 4°C in 0.3 ml of Laemli sample buffer containing protease inhibitors cocktail (Complete, Roche) and 25 μM proteasome inhibitor MG 132 (Calbiochem). After heating at 100 C for 10 minutes the protein suspension was centrifuged at 12000 g for 10 min and the surnatants were assayed for protein concentration.

### *Western blot procedure*

One dimensional electrophoresis was carried out on 10% SDS/polyacrylamide. Proteins were transferred to nitrocellulose membranes, stained by Red Ponceau and then probed with the following antibodies: anti ubiquitin, 1:200 (Sigma) and, after stripping, anti-generic-myosin heavy chain (MHC), 1:10000 (generous gift of Prof. L. Gorza). After reaction with the appropriate secondary antibodies, the blots were revealed using a chemiluminescence detection system.

Densitometry was performed on scanned gels using an NIH image system and the relative percentage of myosin and ubiquitinated myosin was determined. The ration between anti ubiquitin stained myosin and anti MHC stained myosin was taken as an index of ubiquitinisation.

### *Statistical analysis*

The student t-test for unpaired data was applied to see differences between the two groups.

A 5% difference was considered statistically significant.

## RESULTS

CHF animals shower higher levels of both circulating TNFα and SPH, confirming our previous experiments in which we showed the increased levels of this cytokine in heart failure (Table 1).

*Table 1. Serum features of controls and CHF rats. # P< 0.02; * P< 0.04*

| | TNFα (pg/ml) | SPH (nmoles/ml) |
|---|---|---|
| CON | 110±15 # | 0.65 ± 0.12 * |
| CHF | 250 ± 62 # | 1.40 ± 0.35 * |

On the blots of muscle homogenates we could find higher levels of ubiquitinization in the heart failure animals (Figure 1). The ubiquitinised Myosin/anti MHC ratio was in fact higher in the CHF rats (Figure 2).

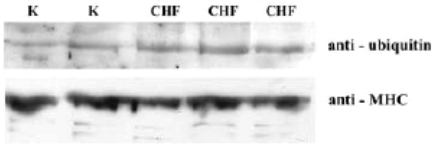


***Figure 1.*** *Myosin ubiquitination in the skeletal muscle of the monocrotaline treated rats with heart failure. K: Control rats. CHF: Heart Failure rats. Top lane: immunoblot with antibodies against ubiquitin. Bottom lane: immunoblot with antibodies against MHC.*

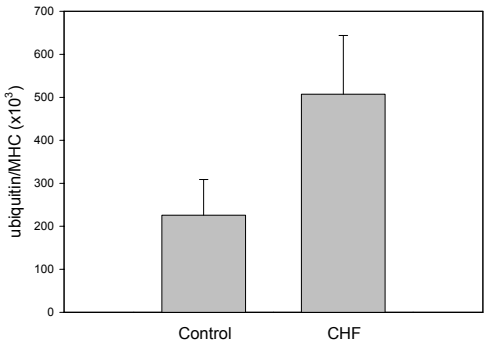


***Figure 2.*** *Quantitation of blot presented in Figure 1. P<0.05.*

## DISCUSSION

There are at least two pathways by which muscle proteins can be degraded:

a) Ubiquitin dependent protein wastage

This process involves protein ubiquitinisation and requires activation of the ubiquitin proteasome system. The process is very much the same of that described for cancer cachexia (6). We have recently demonstrated in the monocrotaline model of right heart failure that fibres atrophy is accompanied by myosin ubiquitinisation (Figure 1 and 2).

Myosin, once bound to ubiquitin, is taken into the proteasome and degraded. It is therefore clear that this mechanisms contributes to the loss of myofibrils and further depresses muscle contractile efficiency. The previously mentioned IGF1-AKT-FOXO-induced system which leads to activation of atrogin-1 (ubiquitin ligase) (23, 26), and the NF-kB induced ubiquitin ligase are other two ubiquitin-dependent protein wastage pathways. This latter one (18) is mediated by the NFkB activation via a TNFα-related pathway. NFkB induces MuRF1 transcription and generation of E3 ubiquitin ligases (Figure 3).

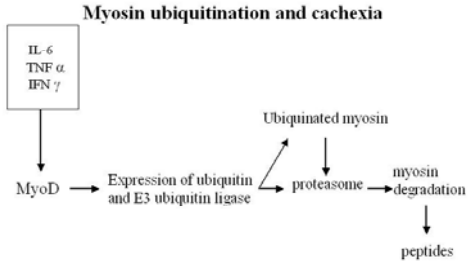


***Figure 3***

Locally acting Insulin-Like Growth Factor 1 inhibits ubiquitin mediated muscle atrophy in chronic left ventricular dysfunction, and this is due to the block of expression of FOXO and ubiquitine ligase, atrogin 1/MAFbx (25).

b) Ubiquitin independent protein wastage

The systems involved in the ubiquitin independent protein wastage are three: the lisosomial, calpain, and caspases systems. The first two are not TNFα related, while the last one is (4).

Recent studies have identified the importance of proinflammatory mediators in the development and progression of heart failure (2, 14, 20). The growing appreciation of the pathophysiological consequences of sustained expression of proinflammatory mediators in preclinical and clinical heart failure models even culminated in a series of multicenter clinical trials that used "targeted" approaches to neutralize TNFα in patients with moderate to advanced heart failure. The portfolio of cytokines that will constitute the focus of inflammation in Heart Failure (CHF) is given by TNF and the interleukin (IL)-1 family (including the recently described member IL-185) and the IL-6 family of cytokines (17). These molecules have been referred to as proinflammatory cytokines, insofar as they were traditionally thought to be derived exclusively from the immune system and were therefore considered to be primarily responsible for mediating inflammatory responses in tissues. However, these inflammatory mediators are now known to be expressed by all nucleated cell types residing in the myocardium, including the cardiac myocyte, thus suggesting that these molecules may do more than simply orchestrate inflammatory responses in the heart. There is increasing evidence that inflammatory mediators play an important role in skeletal muscle wasting and fatigue in a number of clinical settings, including HF. Although the exact mechanisms that are responsible for the expression of pro-inflammatory cytokines in skeletal muscle are not known, it is likely that oxidative stress (5) is an important upstream signal that activates the proinflammatory cascade. Oxidative stress is sufficient to activate nuclear factor kappa-B (NF-KB), an important transcription factor for pro-inflammatory cytokine gene expression.

Several potential triggers for oxidative stress in skeletal muscle have already been identified, including strenuous muscle exercise, muscle injury, ischaemia etc. Inflammatory mediators such as TNFα can provoke apoptosis in skeletal myotubes (19). In experimental models of HF, circulating levels of TNFα are independently correlated with the number of apoptotic myocyte nuclei in tibialis anterior muscle (11). However, this effect appears to be more prominent in fast-twitch than in slow-twitch muscle fibres (12). Studies in patients with HF have yielded similar findings. For example, apoptosis was detected in approximately 50% of patients with HF, whereas skeletal muscle apoptosis was not detected in healthy subjects (1). Patients with apoptosis-positive skeletal muscle myocytes exhibited a significantly decreased maximal exercise capacity compared with patients with apoptosis-negative biopsies (1, 31). Similar findings were reported by Vescovo et al. (31) who reported that peak oxygen consumption was negatively correlated with the number of terminal deoxynucleotidyl-transferase-mediated UTPend labeling-positive nuclei and the skeletal muscle fiber cross-sectional area in patients with HF. Muscle weakness often can also occur in inflammatory diseases without the overt loss of muscle protein (24). Previous studies indicate that cytokines in general, and TNFα in particular, can lead to contractile dysfunction in striated muscle (8, 15). Although the full spectrum of mechanisms that are responsible for inflammation-induced contractile

dysfunction in skeletal muscle are not known, recent studies have suggested an important role for ROS (8). In a transgenic mouse model that overexpresses TNFα, it has been shown that there was a profound weakening of diaphragm muscle force generation that was accompanied by evidence of increased cytosolic oxidative stress (21, 22).

If pro-inflammatory cytokines can produce apoptosis they are also able to produce protein wastage.

However protein degradation can also be a non TNF-ubiquitin-dependent process.

In this series of experiments we have found that the pro-inflammatory cytokine TNFα is increased as it is its second messenger SPH. These cytokines were able to trigger the ubiquitine proteasome pathway and to induce myosin ubiquitinization as demonstrated by the increased Ubiquitinizated myosin anti-MHC ratio. This becomes another important mechanism of myofibrillar wastage and muscle loss. But this is not the only consequence of ubiquitinisation. In fact there are some more consequences of this process and they are mainly functional. Muscle loss and atrophy are per se a cause of decreased muscle performance and force development, but more importantly myosin ubiquitinisation can produce an even worse impairment of muscle efficiency. This is similar to what happens with contractile protein peroxidation which we have demonstrated to occur in CHF and that we have been shown to correlate with skeletal muscle contractile deficiency (8).

This is off course not the only mechanism of skeletal muscle wastage in CHF: In fact, by using the monocrotaline treated rat as a model of heart failure syndrome, we have previously demonstrated an increase in interstitial and myocyte apoptosis in tibialis anterior (TA) muscle (11, 12). In this model when heart failure appears, TNFα plasma levels increase progressively. This increase in TNFα is accompanied by a parallel increase of a sphingolipid, sphingosine (SPH), which is a second messenger of TNFα itself (11). This phenomenon is paralleled by the rise in number of apoptotic nuclei in the soleus and tibialis anterior muscles. Apoptosis does not however occur only in the muscle fibres. When we stained the basal lamina with laminin, we observed that apoptosis occurred also in the interstitial nuclei, that are mainly endothelial capillary cells. The ratio between apoptotic muscle and interstitial cells is about one in two. Apoptosis was accompanied by the expression of activated caspase 3 and 9 at cytosolic level, as demonstrated by the immunoblotting and by the immunostaining/confocal microscope (10) experiments. We have also shown that myocyte apoptosis is ultimately responsible for muscle atrophy, which occurs simultaneously with the rise in the number of apoptotic cells. In fact a statistically significant correlation exists between levels of TNFα, number of apoptotic nuclei and cross sectional area of muscle fibres (11, 12, 31, 32). In the tibialis anterior muscle, a typical fast-twitch muscle, apoptosis occurs non-selectively in both type IIa and IIb fibres, as demonstrated by triple staining with terminal transferase dUTP nickend labelling (TUNEL), antibodies against myosin heavy chain (MHC)2b and 2a and laminin (32).

We also demonstrated the occurrence of apoptosis in the soleus, a typical "slow muscle" (12).

The in vivo observation has been confirmed by in vitro experiments. In fact by adding on cultured myotubes increasing doses of SPH we obtained a dose-dependent number of apoptotic cells (11).

Mitochondria are involved in the apoptotic process. In fact we observed in the muscle of rats with heart failure and increased number of apoptotic cells, a dramatic release of cytochrome c into the cytoplasm, which can be attributed to the mitochondrial damage. This increase in cytosolic cytochrome C could be prevented, by Carnitine (29), ATII receptor antagonists (9) and GH (10) administration. The favourable effect obtained by these drugs on cytochrome c was paralleled by decreased levels of activated Caspases 3 and 9 and by reduced number of apoptotic nuclei. Muscle fibres cross sectional area, which is a precise index of muscle atrophy, was preserved.

The degree of apoptosis correlates with the severity of the disease. The more severe heart failure, the higher the number of apoptosis: This has been shown to be true both in the experimental models of CHF and in man. In fact in rats treated with monocrotaline the number of skeletal muscle apoptosis correlates directly with the degree of right ventricle dilatation, while in man apoptosis correlates inversely with peak oxygen consumption.

## Conclusion

It is known that both in man and in experimental models of heart failure programmed cell death is one of the causes of muscle wastage and atrophy. TNFa and SPH can trigger apoptosis, but they can also activate the ubiquitine proteasome pathway, as demonstrated in this paper. Myosin ubiquitinization can lead to further myosin degradation and atrophy. Muscle function, one of the most important determinants of exercise capacity and symptoms in CHF, is therefore even more profoundly impared.

## Address for correspondence:

Luciano Dalla Libera, CNR Istituto di Neuroscienze, Dipartimento Scienze Biomediche Sperimentali, Viale G: Colombo 3, 35100 Padova (Italy).
Tel +39 049 8276031 Fax + 39 049 827 6040
Email ldl@bio.unipd.it

## REFERENCES

[1] Adams V, Jiang H, Yu J, et al. Apoptosis in skeletal myocytes of patients with chronic heart failure is associated with exercise intolerance. *J Am Coll Cardiol* 1999; 33: 959-65.

[2] Anker SD, Rauchhaus M. Insights into the pathogenesis of chronic heart failure: immune activation and cachexia. *Curr Opin Cardiol* 1999; 14: 211-6.

[3] Anker SD, Swan JW, Volterrani M. et al. The influence of muscle mass, strength, fatigability and blood flow on exercise capacity in cachectic and non-cachectic patients with chronic heart failure. *Eur Heart J.* 1997 18(2): 259-69.

[4] Attaix D, Ventadour S, Codran A. et al. The ubiquitin-proteasome system and skeletal muscle wasting. Essays Biochem. 2005; 41: 173-86.

[5] Baeuerle PA, Baltimore D. NF-kB: ten years after. *Cell* 1996; 87: 13-20.

[6] Chamberlain JS. Cachexia in cancer zeroing in on myosin. *New Engl J Med* 2004; 351-20: 2124-5.

[7] Coats AJS, Clark AL, Piepoli M et al. Symptoms and quality of life in heart failure. The muscle hypothesis. *Br Heart J* 1994; 72: 36-9.

[8] Dalla Libera L, Ravara B, Gobbo V. et al. Skeletal muscle myofibrillar protein oxidation in heart failure and the protective effect of carvedilol. *J Mol Cell Cardiol* 2005; 38: 803-807.

[9] Dalla Libera L, Ravara B, Angelini A. et al. Beneficial effects on skeletal muscle of the Angiotensin II Type 1 receptor blocker Irbesartan in experimental heart failure. *Circulation* 2001; 103: 2195-200.

[10] Dalla Libera L, Ravara B, Volterrani M. et al. Beneficial effects of Gh/IGF-1 on skeletal muscle atrophy and function in experimental heart failure. *Am J Physiol Cell Physiol* 2004; 286: C138-C144.

[11] Dalla Libera L, Sabbadini R, Renken C, et al. Apoptosis in the skeletal muscle of rats with heart failure is associated with increased serum levels of TNF-alpha and sphingosine. *J Mol Cell Cardiol* 2001; 33: 1871-8.

[12] Dalla Libera L, Zennaro R, Sandri M. et al. Apoptosis and atrophy in rat slow skeletal muscles in chronic heart failure. *Am J Physiol* 1999; 277: C982-6.

[13] Drexler H, Riede U, Munzel T et al. Alterations of skeletal muscle in chronic heart failure. *Circulation* 1992; 85: 1751-9.

[14] Farber MO, Mannix ET. Tissue wasting in patients with chronic obstructive pulmonary disease. *Neurol Clin* 2000; 18: 245-62.

[15] Li X, Moody MR, Engel D, et al. Cardiac-specific overexpression of tumor necrosis factoralpha causes oxidative stress and contractile dysfunction in mouse diaphragm. *Circulation* 2000; 102: 1690-6.

[16] Mancini DM, Reichek N, Chance B. et al Contribution of skeletal muscle atrophy to exercise intolerance and altered muscle metabolism in heart failure. *Circulation* 1992; 85: 1364-73.

[17] Mann D, Reid M. Exercise training and skeletal muscle inflammation in chronic heart failure: feeling better about fatigue. *J AmColl Cardiol* 2003; 42: 869-72.

[18] McKinnell IW, Rudnicki MA. Molecular Mechanisms of muscle atrophy. *Cell*, 2004, 119: 907-10.

[19] Meadows KA, Holly JM, Stewart CE. Tumor necrosis factor-alpha induced apoptosis is associated with suppression of insulin-like growth factor binding protein-5 secretion in differentiating murine skeletal myoblasts. *J Cell Physiol* 2000; 183: 330-7.

[20] Moldawer LL, Sattler FR. Human immunodeficiency virus-associated wasting and mechanisms of cachexia associated with inflammation. *Semin Oncol* 1998; 25: 73-81.

[21] Reid MB. Invited review: redox modulation of skeletal muscle contraction: what we know and what we don't. J Appl Physiol 2001; 90: 724-31.

[22] Reid MB, Li YP. Cytokines and oxidative signalling in skeletal muscle. *Acta Physiol Scand* 2001; 171: 225-32.

[23] Sandri M, Sandri C, Gilbert A. et al. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell.* 2004 117(3): 399-412

[24] Simon AM, Zittoun R. Fatigue in cancer patients. *Curr Opin Oncol* 1999; 11: 244-9.

[25] Schulze PC, Fang J, Kassik KA. Et al. Transgenic overexpression of locally acting insulinlike growth factor-1 inhibit ubiquitin-mediated muscle atrophy in chronic left-ventricular dysfunction. *Circ Res*, 2005, 97: 418-26.

[26] Skurk C,Izumiya Y, Maatz H. et al.. The FOXO3 a transcription factor regulates cardiac myocyte size downstream of AKT signaling. *J Biol Chem*. 2005 280(21): 20814-23.

[27] Vescovo G, Ceconi C, Bernocchi P. et al. 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.* 1998 39(1): 233-41.

[28] Vescovo G, Dalla Libera L, Serafini F et al. Improved exercise tolerance after losartan and enalapril in heart failure: correlation with changes in skeletal muscle myosin heavy chain composition. *Circulation* 1998; 98: 1742-9.

[29] Vescovo G, Ravara B, Gobbo V. et al. L-carnitine: a potential treatment for blocking apoptosis and preventing skeletal muscle myopathy in heart failure. *Am J Physiol Cell Physiol* 2002; 283: C802-10.

[30] Vescovo G, Serafini F, Facchin L, et al. 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*. 1996 76(4): 337-43.

[31] Vescovo G, Volterrani M, Zennaro R. et al. Apoptosis in the skeletal muscle of patients with heart failure: investigation of clinical and biochemical changes. *Heart* 2000; 84: 431-7.

[32] Vescovo G, Zennaro R, Sandri M. et al. Apoptosis of skeletal muscle myofibers and interstitial cells in experimental heart failure. *J Mol Cell Cardiol* 1998; 30: 2449-59.