ORIGINAL ARTICLE

Heart failure alters MyoD and MRF4 expressions in rat skeletal muscle

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Summary

Heart failure (HF) is characterized by a skeletal muscle myopathy with increased expression of fast myosin heavy chains (MHCs). The skeletal muscle-specific molecular regulatory mechanisms controlling MHC expression during HF have not been described. Myogenic regulatory factors (MRFs), a family of transcriptional factors that control the expression of several skeletal muscle-specific genes, may be related to these alterations. This investigation was undertaken in order to examine potential relationships between MRF mRNA expression and MHC protein isoforms in Wistar rat skeletal muscle with monocrotaline-induced HF. We studied soleus (Sol) and extensor digitorum longus (EDL) muscles from both HF and control Wistar rats. MyoD, myogenin and MRF4 contents were determined using reverse transcription-polymerase chain reaction while MHC isoforms were separated using polyacrylamide gel electrophoresis. Despite no change in MHC composition of Wistar rat skeletal muscles with HF, the mRNA relative expression of MyoD in Sol and EDL muscles and that of MRF4 in Sol muscle were significantly reduced, whereas myogenin was not changed in both muscles. This down-regulation in the mRNA relative expression of MRF4 in Sol was associated with atrophy in response to HF while these alterations were not present in EDL muscle. Taken together, our results show a potential role for MRFs in skeletal muscle myopathy during HF.

Keywords

heart failure, myogenic regulatory factors, myosin heavy chain, skeletal muscle, Wistar rats

Heart failure (HF) is characterized by a reduced tolerance to exercise due to early fatigue and dyspnea; this may in part be due to skeletal muscle myopathy, with atrophy and shift from type I 'slow' to type II 'fast' fibres (Lipkin *et al.* 1988; Sullivan *et al.* 1990; Mancini *et al.* 1992; De Sousa *et al.* 2000). HF induces skeletal muscle myosin heavy chain (MHC) isoform expression towards the fast isoform (Simonini *et al.* 1996;

Vescovo *et al.* 1998), which is related to HF severity (Spangenburg *et al.* 2002; Carvalho *et al.* 2003).

Different pathways regulate skeletal muscle MHC expression (Allen *et al.* 2001), including myogenic regulatory factors (MRFs), a family of transcriptional factors that control the expression of several skeletal muscle-specific genes. The family has four members: MyoD, myogenin, Myf5 and MRF4. MRFs

form dimers with ubiquitous E proteins (e.g. E12 or E47), resulting in heterodimeric complexes that bind to the E-box consensus DNA sequence (5′-CANNTG-3′) that is found in the regulatory region of many muscle-specific genes (Murre *et al.* 1989), including the *MHCIIB* gene (Wheeler *et al.* 1999). During embryogenesis, MRFs are critical for establishing myogenic lineage and controlling terminal differentiation of myoblasts (for a review, see Parker *et al.* 2003). Previous studies have suggested that myogenin and MyoD may also be involved in establishing and maintaining slow and fast mature muscle fibre phenotype; myogenin is expressed at higher levels compared with MyoD in slow muscles, whereas the opposite is true for fast muscles (Hughes *et al.* 1993; Voytik *et al.* 1993). Similarly, MyoD is associated with the expression of fast type IIX and IIB MHC isoforms (Hughes *et al.* 1993; Hughes *et al.* 1997; Kraus & Pette 1997; Mozdziak *et al.* 1998; Seward *et al.* 2001). Therefore, it is reasonable to suggest that MRFs contribute to mechanisms controlling skeletal muscle MHC expression during HF.

This investigation was undertaken to examine MRF mRNA expression and its potential relationships with changes in MHC composition in young Wistar rat skeletal muscle with monocrotaline (MCT)-induced HF. To compare muscles with different fibre-type composition and function, we performed analyses in the fast phasic extensor digitorum longus (EDL) and slow postural soleus (Sol) muscles.

Methods

Heart failure induction

Seventeen weaned male Wistar rats (3–4 weeks old; 80–100 g) were obtained from the Central Animal House at São Paulo State University. HF was experimentally induced in 10 rats (HF group) by a single intraperitoneal (i.p., 30 mg/kg) injection of MCT, a widely accepted HF model (Vescovo *et al.* 1998; Dalla Libera *et al.* 1999; Dalla Libera *et al.* 2001; Leineweber *et al.* 2002). MCT is a pyrrolizidine alkaloid that induces pulmonary vascular disease with severe right ventricle hypertrophy and failure (Vescovo *et al.* 1989; Reindel *et al.* 1990) without itself producing changes in skeletal muscle MHC composition (Vescovo *et al.* 1998). Preliminary experiments revealed that 30 mg/kg i.p. was an appropriate dose of MCT for our animals with regard to survival and HF induction. MCT-treated rats were allowed to eat freely from a supply of standard rat cubes. Seven control rats (CT group) were injected with saline and were given the same quantity of food as consumed on the previous day by the treated rats. HF and CT rats were studied 22 days after MCT administration when the HF group had developed overt HF. After anaesthesia with i.p. sodium pentobarbital (50 mg/kg), the animals were killed; body weight (BW), Sol weight and EDL weight were evaluated. The Sol/BW and EDL/BW ratios were used as indexes of muscle atrophy. Muscles were immediately frozen in liquid nitrogen and stored at -80 °C. Left ventricle weight (LVW), right ventricle weight (RVW) and atrium weight normalized by BW (LVW/BW, RVW/BW and ATW/BW, respectively) were used as indexes of heart hypertrophy. This experiment was approved by Ethics Committee of Instituto de Biociências, UNESP, Botucatu, SP, Brazil.

Semi-quantitative reverse transcription-polymerase chain reaction analyses of mRNA for MRF genes

Total RNA was extracted from Sol and EDL muscles with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), which is based on the guanidine thiocyanate method. Frozen muscles were mechanically homogenized on ice in 1 ml of ice-cold TRIzol reagent. Total RNA was solubilized in Rnase-free H_2O, incubated in Dnase I (Invitrogen Life Technologies) to remove any DNA present in the sample and quantified by measuring the optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio of approximately 2.0. Two micrograms of RNA was reverse-transcribed with random hexamer primers and Superscript II reverse transcription (RT) in a total volume of 21 μl, according to standard methods (Invitrogen Life Technologies). Control 'No RT' reactions were performed by omitting the RT enzyme. These reactions were then polymerase chain reaction (PCR)-amplified to ensure that DNA did not contaminate the RNA. One microlitre of cDNA was then amplified using 1 μM of each primer (Table 1), ×1 PCR buffer minus Mg, 5 mM $MgCl_2$, 1 mM deoxyribonucleotide triphosphates and 2 units of Platinum® Taq DNA Polymerase (Invitrogen Life Technologies, São Paulo, SP, Brazil) in a final volume of 25 μl. Primer pairs for MyoD were designed from a sequence published in GenBank; myogenin and MRF4 primer sequences were those used by Smith *et al.* (1994). Preliminary experiments were conducted with each gene to determine the number of PCR cycles that represented the linear range of amplification. All PCR products were verified by restriction digestion or by sequencing. The cDNA from each muscle for both CT and HF groups were amplified simultaneously using aliquots from the same PCR mixture. After the PCR amplification, 10 μl of each reaction underwent electrophoresis on 1.0% agarose gels and stained with ethidium bromide. Images were captured and the bands corresponding to each gene were quantified by densitometry as Integrated Optical Density. PCR products were run in duplicate on different gel for each gene, and results averaged. The size (the number of base pairs) of each band corresponded to

the size of processed mRNA. The PCR signals were normalized to the housekeeping gene cyclophilin (Alway *et al.* 2002a).

Electrophoretic separation of MHC

MHC isoform analysis was performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Six to 10 serial cross sections (12 μm thick) were placed in 450 μl of a solution containing 10% (wt/vol) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (wt/vol) SDS and 0.9% (wt/vol) Tris/HCl (pH 6.8) for 10 min at 60 °C. Small amounts of the extracts (6 μl) were loaded on a 7–10% SDS-PAGE separating gel with a 4% stacking gel, run overnight (19–21 h) at 120 V and silver stained. MHC isoforms were identified according to molecular mass, and their relative percentages were quantified by densitometry.

Statistical methods

Data are expressed as mean ± SE. The comparisons between groups were performed using the Student's unpaired *t*-test and multivariate statistical analysis when appropriate. The level of significance was $P < 0.05$.

Results

Presence of heart failure in the monocrotaline-treated rats (Table 2)

After 22 days, all 10 MCT-treated rats showed HF at postmortem, confirmed by atrium and right ventricular hypertrophies (RVW/BW > 0.80), pleural and pericardial effusions and congested liver. No alterations were found in the CT rats.

There was no significant difference in BW between HF and CT groups. Heart weight was increased in HF group compared with CT group, as demonstrated by LVW, RVW and ATW and by the heart hypertrophy indexes (LVW/BW, RVW/BW and ATW/BW).

There was no significant difference in lung wet/dry ratio between CT and HF groups. However, RV, LV, AT and liver wet/dry ratio were greater in HF group than in CT group.

Muscle weight and indexes of muscle atrophy (Table 3)

Sol weight and Sol/BW were significantly decreased in HF group compared with CT group. Both EDL weight and EDL atrophy index (EDL/BW) did not differ between CT and HF groups.

MRF mRNA levels estimated by semi-quantitative RT-PCR

Soleus. MyoD and MRF4 mRNA levels decreased in the HF group compared with its CT group (CT = 0.67 ± 0.04 *vs.* HF = 0.55 ± 0.04 and CT = 0.64 ± 0.05 *vs.* HF = 0.46 ± 0.04, respectively) ($P < 0.05$). Myogenin mRNA level was similar between HF and CT groups (CT = 0.53 ± 0.04 *vs.* HF = 0.46 ± 0.03) (Figure 1a).

EDL. MyoD mRNA level decreased in the HF group compared with its CT group (CT = 1.83 ± 0.30 *vs.* HF = 0.80 ± 0.08, $P < 0.05$). Myogenin and MRF4 expressions were similar between HF and CT groups (CT = 1.01 ± 0.24 *vs.* HF = 0.99 ± 0.12 and CT = 1.14 ± 0.20 *vs.* HF = 1.09 ± 0.12, respectively) (Figure 1b).

MHCs electrophoretic pattern

In Sol muscle, two MHC isoforms were separated, MHC1 and MHC2a; their expressions were not different between HF

Table 1 Oligonucleotide primers used for polymerase chain reaction (PCR) amplification of reverse transcribed RNA

Product	Accession number	Sequence	Start position	T_A (°C)	Cycles	PCR length (bp)	Restriction enzyme	Restriction products (bp)
MyoD	M84176	5′-GACGGCTCTCTC TGCTCCTT	259	60	32	544	Sequenced	
		3′-GTCTGAGTCGCCGCTGTAGT	782					
Myogenin	M24393	5′-TGCCACAAGCCAGACTACCCACC	827	63	31	246	*Pst* I	234,80,65
		3′-CGGGGCACTCACTGTCTCTCAA	1050					
MRF4	M30499	5′-AGAGACTGCCCAAGGTGGAGATTC	491	63	32	272	*Pst* I	118,96,57
		3′-AAGACTGCTGGAGGCTGAGGCATC	1344					
Cyclophylin	M19533	5′-ACGCCGCTGTCTCTTTTC	9	57.7	32	440	*Hind*II	40,400
		3′-TGCCTTCTTTCACCTTCC	431					

Accession number, GenBank accession number; T_A, annealing temperature.

Table 2 Anatomical data of CT and HF groups

	Experimental groups	
	CT ($n = 7$)	HF ($n = 10$)
BW (g)	179.7 ± 2.8	182.2 ± 3.9
LVW (g)	0.42 ± 0.02	0.48 ± 0.01*
RVW (g)	0.14 ± 0.01	0.37 ± 0.01***
ATW (g)	0.06 ± 0.00	0.10 ± 0.01**
LVW/BW (mg/g)	2.32 ± 0.07	2.63 ± 0.08*
RVW/BW (mg/g)	0.77 ± 0.06	2.03 ± 0.07***
ATW/BW (mg/g)	0.32 ± 0.02	0.54 ± 0.03**
RV W/D	4.16 ± 0.06	4.68 ± 0.03***
LV W/D	4.13 ± 0.04	4.53 ± 0.02***
AT W/D	3.53 ± 0.23	5.24 ± 0.23**
Liver W/D	3.37 ± 0.05	3.64 ± 0.07*
Lung W/D	4.57 ± 0.27	4.79 ± 0.17

Values are means ± SE; n, number of animals; CT, control group; RCT, ration control group; HF, heart failure group; BW, body weight; LVW, left ventricle weight; RVW, right ventricle weight; ATW, atrium weight; W/D, wet-to-dry weight.
*$P < 0.05$ statistical significance *vs.* control group.
**$P < 0.001$ statistical significance *vs.* control group.
***$P < 0.0001$ statistical significance *vs.* control group.

and CT groups (HF = 61.6 ± 1.6% *vs.* CT = 63.3 ± 1.7% and HF = 38.4 ± 1.6% *vs.* CT = 36.8 ± 1.7%, respectively) (Figure 2a). In EDL muscle, four MHC isoforms were separated (MHC1, MHC2a, MHC2d and MHC2b). The difference in electrophoretic migration between MHC2a and MHC2d is very small, and it was not possible to quantify them separately. Consequently, we considered the sum (MHC2a/2d) as both isoforms are metabolically similar. In EDL muscle, MHC2a/d and MHC2b were not different between HF and CT groups (HF = 44.5 ± 0.7% *vs.* CT = 42.5 ± 1.2% and HF = 55.5 ± 0.7% *vs.* CT = 57.5 ± 1.2%, respectively) (Figure 2b).

Table 3 Sol and EDL weight of CT and HF groups

	Experimental groups	
	CT ($n = 7$)	HF ($n = 10$)
Sol (mg)	98.7 ± 5.1	86.0 ± 3.4*
Sol/BW (mg/g)	0.55 ± 0.03	0.47 ± 0.01*
EDL (mg)	81.0 ± 7.7	85.3 ± 3.2
EDL/BW (mg/g)	0.45 ± 0.04	0.47 ± 0.01

Values are mean ± SE; n, number of animals; CT, control group; HF, heart failure group; BW, body weight; Sol, soleus weight; EDL, extensor digitorum longus weight.
*$P < 0.05$ statistical significance *vs.* control group.
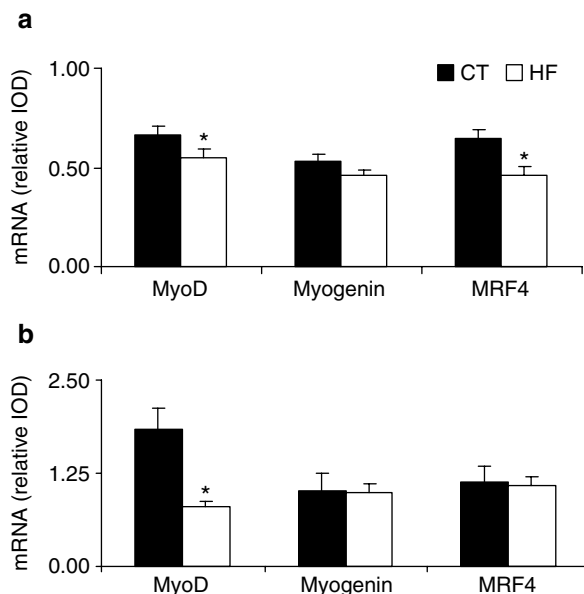
Figure 1 MyoD, myogenin and MRF4 mRNA contents estimated by reverse transcription-polymerase chain reaction (RT-PCR) in control (CT) and heart failure (HF) groups from soleus (a) and extensor digitorum longus (EDL) (b) muscles. Data were run in duplicate on different gels for each gene, and the results were averaged. PCR products were visualized with ethidium bromide staining. Quantification of the PCR signal was obtained by densitometric analysis of the product as Integrated Optical Density (IOD). Gene expressions were normalized to the cyclophilin signal from the same RT product. Normalized data are expressed as means ± SE. *Data are significantly different from CT group at $P < 0.05$.

Discussion

The major finding in this study was that despite there being no change in MHC composition of rat skeletal muscles with MCT-induced HF, the mRNA relative expression of MyoD in Sol and EDL muscles and of MRF4 in Sol muscle were significantly reduced, whereas myogenin was not changed in either muscle.

The pattern of MHC expression during HF from this investigation diverges from previously published data that showed MHC isoform expression towards the fast isoform in chronic HF models such as myocardial infarction in rat (Simonini *et al.* 1996) and rabbit (Spangenburg *et al.* 2002), subtotal constriction of the suprarenal abdominal aorta in rabbit (Coirault *et al.* 1999) and ascending aorta stenosis in rat (Carvalho *et al.* 2003). The short duration of HF in our model could explain this. We could not push experiments further than 22 days because the severity of the MCT-induced HF in young Wistar rats inevitably leads to death in this time

a

b
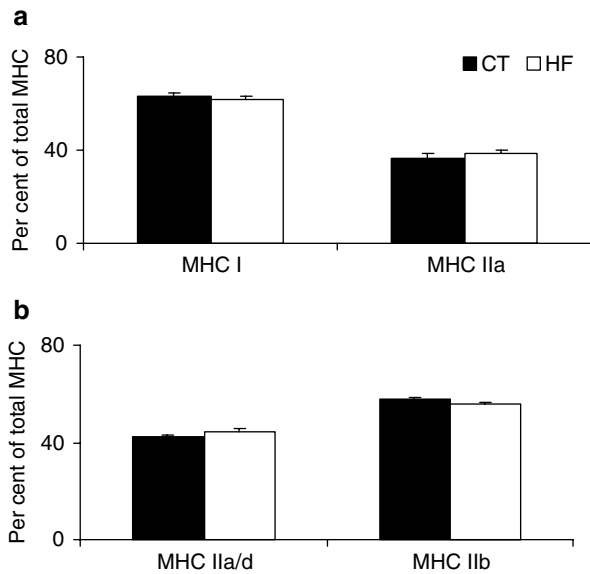
Figure 2 Percentage distribution of myosin heavy chain (MHC) from soleus (a) and extensor digitorum longus (EDL) (B) muscles from control (CT) and heart failure (HF) groups. Data are expressed as means ± SE.

frame. It is noteworthy that the HF period in our study was short and unable to induce change in MHC composition while in other studies, young Sprague–Dawley rats injected with the same dose of MCT as in our experiment (30 mg/kg i.p.) developed HF with consequent changes in MHC composition after 27–30 days (Vescovo *et al.* 1998; Dalla Libera *et al.* 1999; Dalla Libera *et al.* 2001).

The synthesis of muscle-specific proteins may be associated with myogenin and MyoD mRNA levels. Myogenin is expressed at higher levels compared with MyoD in predominantly slow muscle, whereas MyoD is expressed at higher levels compared with myogenin in predominantly fast muscles of mature animals (Hughes *et al.* 1993; Voytik *et al.* 1993). MyoD is associated with the expression of fast type IIX and IIB MHC isoforms (Hughes *et al.* 1993; Hughes *et al.* 1997; Kraus & Pette 1997; Mozdziak *et al.* 1998; Seward *et al.* 2001). Additionally to the role of myogenin in establishing mature slow muscle fibre phenotype (Hughes *et al.* 1993; Voytik *et al.* 1993), it is also involved with oxidative gene expression and metabolic enzyme activity (Coirault *et al.* 1999; Hughes *et al.* 1999; Ekmark *et al.* 2003). Although we did not analyse these metabolic parameters in our study, they probably were not altered because the mRNA relative expression of myogenin was not changed. We also showed that HF induced a reduction in the mRNA relative expression of MyoD in both Sol and EDL muscles suggesting an MHC phenotypic adaptation towards a slower profile in both

muscles, as the mRNA relative expression of myogenin was not changed. It is well known that MHC phenotypic adaptation in limb skeletal muscles during HF occurs towards a faster profile (Simonini *et al.* 1996; Vescovo *et al.* 1998; Spangenburg *et al.* 2002; Carvalho *et al.* 2003).

Interestingly, our results indicate that skeletal muscle fibre phenotype modulation during this syndrome is not exclusively dependent on the MRFs but may also be regulated by distinct pathways, such as those involving activated calcineurin and transcriptional factor nuclear factor of T cells (Chin *et al.* 1998; McCullagh *et al.* 2004), calcium-dependent CaM kinase (McKinsey *et al.* 2000; Wu *et al.* 2000), peroxisome proliferator-activated receptor-gamma coativator 1 (PGC1α) (Wu *et al.* 2001; Lin *et al.* 2002), Ras (Murgia *et al.* 2000) and the transcriptional complex Six1/Eya1 (Grifone *et al.* 2004).

What caused the down-regulation in MyoD mRNA expression during HF needs to be determined; however, cytokine activation (Anker *et al.* 1999) may have been involved. This point has been the subject of considerable debate given that tumour necrosis factor-α (TNF-α) is markedly increased in patients (Levine *et al.* 1990; McMurray *et al.* 1991) and Sprague–Dawley rats with HF (Dalla Libera *et al.* 1999; Dalla Libera *et al.* 2001). One hallmark of TNF-α is the activation of nuclear factor (NF)κB, a ubiquitous transcription factor normally inactive and sequestered in the cytoplasm through association with IκB (Baeuerle & Baltimore 1988). TNF-α exposure leads to the degradation of IκB, allowing NFκB translocation to the nucleus (Israël 2000) where it down-regulates MyoD mRNA at post-transcriptional level (Guttridge *et al.* 2000). This mechanism may partially explain the down-regulation of MyoD that we found in both Sol and EDL muscles. However, further studies need to be conducted to demonstrate this mechanism during HF.

The analysis of MRF4 mRNA expression revealed that, during HF, MRF4 mRNA transcripts were down-regulated in the slow Sol muscle but not in the fast EDL. MRF4 is expressed mainly after birth and is likely to have a role in the maintenance of skeletal muscles rather than the development, differentiation and regeneration processes which may be controlled by MyoD, myogenin and Myf5 expressions (Perry & Rudnick 2000). The concept that MRF4 is involved in regulating maturation events and maintaining adult skeletal muscle is further sustained by the fact that MRF4 is not expressed at substantial levels in limb until very late in fetal development (Hinterberger *et al.* 1991) and is present at higher levels in postnatal muscle (Voytik *et al.* 1993; Hughes *et al.* 1997). Interestingly, in this investigation, a down-regulation in the mRNA relative expression of MRF4 in Sol was associated with atrophy in response to HF while these alterations were not present in EDL muscle. This soleus-specific

down-regulation of MRF4 was also previously observed in response to atrophy disuse of skeletal muscle in adult rats (Loughna & Brownson 1996) indicating a possible function for MRF4 in muscle atrophy. Additionally, it has been demonstrated that an increase in Id repressor protein expression, which forms heterodimers with MRFs and prevents their DNA binding (Benezra *et al.* 1990), is related to muscle atrophy in conditions such as disuse (Gundersen & Merlie 1994), hindlimb (Alway *et al.* 2002a) and ageing (Alway *et al.* 2002b). Taken together, these results shows that the potential of MRFs in muscle atrophy clearly requires further examination. Furthermore, the knowledge of the mechanisms controlling MRF expression during HF may explain many alterations in skeletal muscle-specific gene and protein expression during this syndrome.

Acknowledgements

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