Enhanced matrix metalloproteinase activity in skeletal muscles of rats with congestive heart failure

Hanne M. Schiøtz Thorud,1,2 Annnicke Stranda,3 Jon-Arne Birkeland,1,2 Per K. Lunde,1,2 Ivar Sjaastad,1,2,4 Svein O. Kolset,3 Ole M. Sejersted,1,2 and Per O. Iversen3

1Institute for Experimental Medical Research and 4Department of Cardiology, Ullevaal University Hospital; and 2Center for Heart Failure Research and 3Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

Submitted 1 February 2005; accepted in final form 25 March 2005

Patients with congestive heart failure (CHF) experience decreased skeletal muscle fatigue resistance during exercise. To some extent, this can be explained by reduced cardiac output. However, there is no clear relation between exercise intolerance and left ventricular function (8). Fatigue is also more prominent in heart failure patients than in controls when they exercise only a small muscle group, despite the low workload, which requires only a limited cardiac output (34). These findings have prompted investigators to search for abnormalities within the skeletal muscles that could explain their limited exercise capacity. Skeletal muscle alterations, including reduced fatigue resistance, fiber type shift, contractile protein composition, reduced oxidative capacity, and electrolyte handling, have been observed in CHF patients and in animals in experimental models of heart failure, as reviewed by Lunde et al. (16). Then what is causing the skeletal muscle abnormalities observed in CHF?

The circulatory levels of TNF-α and monocyte chemoattractant protein (MCP)-1 are increased in CHF patients (2, 3). It is possible that increased plasma levels of cytokines can induce acute or chronic changes in skeletal muscle (22). Gielen et al. (7) observed that exercise training reduced the local expression of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in skeletal muscle of CHF patients. Increased serum levels of inflammatory cytokines have also been associated with the magnitude of muscle apoptosis during CHF (13). In vitro experiments have shown that cytokines increase mRNA expression of protein phosphatase 2A, which is upregulated in CHF patients and is an important regulator of intracellular signaling and apoptosis in skeletal muscle (12). Furthermore, Schulze et al. (25) reported elevated levels of inflammatory cytokines and reduced expression of insulin-like growth factor-I in skeletal muscle of rats with heart failure, which together may contribute to a catabolic metabolism that finally results in skeletal muscle atrophy. Possibly, the increased circulating levels of cytokines and chemokines in CHF rats could induce changes in their skeletal muscles that might be related to contractile dysfunction. Libera et al. (13) found that increased serum levels of inflammatory cytokines were associated with increased levels of muscle apoptosis, and Yamamoto et al. (33) showed that MCP-1 enhanced gene expression and synthesis of matrix metalloproteinases (MMPs) in skeletal muscles in vitro. MMPs are enzymes involved in various processes, including the degradation and turnover of extracellular matrix, immune responses, and bioavailability of signaling substances (18). As recently reviewed, very little information is available on the in vivo role of MMPs in skeletal muscle during disease, including CHF (4).

In this study, we tested the hypothesis that MMP activity is increased in skeletal muscle during CHF. Specifically, we studied whether the local MMP activity and MMP protein and mRNA expression in the slow-twitch soleus (SOL) and fast-twitch extensor digitorum longus (EDL) muscle from CHF rats were altered compared with those obtained from sham-operated (Sham) control rats. We also wanted to examine whether the MMP activity could be related to circulating levels of TNF-α and MCP-1 during CHF.
METHODS

Induction of myocardial infarction. This investigation was conducted in accordance with the Norwegian Animal Welfare Act and was approved by the Norwegian Animal Research Authority. Male Wistar rats (Møllegaard Breeding and Research Centre, Skensved, Denmark; ~320 g body wt) were intubated and ventilated with 68% N₂O, 29% O₂, and 2–3% isoflurane. An extensive myocardial infarction was induced by proximal ligation of the left coronary artery. After 6 wk, the rats were anesthetized and ventilated with isoflurane. Left ventricular pressures were measured as described previously (27). Rats were considered to have CHF and were included in the study if left ventricular end-diastolic pressure (LVEDP) was >15 mmHg at 6 wk. At earlier stages, rats with large anterolateral infarctions were included. Sham animals were subjected to the same surgical procedures, but not coronary artery ligation.

Preparation of tissue samples. SOL and EDL muscles were carefully isolated from one hindleg before removal of the tendons. The midportions from a predefined anatomic location of each of the two muscles were used for analyses. Each of these midportions was divided into two equal-sized samples weighing ~20 mg. We also isolated the heart and removed two 20-mg samples, one from the infarcted area and one from viable myocardium of the left ventricle. After this dissection, we immediately transferred the specimens from both skeletal muscles and the myocardium to ice-cold PBS. The samples were used for measurements of gelatinase activity.

Measurement of gelatinase activity. To determine MMP activity, we initially adopted a gelatinase assay and a collagenses assay. Because these two assays yielded similar results (data not shown), we chose to use the gelatinase assay to simplify the procedure. Acid-soluble calf skin collagen (Sigma-Aldrich, St. Louis, MO) labeled with [³H]sodium borohydride by reductive methylation of the amino groups was used as substrate (31). In addition to the tissue sample, each test tube contained a mixture of the [³H]-labeled gelatin solution and HEPES (0.1 mM; pH 7.5), referred to as the test solution. Incubation of this test solution for 3 days at 37°C with 20-mg tissue samples gave maximal enzyme activity (data not shown). Maximal degradation of the [³H]-labeled gelatin was determined by addition of 25 µl of trypsin (1.25 mg/ml; Sigma-Aldrich) to the test solution without tissue sample. Background levels were determined by counting the radioactivity in a test tube containing 25 µl of trypsin inhibitor type 1 (50 mg/ml; Sigma-Aldrich) without tissue sample. After the incubation period, 100 µl of HEPES and 100 µl of trichloroacetic acid (15% final concentration) were added to a 100-µl sample of the test solution and kept on ice for 30 min to precipitate undegraded [³H]-labeled gelatin. This solution was centrifuged for 2 min (12,000 rpm at room temperature). To measure the acid-soluble radioactivity, two 100-µl aliquots were removed from the supernatant. The radioactivity was measured in a liquid scintillation counter (Wallac WinSpectral 1414, PerkinElmer Instruments, Oslo, Norway). Tissue specimens from CHF and Sham rats were always included in the same assay runs.

Zymography. Blood was collected from the aorta immediately before the rat was killed. We added 100 µl of HEPES (1 M, pH 7.5) and 100 µl of CaCl₂ (100 mM) to 1-ml aliquots of plasma sample. The tubes were then stored at ~20°C. The plasma-derived MMP activity was analyzed by zymography, as described elsewhere (29). Briefly, 12- to 15-µl test samples were mixed with 2X or 6X sample buffer before they were loaded onto an 7% SDS-polyacrylamide gel containing gelatin (0.1% final concentration; Sigma-Aldrich). After electrophoresis, the gels were incubated in 2.5% Triton X-100 for 50 min to wash out SDS, with two changes of the solution. The gels were then incubated in assay buffer [0.05 M Tris buffer, pH 8.0, with 0.2 M NaCl, 0.005 M CaCl₂, and 0.02% (wt/vol) Brij 35] overnight at 37°C to allow possible enzymes in the samples to degrade the gelatin matrix. The gel was then stained with Coomassie brilliant blue, and the gelatinase activity was determined as unstained regions using ImageQuant 5.2 (Amersham Biosciences, Oslo, Norway) or Kodak 1D 3.6 (Pedersen & Sønn, Oslo, Norway) software. As positive controls for MMP-2 and MMP-9, conditioned serum-free media from an osteosarcoma cell line and from the monocytic cell line THP-1, respectively, were used (14, 32).

We used reverse zymography to determine metalloproteinase inhibitory activity as a marker of the activity of tissue inhibitors of MMP (TIMP) (14). Briefly, the muscle samples were treated as described above for zymography. This method detects the disappearance of the fluorescence-labeled gelatin, and the presence of inhibitors appears as light fluorescence bands because of reduced degradation of labeled gelatin. Furthermore, its main advantage is that it allows simultaneous detection of MMP and TIMP activity in the same gel.

Western blotting. The SOL and the EDL muscles were removed from one hindleg and immediately quick-frozen in liquid nitrogen. The samples were kept on ice while they were homogenized (Ultra Turrax model T8 IKA, Tamro Medlab, Lørenskog, Norway). The homogenate was mixed with 16% SDS to a final SDS concentration of 1% and incubated at room temperature for 15 min before aliquots were stored at ~70°C. Samples were run on 10% SDS-polyacrylamide gel (Bio-Rad, Oslo, Norway) and then blotted onto a polyvinylidene difluoride membrane (Millipore, Oslo, Norway). The membrane was further incubated in 5% nonfat dry milk in Tris-buffered saline (pH 7.5) with 0.1% Tween 20 for 24–48 h at 4°C and washed before incubation (24 h at 4°C) with primary mouse anti-rat monoclonal antibody raised against MMP-2 or MMP-9 (Oncongene, VWR, Oslo, Norway; Ermatech, Bergen, Norway). The membrane was washed and finally incubated with a secondary anti-mouse antibody conjugated to horseshadish peroxidase (Amersham Biosciences). Immunoreactivity was detected by the enhanced chemiluminescence method (Amersham Biosciences).

Northern blotting of MMP-2 and MMP-9. Poly(A)⁺ mRNA was extracted from ~200 mg of muscle with oligo(dT)-conjugated paramagnetic beads (Dynal, Oslo, Norway). Poly(A)⁺ mRNA was denatured in 60% formamide (vol/vol) and 7.2% formaldehyde (vol/vol), size fractionated on a formaldehyde-agarose gel (1%) with 20 mM sodium phosphate (pH 7.0) using 9 µg poly(A)⁺ mRNA per lane, and transferred to a nylon filter membrane (0.2 µm) by capillary blotting. The RNA was UV cross-linked to the nylon membrane and prehybridized with salmon sperm DNA before hybridization at 42°C overnight with random primed ([α-³²P]dCTP) cDNA probes: pBSbelb32 for MMP-9 and pBSgelα4 for MMP-2 (both kindly provided by S. Tartare-Deckert, Nice, France). After hybridization, the blots were washed, and radioactivity on the membrane was recorded (BAS1800, Fuji Photo Film, Tokyo, Japan). Densitometric analysis was carried out with ImageGauge software (Fuji Photo Film). The mRNA signal was normalized to the signal obtained by hybridization with a GAPDH probe.

Determinations of serum cytokines. Arterial blood samples for cytokine and chemokine measurements were drawn into blood collection tubes without additive (Becton Dickinson, Plymouth, UK), and the tubes were kept at room temperature for 2 h before centrifugation at 2,000 g for 20 min. The supernatant was stored at ~80°C until analyses were performed. ELISA kits (Biosource International, Camarillo, CA) were used to detect serum concentrations of TNF-α and MCP-1. The minimum detectable concentrations of TNF-α and MCP-1 were 0.7 and 8 pg/ml, respectively.

Statistics. Values are means (SD). Differences were evaluated with Kruskal-Wallis test or Wilcoxon’s rank sum test, as appropriate. Statistical significance was assumed for P < 0.05.

RESULTS

Induction of myocardial infarction and development of CHF. At all three time points, the CHF rats had large anterolateral infarcts and showed signs of CHF, including tachypnea, pleural effusion, and pulmonary congestion. The heart weights
were 83–99% higher in CHF than in Sham animals (Table 1). Lung weight increased ~60% in CHF rats at 3 days compared with Sham animals and increased further at 2 and 6 wk: ~75% and 170%, respectively. LVEDP was significantly increased in CHF rats at 3 days, 2 wk, and 6 wk compared with Sham animals: ~275%, 660%, and 478%, respectively.

Increased MMP activity in skeletal muscle, infarcted myocardium, and plasma of CHF rats. To examine whether there was a change in MMP activity in skeletal muscles during development of CHF, we determined gelatinase activity within single SOL and EDL muscles 6 wk after induction of acute myocardial infarction. Gelatinase activity was markedly enhanced in both muscles of CHF rats compared with Sham animals (Fig. 1). In addition, gelatinase activity was ~50% higher (P < 0.05) in EDL than in SOL muscle. In contrast, the MMP activity in both types of muscle from CHF animals was unaltered compared with that from Sham rats 3 days and 2 wk after the primary operation (data not shown), indicating that a chronic heart failure condition might be a prerequisite for the increase in skeletal muscle MMP activity. Interestingly, at 6 wk, we could not detect any differences in TIMP activity between the two groups, suggesting that the increase in MMP activity was not due to impaired TIMP function (Fig. 2).

At 6 wk after induction of myocardial infarction, we did not find any differences in gelatinase activity of viable left ventricular regions between CHF and Sham animals (data not shown). However, the gelatinase activity of the infarcted areas of the left ventricular myocardium was 6.0 (SD 4.0) times higher (P < 0.05, n = 5) than that of viable myocardial regions in CHF rats.

The MMP activity in plasma was determined by zymography 6 wk after induction of myocardial infarction (Fig. 3). The plasma MMP activity was 6.8 (SD 6.2) times greater (P < 0.05, n = 16) in CHF than in Sham animals.

No changes in MMP protein or mRNA in CHF rats. We used Western blotting to determine whether the observed enhancement of gelatinase activity within the EDL and SOL muscles and in the plasma of CHF rats 6 wk after infarction could be explained by increased protein levels of MMP-2 and/or MMP-9, because they are frequently upregulated in the failing heart (1, 23, 28). Although both proteins could be detected in homogenized muscle samples from CHF and Sham animals, the amounts of neither MMP-2 nor MMP-9 differed (P > 0.05) between the two groups in the SOL or EDL muscle (Fig. 4). Nor could we detect altered levels of MMP-2 or MMP-9 proteins in plasma (data not shown).

We used Northern blotting to determine the gene expressions of MMP-2 and MMP-9 in the SOL and EDL muscles 6 wk after infarction. Expression of neither gene was appreciably changed in either muscle (Fig. 5), supporting the finding of unchanged MMP-2 and MMP-9 protein levels within these two muscles in CHF compared with Sham rats.

Unaltered TNF-α and MCP-1 levels during development of CHF. We hypothesized that increased circulatory levels of TNF-α and/or MCP-1 might trigger activation of skeletal muscle MMPs in CHF. Serum concentrations of TNF-α rose in

**R391**

**Table 1. Animal characteristics**

<table>
<thead>
<tr>
<th>Time After Surgery</th>
<th>CHF (n = 4)</th>
<th>Sham (n = 4)</th>
<th>CHF (n = 4)</th>
<th>Sham (n = 4)</th>
<th>CHF (n = 52)</th>
<th>Sham (n = 52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>326 (19)</td>
<td>320 (14)</td>
<td>332 (2)</td>
<td>347 (28)</td>
<td>392 (36)*</td>
<td>407 (28)</td>
</tr>
<tr>
<td>Heart wt, g</td>
<td>2.21 (0.10)*</td>
<td>1.22 (0.0)</td>
<td>2.43 (0.11)*</td>
<td>1.24 (0.0)</td>
<td>2.52 (0.11)*</td>
<td>1.33 (0.0)</td>
</tr>
<tr>
<td>Lung wt, g</td>
<td>2.16 (0.86)*</td>
<td>1.35 (0.10)</td>
<td>2.72 (0.48)*</td>
<td>1.54 (0.19)</td>
<td>4.1 (0.7)*</td>
<td>1.5 (0.1)</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>9 (1.3)*</td>
<td>2.4 (0.8)</td>
<td>20.7 (1.2)*</td>
<td>2.7 (1.5)</td>
<td>27.2 (6.4)*</td>
<td>4.7 (2.1)</td>
</tr>
<tr>
<td>Values are means (SD). CHF, congestive heart failure; Sham, sham operated; LVEDP, left ventricular end-diastolic pressure. *P &lt; 0.05 vs. Sham.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHF and Sham rats after 2 wk (Fig. 6). However, the observed increase was most likely the result of acute surgery and not progression of cardiac dysfunction, because the increases in serum concentrations were similar in the two groups. Furthermore, we could not detect any significant difference in the MCP-1 concentrations between CHF and Sham rats.

DISCUSSION

In this study, we have shown that CHF due to myocardial infarction in rats was accompanied by a marked increase in MMP activity locally within single skeletal muscles as well as in circulating blood. This is apparently the first report examining the possible role of skeletal muscle MMP in CHF. Moreover, the rise in MMP activity in CHF rats was apparently not the result of enhanced MMP gene expression and a subsequent increase in MMP protein synthesis. Nor was it due to altered TIMP activity. Furthermore, the enhanced MMP activity is unlikely to have been triggered by the proinflammatory cytokine TNF-α or the chemokine MCP-1, because the serum concentrations of these proteins exhibited similar time-dependent changes postoperatively in CHF and Sham rats. Possibly, the development of postinfarction CHF causes a subsequent activation of MMPs that leads to contractile dysfunction of skeletal muscle, thereby contributing to decreased fatigue resistance.

If activation of MMPs leads to degradation of the extracellular matrix, skeletal muscle function could be affected in several ways. 1) The passive stress-strain relation will become altered, which could cause an increased load on the intracellular cytoskeleton and the contractile apparatus. 2) It is feasible that diffusion through the extracellular matrix of small substrates and breakdown products might become altered. 3) The proteins of the extracellular matrix are bound to receptors on the cell. Little is known about the intracellular effects of fewer attachments. The intracellular changes in skeletal muscle from patients and animals with heart failure are numerous, and it is
not possible to link activation of MMPs to specific intracellular alterations that can explain reduced fatigue resistance (16).

In this study, we used a model of postinfarction CHF in otherwise healthy adult male rats. Although it does not directly mimic the failing human heart, possible confounding factors, such as coexistent morbidity, age, gender, and a variable response to therapy, were avoided.

At 6 wk after ligation of the left coronary artery, we consistently found elevated lung and heart weights, indicating development of pulmonary congestion and cardiac hypertrophy. Moreover, all rats with myocardial infarction had LVEDP >15 mmHg after 6 wk, a cutoff value that predicts heart failure with ~100% sensitivity and specificity compared with echocardiographic measurements (27). Thus our previous and present findings strongly suggest that the rats had developed severe CHF 6 wk after induction of myocardial infarction.

The MMPs are being increasingly recognized as key players in the remodeling of the left ventricular myocardium that accompanies the progression to heart failure. In line with this, elevated MMP activity has been demonstrated in various settings of the failing heart, e.g., in dilated cardiomyopathy, ischemic and nonischemic heart failure, and congestive and hypertensive heart failure, as well as after oxidative stress in the myocardium (5, 20, 23, 28, 35). Moreover, inhibition of MMP activity attenuated heart failure in several species (11, 23). Thus, as expected, in the present study, we found a substantial increase in MMP activity within infarcted, compared with viable, left ventricular myocardium of CHF rats.

The major and novel finding in the present study was the substantial increase in MMP activity within single skeletal muscles of CHF rats. Interestingly, this increase was more pronounced in the fast-twitch EDL than in the slow-twitch SOL muscle. It is not known why reduced fatigue resistance occurs in skeletal muscles in heart failure. Experiments aimed at testing possible explanations, such as development of atrophy or alterations in skeletal muscle perfusion, have yielded inconsistent results (16). Several long-term studies report a transition toward a faster skeletal muscle phenotype, but this seems not to be the case in short-term studies (16). However, in our rat model of CHF, atrophy and fiber type conversion do not take place (17). On the other hand, we have noted that the fast-twitch flexor digitorum brevis muscle was more fragile during dissection of single fibers in the CHF than in the Sham rat (15), and Perreault et al. (21) found alterations in contractility and Ca$^{2+}$ handling in the fast-twitch EDL muscle in rats 6 wk after induction of heart failure. The higher MMP activity in the fast-twitch EDL than in the slow-twitch SOL muscle merits further attention.

It is known that CHF rats alter their gene expression profiles in skeletal muscles (26). However, in our study, the increase in gelatinase activity probably results from activation or release of preformed MMP molecules, because neither MMP mRNA levels nor protein amounts were appreciably altered in CHF compared with Sham animals. Notably, the MMP activity increased not only locally within single skeletal muscles, but also in circulating blood of CHF rats. Whether this increase in MMP activity stems from MMP molecules derived from muscle fibers or from some other source remains to be determined.

Because the activity of MMP-2 and MMP-9 accounts for most of the gelatinase activity in our assay and these MMPs are frequently elevated in plasma and within the failing myocardium of humans and rats (1, 23, 28), we chose to specifically examine the gene expression and protein synthesis of MMP-2 and MMP-9. Whether the increased gelatinase activity might also be due to other MMPs, however, cannot be excluded. Interestingly, MMP-2 and MMP-9 were increased in experimentally denervated skeletal muscles in animals as well as in humans with degenerative diseases (10, 24).

We detected TIMP activity, but there was no apparent difference between CHF and Sham rats. The TIMP-related activity measured in our assay mostly stems from TIMP-1 and TIMP-2, although some activity of TIMP-3 and TIMP-4 cannot be excluded. Furthermore, the increased MMP activity observed in the present study could also be due to activation of plasmin, cytokines, and/or mitogen-activated protein kinases (6, 19), and this issue merits further attention.

The initiation and progression to overt heart failure occur in combination with increased myocardial expression and release of various proinflammatory cytokines and chemokines. In particular, TNF-α probably contributes to cardiac dysfunction and might compromise the contractile properties of skeletal muscles as well (9, 23, 30). We could, however, not detect any change in TNF-α levels in circulating blood in the CHF rats. Possibly, a diluted TNF-α increment could have been undetectable in the systemic circulation, because we previously measured elevated local TNF-α levels in the venous effluent from the left ventricle of CHF rats (9).

Although MCP-1 reportedly stimulated MMP gene expression in an in vitro skeletal muscle preparation (33), we were not able to demonstrate any differences in the serum concentrations of this chemokine between CHF and Sham rats during the 6-wk observation period, suggesting that the activation of MMP is independent of stimulation with this chemokine.

In this study, MMP activity was examined in muscles that were removed from killed animals with no prior stimulation or activity. Interestingly, in our hands, muscles from CHF rats develop the same maximal force as muscles from Sham rats in the absence of activity before the contractions (17). Thus increased MMP activity apparently does not prevent normal function in a muscle at the onset of exercise. It is only with continued activity that the dysfunction becomes manifest. Also, the fast-twitch EDL muscle, in which MMP activity was increased, seems to be little affected by the CHF condition. In contrast, dysfunction occurs in the slow-twitch SOL muscle during stimulation by greater-than-normal reduction in force and severe slowing of relaxation. Although beyond the scope of the present study, it remains an intriguing possibility that intact extracellular matrix is a requirement for normal function of the working slow-twitch SOL muscle.

In conclusion, the CHF condition was associated with a marked increase in MMP activity in fast- and slow-twitch muscles but was most prominent in the former. Furthermore, this increased MMP activity was apparently not due to altered TIMP activity or increased secretion of TNF-α or MCP-1. We suggest that understanding the signaling pathway leading to the molecular alterations in skeletal muscle due to heart failure might have important consequences for therapy and exercise training for heart failure patients.

ACKNOWLEDGMENTS

The valuable technical advice of J. O. Winberg is gratefully acknowledged.
MUSCLE MATRIX METALLOPROTEINASE IN HEART FAILURE

GRANTS
The study was financed in part by grants from the Throne Holst Foundation, the Norwegian Research Council, the Norwegian Council for Cardiovascular Disease, and Anders Jahre’s Fund for the Promotion of Science.

REFERENCES


