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# Impact of exercise training on myostatin expression in the myocardium and skeletal muscle in a chronic heart failure model

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**Aims** In late-stage chronic heart failure (CHF), elevated cytokines and cachexia are often observed. Several studies have shown that exercise training exerts beneficial effects on skeletal muscle in this setting. Furthermore, it has been shown that the expression of myostatin, a key regulator of skeletal muscle mass, is increased in a variety of cachectic states. This study aimed to investigate the expression of myostatin in CHF, the influence of exercise training on myostatin levels, and regulation of myostatin by tumour necrosis factor-α (TNF-α).

**Methods and results** In an animal model of CHF (LAD-ligation model), protein expression of myostatin was elevated 2.4-fold in the skeletal muscle and more than four-times in the myocardium, compared with control (Co). Exercise training on a treadmill over 4 weeks led to a significant reduction in myostatin protein expression in the skeletal muscle and the myocardium of CHF animals, with values returning to baseline levels. In differentiated C2C12 cells, TNF-α induced the expression of myostatin through a p38MAPK-dependent pathway involving nuclear factor kappa-B (NF-κB). The increased TNF-α mRNA levels in the skeletal muscle of CHF animals correlated significantly with myostatin expression.

**Conclusion** These alterations in myostatin expression in the skeletal and heart muscle following exercise training could help to explain the beneficial anti-catabolic effects of exercise training in CHF.

**Keywords** Myostatin • Exercise training • Chronic heart failure • Heart muscle • Skeletal muscle • TNF-α

## Introduction

Chronic heart failure (CHF) is a major health problem worldwide and its prevalence continues to increase. There is now considerable evidence to suggest that neurohormonal and immune mechanisms may play a central role in the pathogenesis of this syndrome. It has been suggested that when cytokines are expressed at sufficiently high concentrations, as has been shown to occur in heart failure,[1] they may spill over into the circulation and exert endocrine effects.[2] Local inflammation precedes this systemic cytokine release, with local factors like tissue hypoperfusion and oxidative stress as the initial proinflammatory stimulus. Therefore, the local expression of tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) is significantly increased in skeletal muscle biopsies of CHF patients with only mildly elevated serum cytokines.[3]

In the terminal phase of CHF, cachexia is often observed and is associated with a severely reduced quality of life and a poor prognosis. According to data from Anker and Coats, using a weight loss of more than 7.5% over a period of at least 6 months as a cut-off, 16% of CHF patients will be diagnosed as cachectic.[4]

To date, no treatments are available for cardiac cachexia. Great hopes were placed on specific anti-cytokine therapies. However, large scale studies had to be stopped partly because initial analyses indicated an increase in mortality in patients on active treatment.[5]

Interest is now moving towards novel targets to stop muscle wasting and to decrease mortality rates. It has been shown that the expression of myostatin, a key regulator of skeletal muscle mass, is increased in a variety of cachectic states. First evidence came from studies shows that elevations in myostatin expression correlated highly with induced states of muscle loss, such as hind

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limb unloading, AIDS, and steroid treatment.[6–8] Moreover, systemic administration of this negative growth regulator leads to muscle wasting in mice.[9]

Myostatin is a member of the transforming growth factor-β family and is predominantly expressed in skeletal, but also in heart muscle.[10,11] Early studies suggested that myostatin may play an important role in cardiac function. Sharma *et al.*[11] demonstrated that myostatin expression is up-regulated in cardiomyocytes after the induction of myocardial infarction. Cook *et al.*[12] reported that myostatin was dramatically up-regulated in hypertrophied hearts with transgenic over-expression of Akt; a serine–threonine kinase with anti-apoptotic effect. In a rat model of volume overload heart failure, myocardial myostatin mRNA and protein expression were upregulated.[13]

It is known that exercise training has anti-inflammatory effects, with the potential to reduce local cytokine expression and increase anti-apoptotic factors.[3] These beneficial metabolic effects induce an improvement in exercise capacity. The role of myostatin in this setting remains unclear. Exercise training has shown inconsistent effects on myostatin expression in healthy volunteers, dependant on the muscle type and the form of exercise.[14–18] However, the role of myostatin in CHF and the effects of exercise training on myostatin levels have not been elucidated so far.

Therefore, the aim of the present study was to evaluate myostatin expression and the impact of exercise training on myostatin levels, in an established rat model of CHF. In addition, the effect of TNF-α on the expression of myostatin and possible intracellular pathways was investigated in a cell culture model.

# Methods

## Rat model of chronic heart failure

Wistar Kyoto rats underwent LAD-ligation to induce CHF ($n = 26$) or sham operation control (Co) ($n = 26$) as described previously.[19] CHF was verified by echocardiography 1 month after the operation. Following confirmation of the presence of CHF, the two groups were divided into either an exercise training group (CHF-E; $n = 13$/Co-E; $n = 13$) or a sedentary group (CHF; $n = 13$/Co; $n = 13$). Rats allocated to the exercise groups were trained for 4 weeks on a treadmill (2 sessions/day, each lasting 30 min). The speed of the treadmill was 30 m/min. After 8 weeks, the animals were sacrificed and the gastrocnemius muscles were collected. The heart was dissected out and in CHF animals remote parts of the infarcted myocardium were used. The harvested tissue was immediately snap frozen in liquid nitrogen and stored at −80°C.

The investigation conforms the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The local Council for Animal Research approved the experimental protocols.

## Echocardiographic assessment of left ventricular function

Echocardiographic measurements were performed using a 12 MHz transducer connected to a Hewlett–Packard Sonos-5500. A short-axis two-dimensional image-guided M-mode view of the left ventricle was acquired. Left ventricular end-diastolic and end-systolic dimensions were measured in the M-mode tracing in the parasternal short axis according to the leading-edge technique. Measurements from five consecutive cardiac cycles were averaged and used for further analysis. Fractional shortening (FS) was calculated according to the formula: $FS = [(LVEDD - LVESD)/LVEDD] \times 100$. Left ventricular end-diastolic and end-systolic volumes and ejection fraction were calculated in the parasternal long-axis view using the disk method.

## RNA isolation and quantification of mRNA expression

Total RNA was isolated from the gastrocnemius muscle and heart tissue using RNeasy (Qiagen, Hilden, Germany). One hundred nanograms of total RNA were reverse transcribed into cDNA using random hexamers and Sensiscript reverse transcriptase (Qiagen). An aliquot of the cDNA was used for quantitative RT-PCR applying the Light Cycler system (Roche Diagnostics Inc.). For the detection of myostatin, specific primers and internal probes were used. The expression of specific genes was normalized to the expression of 18S rRNA. The following primers and conditions were used: 18S rRNA (5′-ATACAGGACTCTTTCGAGGCCC-3′; 5′-CGGGACACTCAGCTAAGAGCAT-3′; 61°C annealing); myostatin (5′-GTCTTCACATCAATACTCTGCCA-3′; 5′-CATGCCTACCGAGTCTGACTT-3′ 55°C annealing); myostatin probes (5′-LC640-GTGCAAATCCTGAG ACTCATCAAACCCATG–PH-3′; 5′-GAGAGCCGTCAAGACT CC TACAACAGTGT–FL-3′).

For the detection of TNF-α, specific primers (5′-GGTCCCAACAAGGAGGAGAAGT-3′; 5′-GGTTGTCTTTGAGATCCATGCC-3′) in conjunction with a specific TaqMan-probe (5′-6FAM-ACGTGGG CTACGGGCTTGTCACT–BBQ) were used.

## Quantification of protein expression

Frozen tissue samples were homogenized in lysis buffer[20] and western blot analysis was performed as described previously.[21,22] Myostatin protein expression was quantified using specific antibodies (R&D Systems, Heidelberg, Germany). To compensate for blot-to-blot variations, an internal standard was loaded on each SDS–polyacrylamide gel, and the densitometry results were expressed as the ratio between the sample and the standard intensity. Loading differences were controlled by re-probing the blot with an antibody against GAPDH (Hytest, Turku, Finland). All samples were analysed in duplicate.

## Cell culture studies

Mouse C2C12 myoblasts (ATCC CRL-1772) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Biochrom KG, Berlin, Germany) supplemented with 10% foetal calf serum (FCS) (Biochrom KG), 100 U/mL penicillin, and 100 μg/mL streptomycin. For differentiation into myotubes, myoblasts were grown until they reached 60–80% confluence, then the medium was changed to DMEM supplemented as above with 2% horse serum instead of FCS. After 4 days in differentiation medium, cells were incubated with different concentrations of TNF-α (5–100 ng/mL) (R&D Systems) in serum-free media for up to 24 h. Experiments were repeated using IL-1β (5–100 ng/mL) (Upstate Biotechnology, Lake Placid, New York) or IL-6 (5–100 ng/mL) (Sigma, St Louis, MO, USA) instead of TNF-α. Myostatin expression was quantified by qRT–PCR and western blot. To elucidate the signal transduction pathways involved in the TNF-α-mediated activation of myostatin, the cells were pre-incubated for 1 h with the following specific inhibitors before the addition of TNF-α: SB203580 (10 μM; Calbiochem, La Jolla, CA, USA) a highly specific, cell-permeable inhibitor of p38MAPK;[23] PD98059 (50 μM; Calbiochem), a specific blocker for the activation of p42/44MAPK;[24]

pyrrolidine dithiocarbamoate (PDTC, Sigma), an antioxidant often used to inhibit the activation of nuclear factor kappa-B (NF-κB).[25]

### Statistical analysis

Values are given as mean ± SEM for all variables. Inter-group comparisons were performed with an unpaired *t*-test or a one-way ANOVA, where appropriate. A probability value of $<0.05$ was considered statistically significant.

## Results

### Echocardiography

End-diastolic diameter (EDD), end-systolic diameter (ESD), end-diastolic volume (EDV), and end-systolic volume (ESV) were significantly enlarged in the CHF rats compared with the controls, consistent with severe left ventricular dilation as a result of the remodelling process. Fractional shortening and ejection fraction (LVEF) were severely impaired in the CHF rats, indicative of a blunted contractile function (*Table 1*). In the CHF-E group, there was a significant improvement in FS and LVEF as well as a reduction in ESD, EDV, and ESV in comparison to the sedentary CHF group.

### Expression of myostatin in skeletal muscle after induction of heart failure

In the gastrocnemius muscle of animals with CHF, the mRNA expression of myostatin (*Figure 1A*) was not significantly increased as compared with controls (Co: 6.1 ± 1.2 vs. CHF: 7.3 ± 1.5 arbitrary units; $P$ = ns). In contrast, protein expression of myostatin was elevated by 140% in animals with CHF as compared with controls (Co: 0.87 ± 0.1 vs. CHF: 2.09 ± 0.5 arbitrary units; $P < 0.01$) (*Figure 1B*).

### Expression of myostatin in heart muscle after induction of heart failure

Animals with CHF expressed slightly higher mRNA levels of myostatin (*Figure 2A*) in the myocardium compared with controls, although this did not reach significance (Co: 1.6 ± 0.2 vs. CHF: 4.3 ± 1.9 arbitrary units; $P$ = ns). However, protein expression of myostatin was more than four times higher in the heart muscle of animals with CHF compared with controls (Co: 2.5 ± 0.7 vs. CHF: 11.7 ± 3.4 arbitrary units; $P < 0.01$) (*Figure 2B*).

**Table 1 Echocardiographic characteristics of the animals**

| | Co | Co-E | CHF | CHF-E |
|---|---|---|---|---|
| EDD (mm) | 5.1 ± 0.2 | 4.7 ± 0.1 | 8.6 ± 0.5* | 7.4 ± 0.4* |
| ESD (mm) | 2.7 ± 0.1 | 2.4 ± 0.1 | 7.1 ± 0,6* | 5.6 ± 0.4*$ |
| EDV (μL) | 203 ± 10 | 178 ± 9 | 602 ± 67* | 368 ± 40*$ |
| ESV (μL) | 56 ± 4 | 47 ± 3 | 389 ± 60* | 173 ± 27*$ |
| FS (%) | 47 ± 1 | 46 ± 1 | 18 ± 3* | 25 ± 2*$ |
| LVEF (%) | 72 ± 1 | 75 ± 1 | 32 ± 4* | 50 ± 3*$ |

EDD, end-diastolic diameter; ESD, end-systolic diameter; EDV, end-diastolic volume; ESV, end-systolic volume; FS, fractional shortening; LVEF, left ventricular ejection fraction
*$P < 0.05$ vs. Control, $^{\$}P < 0.05$ vs. CHF.

### Impact of tumour necrosis factor-α, interleukin-1β, and interleukin-6 on the expression of myostatin

It is known that CHF leads to elevated concentrations of TNF-α in skeletal muscle biopsies. To verify whether TNF-α has a direct stimulatory effect on myostatin expression, C2C12 myocytes were incubated with TNF-α, and myostatin expression was determined. The optimal concentration of TNF-α in the C2C12 cell culture was evaluated in screening experiments, which showed that 50 ng/mL of TNF-α was the lowest concentration able to significantly induce myostatin expression in C2C12 myocytes (*Figure 3A*). Incubation of skeletal muscle myotubes with 50 ng/mL TNF-α for a period of 24 h resulted in an eight-fold increase in myostatin protein content (control: 0.16 ± 0.02, TNF-α: 1.4 ± 0.3 arbitrary units; $P < 0.001$ vs. control) (*Figure 3B*). There was no dose-dependent effect of IL-6, but there was a trend for the 10 and 50 ng/mL concentrations of IL-1β to increase the myostatin mRNA expression (data not shown).

To elucidate the signalling pathway involved in the TNF-α-mediated activation of myostatin expression, cells were stimulated with TNF-α in the presence or absence of selective inhibitors. Pre-incubation of the C2C12 myocytes with SB203580 and PDTC blunted the TNF-α induced the up-regulation of myostatin. However, the selective p42/44 MAPK inhibitor PD98059 was unable to prevent of the increase in myostatin induced by TNF-α (*Figure 4*).

### Expression of tumour necrosis factor-α in skeletal muscle after induction of heart failure

There was an increased expression of TNF-α mRNA in the gastrocnemius muscle of CHF rats compared with controls (Co: 2.4 ± 0.3 vs. CHF: 4.3 ± 0.6 arbitrary units; $P < 0.05$) (*Figure 5A*). This increased TNF-α level correlated significantly with myostatin expression ($r = 0.57$; $P < 0.01$; $n = 11$ in each group) (*Figure 5B*).

### Impact of exercise training on myostatin expression in chronic heart failure

In the CHF-E group, the protein expression of myostatin in the gastrocnemius muscle was reduced by 55% as compared with the sedentary CHF animals (CHF: 2.1 ± 0,5 vs. CHF-E: 0.95 ± 0.2 arbitrary units; $P < 0.05$) (*Figure 6A*). A similar effect was observed in the heart muscle, with the exercise-trained CHF animals showing a five-fold reduction in myostatin protein expression compared with the sedentary CHF animals (CHF: 11.7 ± 3.4 vs. CHF-E: 2.3 ± 1.0 arbitrary units; $P < 0.01$) (*Figure 6B*). There were no differences in myostatin protein expression in the heart or skeletal muscle between the sedentary or exercise-trained control animals (*Figure 6A* and *B*).

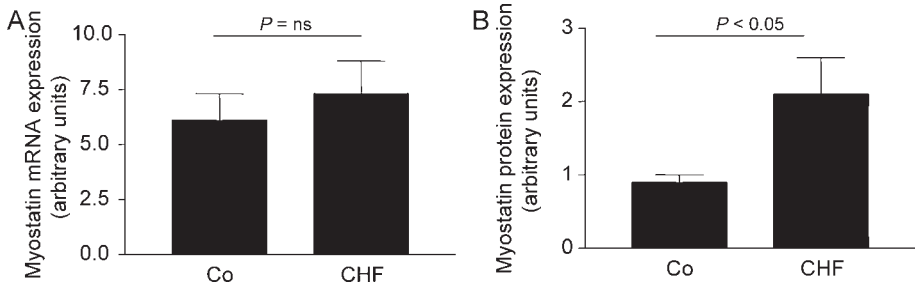


**Figure 1** Quantification of myostatin mRNA (*A*) and myostatin protein expression (*B*) in the gastrocnemius muscle of sham-operated animals (Co, $n = 13$) and rats with CHF ($n = 13$). The values are depicted as a ratio of the expression of 18S rRNA or GAPDH. Results are expressed as mean ± SEM.

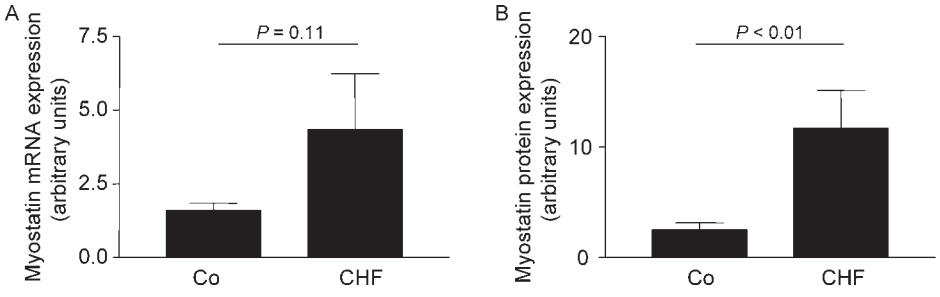


**Figure 2** Quantification of myostatin mRNA (*A*) and myostatin protein expression (*B*) in the heart muscle of sham-operated animals (Co, $n = 13$) and rats with CHF ($n = 13$). The values are depicted as a ratio of the expression of 18S rRNA or GAPDH. Results are expressed as mean ± SEM.

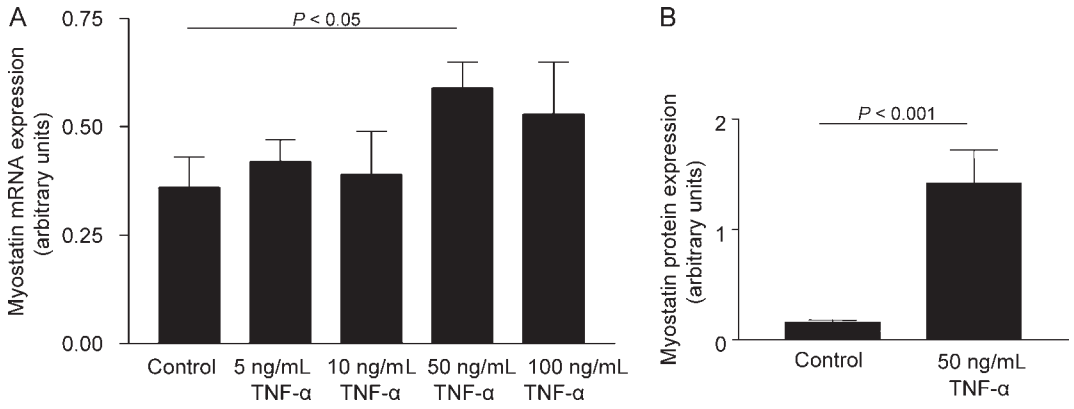


**Figure 3** (*A*) Expression of myostatin mRNA with increasing concentrations of TNF-α in a cell culture model of differentiated C2C12 cells compared to control. (*B*) Protein levels of myostatin in a cell culture model of differentiated C2C12 myocytes. TNF-α induces the expression of myostatin. Results are expressed as mean ± SEM.

# Discussion

Cardiac cachexia is often observed in the terminal stages of CHF and is associated with a severely reduced quality of life and a poor prognosis. Myostatin, a key regulator of muscular growth, is increased in patients with cachexia and muscle wasting, for example, due to HIV infection.[8] However, the role of myostatin in CHF is less clear. Three important messages emerge from this study. First, myostatin is significantly up-regulated in the myocardium and skeletal muscle after induction of CHF. Secondly, the

proinflammatory cytokine TNF-α has the potency to induce the expression of myostatin in C2C12 skeletal muscle myocytes via the activation of p38MAPK and NF-κB. Thirdly, in CHF exercise training leads to a reduction in myostatin to normal levels in heart and skeletal muscle.

## Chronic heart failure and the expression of myostatin in heart and skeletal muscle

Only 2 years after the first description of myostatin in skeletal muscle, Sharma *et al.*[11] reported that this key regulator of muscle growth could also be detected in foetal and adult heart tissue, but at very low concentrations. In a study using a rat model of volume-overload heart failure induced by an aorto-caval shunt, myocardial myostatin mRNA and protein expression were up-regulated.[13] Myostatin expression was increased 2.7-fold at 4 weeks. Interestingly, five of the eight animals with volume-overload CHF developed skeletal muscle wasting, as assessed by the cross-sectional area of the semi-membranous muscle in the rat hind limb, and this effect was reversed with carvedilol.[13] In the present study, we also observed a significant increase in myostatin protein expression in heart and skeletal muscle after LAD-ligation in a rat model of ischaemic cardiomyopathy. These results are in line with the substantial increase in myostatin protein in the border zone of the infarcted myocardium, which were detected for at least 30 days after infarction.[11] However, as mRNA levels were only slightly but non-significantly increased in our study, there might be post-transcriptional and post-translational modifications, which need further elucidation.

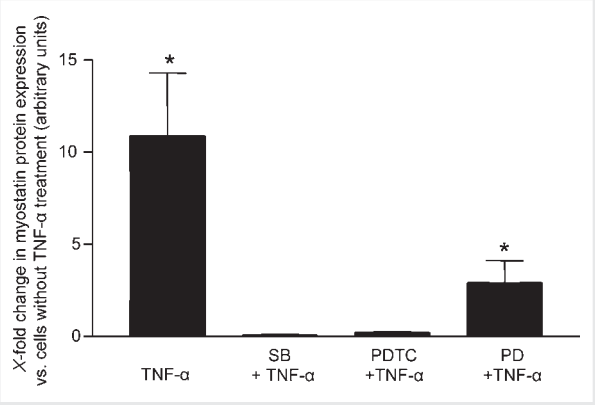


**Figure 4** Changes of protein levels of myostatin in differentiated C2C12 myocytes after TNF-α treatment. Cells were pre-incubated for 1 h with inhibitors of different signalling cascade molecules before TNF-α (50 ng/mL) was added to the medium. After 24 h, the cells were harvested and the expression of myostatin protein was determined by western blot. The columns represent the changes compared with the cells not treated with TNF-α, which were set as 1. Results are expressed as mean ± SEM. *$P < 0.05$ vs. cells without addition of TNF-α ($n = 3$ per data point). PD = PD98059, SB = SB203580.

## Tumour necrosis factor-α and the induction of myostatin

It has been reported that both serum levels and local concentrations of inflammatory cytokines,[2] especially TNF-α, are significantly increased in heart and skeletal muscle in CHF.[3,26] TNF-α concentrations correlate with the degree of functional impairment as assessed by NYHA functional class[27,28] or 6-min walk test.[29] Since TNF-α seems to mediate muscle wasting,[30] it is tempting to speculate that TNF-α may be involved in the induction of myostatin expression. This hypothesis is supported by our results, which demonstrate significantly increased levels of myostatin mRNA and protein levels after incubation of C2C12 myocytes with TNF-α. As there is an NF-κB DNA consensus sequence in the promoter region of myostatin, inhibitors of NF-κB and other upstream kinases were tested to explore possible pathways of signal transduction. Inhibition of both NF-κB and p38 MAPK blunted the TNF-α-induced increase in myostatin. However, inhibition of p42/44 MAPK induced only a mild and non-significant suppression of myostatin expression. Taken together, these data suggest the

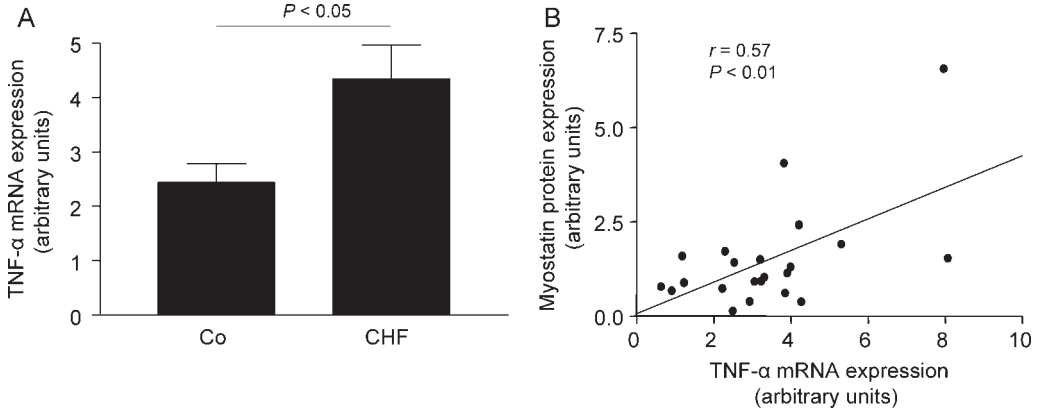


**Figure 5** (*A*) Quantification of TNF-α mRNA expression in skeletal muscle of control (Co, $n = 11$) and chronic heart failure animals (CHF, $n = 11$). (*B*) Correlation between TNF-α mRNA expression and myostatin protein levels in skeletal muscle. The values are depicted as a ratio of the expression of 18S rRNA or GAPDH. Results are expressed as mean ± SEM.

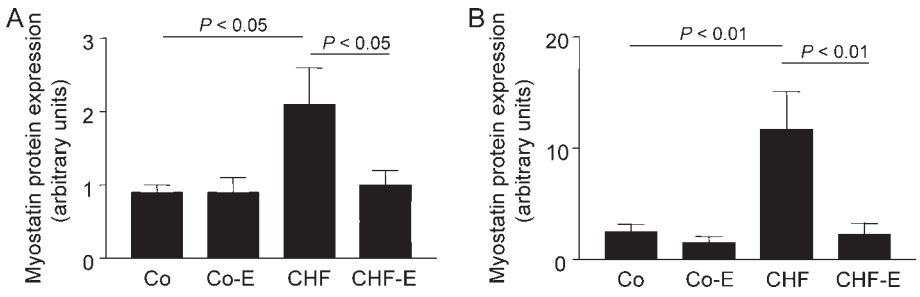


**Figure 6** Quantification of myostatin protein in skeletal (*A*) and in heart muscle (*B*) of sham-operated animals with (Co-E) or without (Co) exercise training and rats with CHF after LAD-ligation with (CHF-E) or without (CHF) exercise training. Exercise training reduced elevated myostatin levels in CHF animals significantly. No changes regarding the myostatin protein concentration were seen in control animals after exercise training neither in skeletal nor in heart muscle (A and B). Results are expressed as mean ± SEM.

possibility of myostatin stimulation by elevated TNF-α levels via NF-κB and p38 MAPK activation. This may at first appear to be contradictory, as the promoter of the TNF-α gene comprises a *cis*-activating element responsive to the transcriptional factor NF-κB,[31] but on the other hand, the activation of the TNF-α receptor by TNF-α also activates NF-κB.[32] This would theoretically lead to a self-supporting positive feedback. Further investigations are therefore necessary to address this complex process in more detail.

The results of our cell culture experiments contradict a report from Bakker *et al*. who failed to induce myostatin expression by TNF-α stimulation of pre-differentiated myotubes, these differences may be due to the divergent cell states.[33] There is no doubt that gene regulation follows different pathways depending on the stage of cell development. Moreover, the concentration of TNF-α used by Bakker was less than half of the concentration used in our cell culture model. Our screening experiments demonstrated that lower TNF-α concentrations are not able to sufficiently stimulate myostatin expression.

TNF-α mRNA levels in the gastrocnemius-muscle of CHF animals were significantly higher compared with controls, which is in agreement with previously published data.[3] Interestingly, there was a significant correlation between these raised TNF-α levels and myostatin concentration, supporting the hypothesis of a relationship between TNF-α and myostatin.

## The effects of exercise training on myostatin

Although several studies have investigated the effects of exercise training on myostatin expression in skeletal muscle, the results remain inconclusive.[14–18] In healthy subjects for example, both increasing and decreasing levels of myostatin mRNA have been observed after different types of exercise training. Several studies using heavy resistance training for several weeks or one bout of resistance exercise have shown a reduction in myostatin mRNA expression.[14,15,17] In contrast, other studies have shown an increase in myostatin mRNA and protein expression following heavy resistance training in healthy individuals.[16] Heavy endurance training in rats, however, reduced myostatin mRNA content in skeletal muscle but increased the mRNA levels in the heart muscle. Western blots revealed no differences in the myostatin protein levels.[18] The results of the present study support the findings of this last report, with no detectable difference in protein levels in skeletal muscle of healthy rats after exercise training. As suggested previously in the setting of CHF, post-translational and post-transcriptional modifications might be responsible for the discrepancy between mRNA and protein findings and further investigations are required. Endogenous inhibitors that can potentially modify myostatin protein concentration, namely follistatin, follistatin-like related gene (FLRG), and growth differentiation factor associated protein-1 (GASP-1), without influencing the mRNA level, have been described in the literature.[34] Recent studies suggest an autoregulatory feedback loop that determines an inverse relationship between myostatin protein activity and mRNA transcription.[35] However, physiological effects will depend on the local protein content, irrespective of mRNA concentrations. In the heart muscle, changes are similar to the findings reported in skeletal muscle. The described increase in myostatin mRNA levels in the above-mentioned study[18] might be linked to cardiac tissue damage, such as stress-induced infarctions caused by heavy endurance training (swimming with external weights).

In CHF, the situation is different. To our knowledge, there is currently no information about the effects of physical exercise on skeletal and heart muscle myostatin levels in CHF. Surprisingly, the increase in myostatin protein detected in the CHF rats in our study was reduced to baseline levels after 4 weeks of exercise training. In a previous study, we demonstrated that exercise training reduced local expression of TNF-α in patients with CHF.[3] Therefore, the significant reduction in myostatin levels in CHF after exercise training could be linked to the reduction in the levels of the stimulating agent TNF-α.

In summary, the induction of CHF by LAD-ligation led to a significant increase in myostatin protein expression in skeletal and heart muscle. Exercise training significantly reduced these levels back to baseline values. The proinflammatory cytokine TNF-α has the potency to induce the expression of myostatin *in vitro* via the activation of NF-κB and p38MAPK. The beneficial anti-catabolic effects of exercise training in CHF might be mediated by the suppression of inflammatory cytokines leading to normal myostatin levels.

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**Conflict of interest:** none declared.

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