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

Hanne M. Schiøtz Thorud,1,2 Annicine 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 electrosynaptic 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 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.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
METHODS

Induction of myocardial infarction. This investigation was con-
ducted 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 infar-
cation 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 proce-
dures, but not coronary artery ligation.

Preparation of tissue samples. SOL and EDL muscles were care-
fully 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
each 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 collagenase 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 count-
ning 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 Win Spectral
4141, 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 assayed 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 a 7.5% 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 ID
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 in-
hibitory 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 disappear-
ance 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-polyacryl-
amide gel (Bio-Rad, Oslo, Norway) and then blotted onto a polyvi-
nylidene difluoride membrane (Millipore, Oslo, Norway). The mem-
brane 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 mono-
clonal antibody raised against MMP-2 or MMP-9 (Oncogene, VWR,
Oslo, Norway; Ertamech, Bergen, Norway). The membrane was
washed and finally incubated with a secondary anti-mouse antibody
conjugated to horseradish peroxidase (Amersham Biosciences).

Northern blotting of MMP-2 and MMP-9. Poly(A)+ mRNA was
extracted from ∼200 mg of muscle with oligo(dT)-conjugated para-
magnetic beads (Dynal, Oslo, Norway). Poly(A)+ mRNA was dena-
tured 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 blitting.
The RNA was UV cross-linked to the nylon membrane and prehy-
bridized with salmon sperm DNA before hybridization at 42°C
overnight with random primed (neo32P)dCTP cDNA probes:
pBSbelb32 for MMP-9 and pBSgel4 for MMP-2 (both kindly pro-
vided by S. Tartare-Deckert, Nice, France). After hybridization, the
membrane was further incubated in 5% nonfat dry milk in Tris-buffered
saline, and the presence of inhibitors as a marker of activity.

Determinations of serum cytokines. Arterial blood samples for
cytokine and chemokine measurements were drawn into blood col-
lection 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, Cam-
arillo, 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 antero-
larateral infarcts and showed signs of CHF, including tachypnea,
pleural effusion, and pulmonary congestion. The heart weights

Downloaded from http://ajpregu.physiology.org/ by 10.200.32.247 on August 15, 2017
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
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

Fig. 3. Increased MMP activity in plasma from CHF rats. Plasma from CHF and Sham rats 6 wk after induction of myocardial infarction were subjected to zymography to determine gelatinase activity. Arrows mark degradation products. For comparison, migration positions of MMP-2 and MMP-9 standards are shown. Data from 2 CHF and 2 Sham rats are shown.

![Graph](Fig. 3)

Fig. 4. Unchanged MMP-2 and MMP-9 proteins in skeletal muscles of CHF rats. Samples from EDL and SOL muscles were solubilized and subjected to SDS-PAGE followed by Western blotting using antibodies against rat MMP-2 and MMP-9. Data from 1 CHF and 1 Sham rat are shown and are representative of 4 other CHF and Sham rats.

![Graph](Fig. 4)

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

Fig. 5. Unaltered gene expressions of MMP-2 (A) and MMP-9 (B) in EDL and SOL muscles from CHF rats. Northern blotting was used to determine mRNA for both proteins, and values were normalized to GAPDH. Values are means (SD); n = 7.

![Graph](Fig. 5)

Fig. 6. Similar postoperative changes in serum concentrations of TNF-α (A) and monocyte chemotractant protein-1 (MCP-1, B) in CHF and Sham rats. Values are means (SD).

![Graph](Fig. 6)
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.
REFERENCES


