Temporary fatigue and altered extracellular matrix in skeletal muscle
during progression of heart failure in rats

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Running head: Skeletal muscle fatigability and ECM in CHF

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Abstract

Patients with congestive heart failure (CHF) experience increased skeletal muscle fatigue. The mechanism underlying this phenomenon is unknown, but a deranged extracellular matrix (ECM) might be a contributing factor. Hence we examined ECM components and regulators in a rat post-infarction model of CHF. At various time points during a 3.5 month-period after induction of CHF in rats by left coronary artery ligation, blood, interstitial fluid (IF) and muscles were sampled. Isofluran anesthesia was employed during all surgical procedures. IF was extracted by wicks inserted intermuscularly in a hind limb. We measured cytokines in plasma and IF whereas MMP activity and collagen content as well as the level of glycosaminoglycans and hyaluronan were determined in hind limb muscle. In vivo fatigue protocols of the soleus muscle were performed at 42 and 112 days after induction of heart failure. We found that the MMP activity and collagen content in the skeletal muscles increased significantly at 42 days after induction of CHF, and these changes were time-related to increased skeletal muscle fatigability. These parameters returned to Sham levels at 112 days. Vascular endothelial growth factor (VEGF) in IF was significantly lower in CHF compared to Sham-operated rats at 3 and 10 days, but no difference was observed at 112 days. We conclude that temporary alterations in the ECM, possibly triggered by VEGF, are related to a transient development of skeletal muscle fatigue in CHF.

Key words:
Fatigue, extracellular matrix, interstitial fluid, ischemic heart failure
**Introduction**

Patients with congestive heart failure (CHF) frequently experience increased skeletal muscle fatigue. Also, and in contrast to the healthy individual, cardiac output relates poorly to the exercise capacity of CHF patients (10, 45). Both animal studies and clinical studies have identified structural, biochemical and functional abnormalities within skeletal muscle in CHF. For example, a fibre type switch towards a less fatigue resistant phenotype (36, 44), impaired calcium handling (18, 26), altered myocyte calcium sensitivity (18) and reduced oxidative capacity (5, 20) have been reported. Consistently, increased fatigability of the rat skeletal muscle in CHF has been demonstrated both in vivo (16) and on a single fibre level (18). Importantly, in these CHF rats, blood flow to the exercising soleus muscle was unaltered compared with Sham rats (30).

Research on muscular fatigue in CHF has focused on the skeletal muscle fibre itself, while possible alterations of the adjacent extracellular matrix (ECM) and its components have apparently not been explored in detail. Interestingly, biomarkers of ECM remodelling and collagen turnover were recently demonstrated to be positively correlated to both mortality and morbidity in CHF patients (27). In a previous study (17) we noted that the flexor digitorum brevis muscle (a fast-twitch muscle) in post-infarction CHF rats was more fragile during dissection compared to Sham operated rats, despite the absence of atrophy and edema (16). This led to the assumption that ECM might be altered in CHF and we therefore investigated the role of matrix metalloproteinases (MMPs) in the skeletal muscle of CHF rats. We found increased MMP-activity in both fast and slow skeletal muscles of CHF rats compared to Sham 6 weeks after induction of myocardial infarction (31). These findings were later supported by others (3).

Force transmission from the myocytes to the bone is dependent on the structural integrity between individual muscle fibres and the ECM (2). The connective tissue, dominated
by collagen, is of particular importance for this force transmission as it interacts closely with the contractile elements of the muscle (14). Furthermore, proteoglycans are of importance in linking together the fibrous structures of the ECM (32, 42). Alterations in ECM components can therefore contribute to decreased resistance to fatigue.

CHF is characterised by elevated plasma levels of pro-inflammatory cytokines (9, 23) that increase with disease severity and have prognostic value (4, 24, 38). This supports the notion that elevated levels of cytokines may reflect important pathogenic mechanisms in this disease. What causes the skeletal muscle alterations in CHF is poorly understood, but humoral factors, like pro-inflammatory cytokines, might be the link between the failing heart and the altered skeletal muscle. Several cytokines and growth factors have the potential to alter skeletal muscle ECM. For example, interleukin (IL)-1β can induce a marked increase in MMP activity (1) and transforming growth factor (TGF)-β has been shown to stimulate collagen formation and reduce its degradation (7, 25) as well as increasing the synthesis of proteoglycans (28). IL-6, known to be produced by skeletal muscle fibres, is also released by fibroblasts (41) and can stimulate their production of collagen, sulfated glycosaminoglycans and hyaluronan (6).

On this basis, we now wanted to examine MMPs, relevant cytokines, both in plasma and locally within skeletal muscle, as well as possible alterations of structural components of the ECM during progression of ischemic heart failure in rats.

We hypothesized that induction of ischemic heart failure in rats would lead to an early cytokine response that would alter the structural integrity of skeletal muscle-ECM and thereby contribute to decreased resistance to fatigue.
Materials and Methods

Experimental model

The investigation was conducted in accordance with APS’s *Guiding principles in the Care and Use of Animals* and was approved by the Norwegian Animal Research Authority. Two animals were housed per cage and given access to food and water *ad libitum* in a temperature-regulated room on a 12:12-h day/night cycle.

Under anesthesia (68 % N2O/29% O2/1.5 to 2% Isofluran; Abbot Scandinavia, Solna, Sweden), male Wistar rats (Taconic, Skensved, Denmark; ~320 g body wt) were subjected to ligation of the left coronary artery (CHF) or a Sham operation as described (34). At 3, 10, 42 and 112 days after this primary operation, rats were again anesthetized and echocardiography was performed using a Vivid 7 echocardiograph (GE Vingmed Ultrasound, Horten, Norway) with a 12-MHz linear array transducer (M12L) (34). Left ventricle end-diastolic pressure (LVEDP) was determined by a 0.67 mm microtip pressure catheter (SPR-407, Millar Instruments Inc., Houston, TX) inserted through the right carotid artery and into the left ventricle. Only rats with LVEDP ≥ 15 mm Hg were included in the CHF group in subsequent experiments (34). After these measurements, the soleus (SOL), tibialis anterior and extensor digitorum longus (EDL) muscles of one limb were carefully removed. Since intermuscular wicks were inserted in one hindlimb of each animal, only one set of hindlimb muscles were available for the remaining analysis. We considered using different muscles for different analysis to be appropriate in order to restrict the use of animals to an acceptable level. Blood was sampled from the abdominal aorta into a vacutainer citrate tube and added 100 µl of 1 M Hepes (pH 7.2-7.4) and 100 µl of mM CaCl2 before centrifugation at 1100 x g for 10 min at 4 ºC. The animal was sacrificed after the heart and lungs were removed during deep anesthesia.
Collection of wick interstitial fluid from hind limb muscle

Muscle interstitial fluid (IF) was sampled by intermuscular wick implantation as detailed (43). Three-stranded nylon wicks (diameter, 1 mm; length, 5-7 cm) pre-washed in acetone, ethanol and distilled water were used. Dry wicks were inserted intermuscularly post mortem in hindlimb muscle between the adductor magnus and gracilis muscles and the semimembranosus and semitendinosus muscles rather than the SOL and EDL muscles, as the latter two were used for other measurements. The wicks where then left to absorb IF for 15-20 min before removal and transferral to centrifuge tubes with funnels containing 1.5 ml mineral oil with 5 µl protease inhibitor cocktail (Roche, Mannheim, Germany) to avoid degradation of cytokines in the IF. To prevent evaporation, all wick handling took place in an incubator kept at 100 % relative humidity. Wick fluid was isolated under mineral oil by centrifugation at ~21,000 x g for 10 min, aspirated by a pipette and stored at -80 °C until further analyses.

Cytokine analysis

A panel of cytokines were quantified in IF and plasma. A multiplex fluorescent bead immunoassay kit (Linco Research Inc, St. Charles, MI) was used for the simultaneous quantification of the following cytokines: IL-1α, IL-1β, IL-2, IL-6, IL-10, growth regulated oncogene-KC (GRO-KC), interferon-γ (IFN-γ), tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), Chemokine (C-C motif) ligand 2 (CCL2) [monocyte chemotactic protein 1 (MCP-1)], and CCL3 [macrophage inflammatory protein-1α (MIP-1α)]. The samples were prepared for analysis by dilution with serum matrix diluent and the analysis performed as specified by the manufacturer. Total surface fluorescence was measured with a flow-based dual laser system (Luminex100, Luminex Corporation, Austin, TX). The lower detection level was 4.8 pg/ml.
Transforming growth factor -β1

TGF-β1 in plasma and IF were measured with an ELISA kit (Bender MedSystems, Vienna, Austria) according to specifications given by the manufacturer. The plasma and wick fluids were first diluted 1:20 with assay buffer. For each well the optical density at 450 nm was recorded with Spectramax pluss 384 microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The lower detection level was 0.47 ng/ml.

Collagen content

Assessment of total collagen content in the tibialis anterior muscle was based on the hydroxyproline content (46). Muscles were collected as described above. Approximately 20-50 mg of freeze-dried and homogenized muscle was placed in glass vials and added 1 ml 6 M HCl and left to hydrolyze overnight at 120 °C. After cooling, 3 ml of distilled water was added. The solution was pipetted into a total volume of 4.5 ml, so that the tissue concentration in all samples reached 0.25 mg/ml. Five-hundred µl of diluted hydrolysed sample was pipetted into a test tube, and added 250 µl of 0.05 M Chloramine-T reagent before they were mixed and incubated at room temperature for 20 min. Two-hundred and fifty µl of 3.15 M perchloric acid was added, and left to incubate at room temperature for 5 min, before adding 250 µl of 20 % (wt/vol) of p-dimethylaminobenzaldehyde dissolved in ethylenglycolmonoethylether. After mixing, the tubes were placed in a waterbath at 60 °C, and then in ice water for 5 min before the absorbance was read at 557 nm with the Spectramax plus 384 spectrophotometer. The concentration of hydroxyproline was quantified by comparison with a standard curve in the range of 0.5-6 µg/ml, based on a stock solution of 25 mg L-4-hydroxyproline (Fluka Chemie GmbH, Buchs, France) dissolved in 250 ml 1 mM HCl. Collagen content was determined using an estimate of 6.94 µg collagen/µg hydroxyproline (13).
Assessment of collagen type III was performed on muscle tissue samples from EDL and SOL. Separation of proteins were performed on 10 % sodium dodecyl sulfate (SDS) gels. After blotting the PVDF-membranes were incubated with primary antibody against collagen III (1:5000, #600-401-105, Rockland, Gilbertsville, PA) overnight at 4 °C. Secondary antibody was NA934V (Amersham Biosciences/GE Healthcare Bio-Sciences, NJ). The signal from the immunoactive spots was visualized with ECL Plus detection system (RPN 2132, Amersham, Oslo, Norway). Detection of bands was done with LAS 4000 (Fujifilm, Sweden) and quantified with Imagequant (GE Healthcare).

**MMP-activity**

MMP activity was measured with a gelatinase assay as detailed (31). Briefly, SOL and EDL muscles were carefully isolated and stripped for tendons and connective tissue before immediate transfer to ice-cold phosphate-buffered saline. In the gelatinase assay calf skin ³H-collagen (Sigma-Aldrich, Steinheim, Germany) was used as substrate. The acid-soluble radioactive protease products were measured in a liquid scintillation counter (Wallac Win Spectral 1414, PerkinElmer Instruments, Oslo, Norway). Due to slight variations in the incorporation of radioactivity when preparing the different gels, the results had to be standardized (31). To this end the gelatinase activity obtained from muscle samples of CHF rats were normalized to those obtained from Sham rats in the same analytical run (i.e. run on the same gel) to yield a relative value. Sham value was set to 1.0.

**Hyaluronan analysis**

Hyaluronan in EDL muscle was analysed using an ELISA kit (Corgenix UK Ltd, Peterborough, UK). Approximately 25 mg of freeze-dried and homogenized muscle was placed in glass vials and added 1 ml of 0.5 mg/ml Pronase E (Sigma-Aldrich St. Louis, MI)
dissolved in TrisHCl/CaCl₂ buffer (0.05/0.01 M, pH 7.2). The vials were incubated for approximately 24 h at 56 °C, and then boiled in 13 min in a water bath, followed by cooling and centrifugation in 10 min at 464 x g. The sample solution was then diluted 1:500 with assay buffer, before further analysis was performed according to specifications given by the manufacturer. The plates were precoated with hyaluronan antibodies, and standards and experimental samples were added. For each well the optical density at 450 nm was read using the Spectramax plus 384 microplate reader. The lower detection level was 50 ng/ml.

Isolation of glycosaminoglycans (GAGs)

Isolation of GAGs was based on the method of Ledin et al. (15). Frozen muscle samples (EDL and SOL) were cut into small pieces (14-43 mg dry weight) and treated with Pronase E (Sigma Aldrich, St. Louis, MI, 0.8 mg/ml) in 0.5 ml of buffer (50 mM Tris-HCl, pH 8.0, 1 mM CaCl₂, and 1 % Triton X-100) at 55 °C overnight. Subsequently, 0.4 mg of Pronase E was added, and the samples were incubated for 2 h. After inactivation by boiling and adjustment of the samples to 2 mM MgCl₂, 12 mU of endonuclease (Sigma-Aldrich, St. Louis, MI) were added and incubated for 2 h at 37 °C and, after heat inactivation of the enzyme, adjusted to a final concentration of 0.1 M NaCl. Subsequently, the samples were centrifuged at 14,000 x g for 10 min. The GAG chains were desalted and purified by ion-exchange chromatography on 0.3 ml DEAE-Sephacel columns. The ion-exchange columns were primed by washing with 2 M NH₄HCO₃ and a loading buffer of pH 8.0 (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 0.1 % Triton X-100). The supernatants from the digestions were applied, and the columns were washed successively with a loading buffer of pH 8.0, a washing buffer of pH 4.0 (50 mM sodium acetate, 0.1 M NaCl, and 0.1% Triton X-100), and finally with 0.2 M NH₄HCO₃. The GAG chains were eluted in 3 x 0.3 ml portions of 2 M
NH₄HCO₃. The eluates were collected in microcentrifuge tubes and repeatedly freeze dried until the pH values were close to 7.

For chondroitin sulfate (CS) degradation, the GAG pools were digested with 50 mU of chondroitinase ABC (cABC) (Seikagaku Corporation, Tokyo, Japan) in 50 µl of 40 mM Tris-acetate buffer, pH 8.0. CS digestion was performed for 3 h at 37°C, followed by heat inactivation. cABC will also degrade hyaluronan to disaccharides, so the disaccharide analysis of CS will therefore contain both hyaluronan disaccharides and CS disaccharides. Samples were dried and dissolved in 100 µl H₂O. From the CS digests, 10 µl was removed for analysis of CS disaccharides by reverse phase ion pair high performance liquid chromatography (RPIP-HPLC; see below). For heparan sulfate (HS) disaccharide preparation, 90 µl of the CS digest was filtrated with a 3 kDa cut-off Microcon filter (Millipore, Billerica, MA). The concentrate containing intact HS-GAGs was freeze-dried and dissolved in 20 µl of H₂O. Two aliquots were dried and prepared for heparitinase digestion. One aliquot was treated with 0.4 mU of heparitinases I, II, and III (Grampian Enzymes, Orkney, Scotland, UK) in 15 µl of heparitinase buffer (5 mM Hepes, pH 7.0, 50 mM NaCl, 1 mM CaCl₂, 0.7 mg/mL BSA) and incubated for 16 h at 37°C. The other aliquot was incubated without enzymes. The reaction was stopped by heat inactivation. All the samples were dried and dissolved in 45 µl H₂O before analysis with RPIP-HPLC.

**Analysis of disaccharides**

Quantitative analysis of disaccharides derived from GAGs was performed by RPIP-HPLC (35) on a Luna 5 µ C18 reversed phase column (4.6 x 150 mm; Phenomenex, Torrance, CA, USA) in 8.5 % acetonitrile and 1.2 mM hydrogen sulfate (Fluka, St. Louis, MO) by applying a stepwise gradient from 1 to 53 % of 0.2 M NaCl (39). The flow rate was 1.1 ml/min, and the fluorescent labeling reaction was performed by addition of 2-cyanoacetamide
(0.25%; Sigma) in NaOH (0.5%; 0.35 ml/min). Signals were quantified against standards (Sigma and Grampian Enzymes). The chromatography software used was Chromeleon (Dionex, Sunnyvale, CA).

Fatigue protocol

At 42 and 112 days we performed an in vivo isometric fatigue protocol on SOL of CHF and Sham rats as described in detail elsewhere (16). In short, the rats were anesthetized and blood pressure and LVEDP were monitored by a catheter inserted into the left ventricle as described above. SOL was denervated and then electrically stimulated at 5 Hz for 6 s at 10 s intervals for 30 min with 1 ms pulses. Blood supply to the working muscle was intact and muscle core temperature was maintained at 39 °C. The foot and the tibia of the rat were fixed by two clamps and the distal tendon of the muscle was attached to a force transducer and positioned in parallel to the tibia. The muscle was stretched and stimulation voltage was adjusted to give maximal force of tetanic contractions. Maximum force during the stimulation train as well as contraction rate of the first contraction and relaxation rate of the last contraction of each train were calculated.

Statistics

Values are means and standard error of the mean (SEM). Statistical significant differences were assumed for P < 0.05. To evaluate time-dependent differences between CHF and Sham, a two-way ANOVA with CHF and time as variables was used, whereas the Bonferroni correction was used for post hoc analysis. For the GAG analysis, a Student’s t-test was used since the time points were pooled and only the 2 study groups were compared. The MMP activity in muscle samples of CHF rats were normalized to those obtained from Sham rats in the same analytical run to yield a relative value. Accordingly, a one sample t-test was
used at each time point followed by a Bonferroni correction to see whether the MMP activity in CHF muscles was significantly different from 1.
Results

Induction of post-infarction heart failure

As shown in Table 1, we found effects of CHF (P<0.0001) and time (P<0.05) on lung weight (LW), LVEDP and left atrial diameter (LAD), and there was interaction (P<0.05) between the two variables for both LVEDP and LAD. Post hoc analysis showed that LW of CHF animals were elevated (P<0.0001) at all time points (Table 1) indicating pulmonary congestion. In line with this, LVEDP and LAD also increased (P<0.0001) among CHF rats compared to Sham at all time points. In addition, cardiac output decreased in the CHF animals at these time points (P<0.0001). There was an overall effect of time, but not of CHF on body weight (Table 1). For additional echocardiographic parameters and animal characteristics we refer to online supplemental material.

Temporary increase of MMP activity and collagen content

Gelatinase activity, as a measure of MMP-related activity, within isolated SOL (a slow-twitch muscle) and EDL (a fast-twitch muscle) was determined at 3, 10, 42 and 112 days after induction of CHF. There was no difference (P>0.05) in MMP activity between CHF and Sham at 3 days (Figure 1). However, the CHF group exhibited a significantly higher MMP activity in the SOL muscle after 10 days and in the SOL and EDL muscles after 42 days, compared to Sham. The MMP activity had returned to Sham level by 112 days.

At the same time points collagen content of the tibialis anterior muscle (predominantly a fast twitch muscle) from the same animals was assessed. There were significant effects of both time and CHF, but no significant interaction between the two variables. Post hoc analysis revealed that collagen content of this muscle, as expressed in percent of dry weight, was moderately elevated in the CHF group at 42 days (P<0.05), coinciding in time with the up-regulation of MMP activity (Figure 2). At the other specific time points there were no
significant differences between the two groups. There was no difference in content of collagen type III between CHF and Sham rats for either EDL or SOL muscle (Figure 3).

**Muscle structures of sulfated GAGs and hyaluronan**

To investigate whether the composition of muscle GAGs was altered in CHF animals, GAGs were isolated from SOL and EDL muscles at 3, 10, 42 and 112 days after the primary operation. The results showed that heparan sulfate (HS), as well as chondroitin sulphate (CS) + hyaluronan, from both SOL and EDL muscles consisted of similar disaccharides and in similar ratios. Furthermore, the distribution (in percent of total) of the different disaccharides in HS and CS + hyaluronan was not affected in CHF animals in the two types of muscle analysed (data presented in supplemental material). From the HPLC chromatogram it was also possible to calculate the ratio of CS + hyaluronan / HS in each preparation. The ratio was 3:1 in all muscles analysed, and no apparent difference was observed between Sham and CHF muscles. The hyaluronan content of EDL muscles, as analysed by ELISA, was also similar in the two groups at all time points (Data presented in supplemental material).

**Reduced interstitial levels of VEGF in CHF**

To detect changes in cytokine concentrations and their gradients during progression of CHF, cytokines were measured in both plasma and IF at all four time points. For VEGF there was an effect of CHF (P<0.05), and interacting with time (P<0.05). Post hoc analysis revealed a lower concentration of VEGF in IF of CHF animals compared to Sham at the two earliest time points (P<0.0001 and P<0.05 at 3 and 10 days, respectively). By measuring both plasma and IF concentrations we were able to determine the cytokine concentration gradients between these two compartments. This revealed a large concentration gradient of VEGF from IF towards plasma in both groups. For all the other cytokines analysed (IL-1α, IL-1β, IL-2,
IL-6, IL-10, GRO-KC, IFN-γ, TNF-α, CCL2, CCL3, and TGF-β1) there were no significant differences, neither in plasma nor in the IF, between the two groups (for details see online supplemental material).

**Temporarily increased fatigability in CHF**

Maximal force development as well as contraction and relaxation time were obtained for CHF and Sham animals at 42 and 112 days. At 42 days we found effects of CHF on these three parameters (P<0.0001), and for maximal force there was also effect of time (P<0.05). Despite the overall higher maximal force development in the CHF group at 42 days, post hoc tests did not reveal any significant differences at the specific time points during the fatigue protocol (Figure 5A). Initial maximal force at 42 days was 99.4 ± 1.4 g and 90.1 ± 6.6 g for the CHF and Sham group respectively. Hence the CHF rats, although having an overall higher force production than Sham rats, displayed a reduction in force towards the end of the fatigue protocol, obtaining values similar to that of Sham rats (Figure 5A). There was an overall increased relaxation time in the CHF group compared with Sham (P<0.05) and with significant interaction with time. Post hoc analysis detected significantly slower relaxation rates at the two latest time points in the fatigue protocol (Figure 5C). The CHF rats also displayed overall slower contraction rates compared with Sham. Post hoc tests revealed significant slower contraction rates at the three latest time points (Figure 5B). At 112 days there were no differences in maximum force, contraction rates or relaxation rates compared with Sham (Figure 5D-F).
Discussion

The underlying hypothesis of the present study was that the increased muscular fatigue associated with CHF, confirmed both by experimental rat studies (16, 18, 26) as well as clinical studies (19, 21), might be caused by changes in ECM. In line with this, the progression of CHF and skeletal muscle fatigability was accompanied by an increase in collagen content and MMP activity at 42 days. At 112 days, however, both the collagen content and MMP activity had returned to Sham levels, and there were no differences with respect to fatigability at this time point. Furthermore, the CHF animals exhibited a reduced level of VEGF in muscle IF at early time points. Collectively these findings suggest that although a marked and stable post-infarction heart failure condition develops in the CHF rats, skeletal muscle fatigue and alterations in skeletal muscle ECM are temporary.

Induction of heart failure by ligation of the left coronary artery in rat is an established model (34) that mimics the development of human post-infarction heart failure. However, the possible impact of e.g. atherosclerosis can not be accounted for in this model. Based on previous studies (16, 31) it is our opinion that 8 to 10 samples for the different analysis would be sufficient to detect physiologically relevant differences between the groups. We were not however, able to do a formal power analysis. Technical and logistic circumstances resulted in some variation in sample size for the different analysis as well as for the different time points. Neither muscle weights nor changes in specific tension of the muscles were measured, and could be regarded as a limitation of the study. However, we have previously demonstrated that there is no difference in muscle weight or muscle water content, neither in fast nor in slow skeletal muscle, between CHF and Sham animals up until 17 weeks in this model (18). This indicates that there is no muscle atrophy or muscle edema in the CHF animals.

The present method for IF isolation has been thoroughly validated (43). By implanting wicks post mortem after circulatory arrest, we avoided net transcapillary transport of fluid and
protein between tissue and the vascular compartment, thereby avoiding any vascular reactions to the wick implantation. Furthermore, by placing wicks between adjacent muscles we minimized the possibility of cell damage and contamination of interstitial fluid with cell proteins. In our opinion, fluid from wicks implanted intermuscularly post mortem reflects the composition of native IF (43).

Previously we have reported that CHF is accompanied by increased MMP activity in both the EDL and the SOL muscles at 42 days (31). In that study we found no differences in mRNA expression neither at protein level of MMP-9 or MMP-2, nor in the activity of tissue inhibitors of MMPs (TIMP-1 and TIMP-2) (31). Carvalho et al. (3) have also reported increased MMP activity in skeletal muscle of rats with CHF induced by monocrotaline, a toxic compound causing right-sided heart failure secondary to pulmonary hypertension, but they did not investigate to what extent the toxin per se could affect skeletal muscle properties. Moreover, they performed their analysis at an early time point (22 days after administration of the drug). In addition to the increased MMP activity at 42 days we found a concomitant moderate increase in collagen content of the tibialis anterior muscle in the CHF animals. These alterations, although also temporary, may reflect a transient disruption of the structural integrity of ECM in the skeletal muscle of CHF animals, initially caused by ligation of the left coronary artery. Among the about 30 subtypes of hitherto described collagens, most with different functions, we chose to examine collagen type III since the plasma level of N terminal type III collagen reportedly is a strong predictor of functional capacity and clinical outcome in CHF patients (27), indicating that turnover of collagen III in ECM might be of pathophysiological importance in CHF. In the present study we found no alterations in collagen III content of fast or slow skeletal muscle. It is therefore likely that the measured increase in total amount of collagen is due to alterations of other collagen types. A candidate
initiator of the increased collagen content could be TGF-β, a well known pro-fibrotic stimulus (29, 33), but it was not increased in IF of CHF rats at any time point in the present study.

All previous studies on muscle fatigue in CHF animals have been performed about 6 weeks after primary surgery and they all suggest reduced contractile function and increased fatigability of skeletal muscle in CHF as compared to Sham (16-18, 26, 37). We are apparently the first to assess contractile function and fatigability of skeletal muscle in CHF animals beyond 6 weeks after induction of heart failure. In line with previous studies we found that SOL from CHF animals displayed An apparent fall in maximal force development during the protocol as well as slower contraction and relaxation rates compared to Sham at 42 days. However, at 112 days there were no differences in contractile function and CHF animals showed no sign of fatigue as compared to Sham. Previous findings of increased skeletal muscle fatigability have not considered this as a possible temporary phenomenon. We have previously shown that the higher maximal force in CHF rats compared to Sham is probably due to the increases in contraction and relaxation time in these animals (16). As the length of the contraction cycle increases, the muscle is not allowed to fully relax before the next stimulation. Consequently, there will be a slight summation and measured maximal force increases.

Heparan sulfate proteoglycans (HSPGs) are important in linking together the fibrous elements of the ECM (32, 42). The HS GAG chains can interact with several molecules, such as VEGF and other growth factors, chemokines and enzymes to modulate their functions (8). The down-regulation of VEGF in IF of CHF rats seen at day 3 and 10 was apparently unrelated to the HSPGs as the content of disaccharides was similar for HS chains for the CHF and Sham groups. The ratio of CS + hyaluronan / HS was also unaltered in skeletal muscles of CHF rats as compared to Sham, indicating no differences between the two groups in the total amount of GAGs.
VEGF is present in the skeletal muscle interstitium of humans (12) and rats (22), and previous studies have suggested a role for VEGF in the regulation of MMP activity (40, 47). Here we hypothesized that an early activation of a cytokine response might trigger skeletal muscle ECM alterations. A main finding was a reduced level of VEGF in the IF of CHF rats at 3 and 10 days. Notably, we previously reported unaltered capillarisation 6 weeks after CHF induction (30) and here we report a highly significant reduction of VEGF in the IF of CHF rats followed by an increased MMP activity. VEGF signaling occurs by binding to VEGF receptors (VEGFRs) and the ligand-receptor complex may be internalized, thus removing VEGF from the IF. Interestingly therefore, the large concentration gradient of VEGF from the muscle IF to plasma in both CHF and Sham animals at all time points indicates a net production of VEGF in the muscle. Furthermore, VEGFRs have been shown to induce MMP-9 (11), that besides MMP-2, accounts for most of the gelatinase activity. Although not formally tested, an up-regulation of VEGFRs could potentially explain both the initially reduced VEGF and the subsequent increase in MMP activity.

In conclusion, we found little evidence to support a role for any sustained alteration of muscular ECM in overt CHF. However, we found early changes in muscular VEGF levels, and the subsequent temporary alterations in MMP activity and collagen content were time-related to increased skeletal muscle fatigability.

**Perspectives and significance**

Increased skeletal muscle fatigability related to intrinsic alterations in the muscle in CHF patients seems thoroughly documented (5, 20, 36, 44). However, in the present study we could not demonstrate skeletal muscle fatigue or ECM alterations at a late stage after induction of heart failure in rats by coronary ligation. Possibly this post-infarction rat model of CHF might be less biologically useful than previously assumed to investigate the
mechanisms of skeletal muscle fatigue relevant for CHF patients. Alternatively, the reduced resistance to fatigue in skeletal muscles in CHF patients may not be directly mechanistically related to the heart failure condition, but rather to some epiphenomenon, e.g. deconditioning. Consequently both basic research and clinical studies are needed to unravel the possible direct links between early-onset alterations in ECM and the development of fatigue within individual skeletal muscle fibers.
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Figure legends

Fig. 1. MMP activity of the SOL and EDL muscles in CHF and Sham animals. Asterisks denote a significantly increased MMP activity in CHF compared to Sham at the specified time points as measured by one sample t-test followed by a Bonferroni correction. Values are mean + SEM and the number of rats per time point is noted in parentheses.

Fig. 2. Total collagen content of the tibialis anterior muscle in CHF and Sham animals. CHF rats had higher overall collagen content as well as a higher collagen content at 42 days as evaluated by post hoc tests compared to Sham. Values are mean + SEM and the number of rats per time point is noted in parentheses. Asterisk denotes significant change.

Fig. 3. No difference in Collagen type III in EDL and SOL in CHF and Sham rats as measured by Western blots. N = 4 for all time points and for Sham and CHF rats. Values are mean + SEM.

Fig. 4. VEGF in IF (Panel A) and plasma (Panel B) of CHF and Sham animals. There was a time-dependent reduction of VEGF in IF of CHF animals compared to Sham. Post hoc tests revealed reduced levels of VEGF at 3 (P<0.0001) and 10 (P<0.05) days after primary surgery. Values are mean + SEM and the number of rats per time point is noted in parentheses. Asterix denotes P<0.05 and double asterix denotes P<0.0001.

Fig. 5. Contractile properties of SOL in CHF and Sham animals during an isometric fatigue protocol at 42 (panels A, B, C) and 112 (panels D, E, F) days. Panels A and D: maximum force during the stimulation train. Panels B and E: contraction rate of the first contraction in
each stimulation train. Panels C and F: relaxation rate of the last contraction of each
stimulation train. The asterix denotes significance for CHF compared with Sham as measured
by two-way ANOVA followed by the Bonferroni correction whereas # denotes a significant
overall higher maximal force compared with Sham. Values are mean and SEM and the
number of rats included for each analysis is noted in parentheses.

Table 1. Animal characteristics.

<table>
<thead>
<tr>
<th></th>
<th>3 days</th>
<th></th>
<th>10 days</th>
<th></th>
<th>42 days</th>
<th></th>
<th>112 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHF</td>
<td>Sham</td>
<td>CHF</td>
<td>Sham</td>
<td>CHF</td>
<td>Sham</td>
<td>CHF</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>287 ± 3 (10)</td>
<td>297 ± 4 (10)</td>
<td>303 ± 4 (13)</td>
<td>320 ± 3 (10)</td>
<td>370 ± 9 (20)</td>
<td>378 ± 9 (14)</td>
<td>445 ± 14 (16)</td>
</tr>
<tr>
<td>Lung weight (g)</td>
<td>3.0 ± 0.2* (10)</td>
<td>1.3 ± 0.03 (10)</td>
<td>3.2 ± 0.2* (10)</td>
<td>1.3 ± 0.1 (10)</td>
<td>3.7 ± 0.2* (10)</td>
<td>1.3 ± 0.0 (10)</td>
<td>3.8 ± 0.2* (10)</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>16 ± 1.6* (10)</td>
<td>4 ± 0.6 (10)</td>
<td>22 ± 2.5* (10)</td>
<td>2 ± 0.6 (10)</td>
<td>26 ± 2.2* (10)</td>
<td>3 ± 0.3 (10)</td>
<td>25 ± 1.6* (10)</td>
</tr>
<tr>
<td>LAD (mm)</td>
<td>5.3 ± 0.2* (10)</td>
<td>3.3 ± 0.1 (10)</td>
<td>5.6 ± 0.1* (10)</td>
<td>3.5 ± 0.1 (10)</td>
<td>6.5 ± 0.2* (10)</td>
<td>3.6 ± 0.1 (10)</td>
<td>6.7 ± 0.2* (10)</td>
</tr>
<tr>
<td>CO (ml/min)</td>
<td>65 ± 3* (10)</td>
<td>134 ± 8 (10)</td>
<td>82 ± 3* (10)</td>
<td>131 ± 7 (10)</td>
<td>81 ± 4* (10)</td>
<td>138 ± 9 (10)</td>
<td>77 ± 4* (10)</td>
</tr>
</tbody>
</table>

Body weight, lung weight, left ventricle end-diastolic diameter (LVEDP), left atrial diameter (LAD) and cardiac output (CO) in CHF rats compared to Sham at 3, 10, 42 and 112 days after primary surgery. Asterisks denote P < 0.05 compared with respective Sham as measured by two-way ANOVA followed by the Bonferroni correction. Values are mean ± SEM. Number of animals in parenthesis.
A  SOL

B  EDL

MMP activity (normalised to Sham)

(8) (7) (10) (8) (7) (10) (8)

Time after primary operation (days)
Time after primary operation (days)

Collagen (% of dry muscle weight)

CHF
Sham

(8) 10 42 112
(8) 13 (9) (14)
(8) 9 (9)
(8) (5)