Effects of Congestive Heart Failure on Ca$^{2+}$ Handling in Skeletal Muscle During Fatigue

Per Kristian Lunde, Ole M. Sejersted, Hanne-Mari Schiøtz Thorud, Theis Tønnessen, Unni Lie Henriksen, Geir Christensen, Håkan Westerblad, Joseph Bruton

Abstract—Skeletal muscle weakness and decreased exercise capacity are major symptoms reported by patients with congestive heart failure (CHF). Intriguingly, these skeletal muscle symptoms do not correlate with the decreased heart function. This suggests that CHF leads to maladaptive changes in skeletal muscles, and as reported most markedly in slow-twitch muscles. We used rats at 6 weeks after infarction to measure expression of key proteins involved in SR Ca$^{2+}$ release and uptake in slow-twitch soleus muscles. We also measured force and myoplasmic free [Ca$^{2+}$] ([Ca$^{2+}$]) in intact single fibers of soleus muscles. CHF rats showed clear signs of severe cardiac dysfunction with marked increases in heart weight and left ventricular end-diastolic pressure compared with sham operated rats (Sham). There were small, but significant, changes in the content of proteins involved in cellular Ca$^{2+}$ handling in CHF muscles: slight increases in SR Ca$^{2+}$ release channels (ie, the ryanodine receptors) and in SR Ca$^{2+}$-ATPase. Tetanic force and [Ca$^{2+}$], were not significantly different between CHF and Sham soleus fibers under resting conditions. However, during the stimulation period there was a decrease in tetanic force without changes in [Ca$^{2+}$], in CHF fibers that was not observed in Sham fibers. The fatigue-induced changes recovered rapidly. We conclude that CHF soleus fibers fatigue more rapidly than Sham fibers because of a reversible fatigue-induced decrease in myofibrillar function. (Circ Res. 2006;98:1514-1519.)

Key Words: skeletal muscle ▪ congestive heart failure ▪ muscle fatigue ▪ Ca$^{2+}$

Patients with congestive heart failure (CHF) frequently report skeletal muscle weakness and decreased fatigue resistance. These skeletal muscle symptoms are not correlated with the observed decrease in heart function.1 This suggests that CHF leads to intrinsic defects in skeletal muscles, and several authors have described important functional and biochemical changes in skeletal muscle cells (for recent reviews see references 2–4). Even so, the mechanism(s) underlying the impaired skeletal muscle function in CHF remains unclear.

Impaired sarcoplasmic reticulum (SR) Ca$^{2+}$ handling has been observed in skeletal muscle cells of rats with CHF induced by myocardial infarction.5–7 Using a rat CHF model at 6 weeks after infarction, we could only detect a subtle slowing of the SR Ca$^{2+}$ reuptake in single fast-twitch fibers isolated from a foot muscle.8 However, in our rat model of CHF, changes in contractile function during stimulation are more pronounced in slow-twitch than in fast-twitch skeletal muscle; slow-twitch soleus muscles of CHF rats showed a marked slowing of relaxation followed by a decrease in force development that was not seen in control muscles.8,9 The aim of the present study was to determine whether skeletal muscle Ca$^{2+}$ handling and force production in CHF animals were changed at an early time point when changes in protein expression were minimal. Therefore we measured the expression of key proteins involved in SR Ca$^{2+}$ release and uptake in slow-twitch soleus and fast-twitch extensor digitorum longus (EDL) muscles of CHF and Sham rats. Furthermore, we measured myoplasmic free [Ca$^{2+}$] ([Ca$^{2+}$]) and force in intact single cells isolated from soleus muscles of CHF and Sham rats; these are the first simultaneous measurement of [Ca$^{2+}$], and force in single slow-twitch fibers of rat muscles. The results show that at six weeks after infarction, there were only small changes in protein levels in CHF muscles. In accordance with these findings, [Ca$^{2+}$], and force were not significantly different in unfatigued CHF and Sham soleus fibers. However, during fatigue there was a decrease in tetanic force without changes in [Ca$^{2+}$], in CHF fibers that was not observed in Sham fibers.

Materials and Methods

An expanded Materials and Methods section is given as a data supplement (available online at http://circres.ahajournals.org).

Original received October 22, 2004; first resubmission received March 3, 2005; second resubmission received February 8, 2006; revised second resubmission received April 11, 2006; accepted May 3, 2006.

From the Institute for Experimental Medical Research (P.K.L., O.M.S., H.-M.S.T., U.L.H., G.C.), Ullevål University Hospital, Oslo, Norway; the Center for Heart Failure Research (P.K.L., O.M.S., H.-M.S.T., T.T., U.L.H., G.C.), University of Oslo, Norway; the Department of Physiology and Pharmacology (H.W., J.B.), Karolinska Institutet, Stockholm, Sweden; and the Department of Cardiothoracic Surgery (T.T.), University of Oslo, Ullevål University Hospital, Oslo, Norway.

Correspondence to Per Kristian Lunde, Institute for Experimental Medical Research, Ullevål University Hospital, N-0407 Oslo, Norway. E-mail p.k.lunde@medisin.uio.no

© 2006 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/01.RES.0000226529.66545.e5

1514
Experimental Model
Under gas anesthesia, Wistar rats (250 to 300 g; Scanbur BK, Sollentuna, Sweden and Møllegaard, Ry, Denmark) were subjected to ligation of the left coronary artery (CHF) or a Sham (Sham) operation as described previously.10,11 One, 6, or 17 weeks after the primary operation, rats were again anesthetized and systolic blood pressure and left ventricle end-diastolic pressure (LVEDP) were determined by a 0.67-mm (2F) microtip pressure catheter (SPR-407, Millar Instruments Inc) inserted through the right carotid artery. Only rats with LVEDP ≥15 mm Hg were included in the CHF group in subsequent experiments.12 After determination of LVEDP, soleus and EDL muscles were dissected free and the rats were euthanized by cervical dislocation. The experiments were approved by the Norwegian Animal Research Authority and the Stockholm North local ethical committee.

Protein Immunoblot Analysis and mRNA Quantification
Muscle membrane proteins were isolated as previously described.13 Antibody specificity was tested by a standard Western blot protocol of 50 μg of muscle membrane protein. For quantification of the different proteins a slot blot technique was used. The isolation of mRNA was done with oligo(dT)-conjugated paramagnetic beads, and mRNA expression was determined by standard Northern blot technique.

Isolation of SR Membranes and Quantification of Membrane Proteins and Tissue Electrolytes
SR membranes were isolated for determination ofryanodine receptor (RyR) and Ca\(^{2+}\)-ATPase content as described elsewhere.14 Ca\(^{2+}\)-ATPase was determined by Ca\(^{2+}\) dependent steady-state incorporation of [\(^{32}\)P] in the SR preparation.15 RyR was quantified by binding of [\(^{3}H\)]ryanodine.14 The Na\(^{+}\)-K\(^{+}\)-ATPase content was determined by vanadate supported binding of [\(^{3}H\)]ouabain in muscle samples.16 Electrolyte content was determined in tissue specimens as described previously.17

Experiments on Intact Single Fibers
Six weeks after the primary operation, single fibers were manually dissected from soleus muscles as described previously.18 Platinum clips were attached to the tendons and the isolated fiber was suspended horizontally between an adjustable hook and an Akeres AE801 force transducer in a stimulation chamber. The fiber was superfused with a standard Tyrode solution; experiments were performed at 24 to 26°C. The fluorescent indicator indo-1, which was injected into the cell, was used to measure [Ca\(^{2+}\)]. Successful [Ca\(^{2+}\)] measurements were performed in 9 fibers from CHF rats and 10 fibers from Sham rats. In addition, 1 Sham fiber and 3 CHF fibers not injected with indo-1 were used to observe the effect of caffeine at the end of induction of fatigue. To establish the force-frequency and force-[Ca\(^{2+}\)] relationships under control conditions, contractions were evoked at 1 minute intervals using 1 s trains of pulses at 10 to 100 Hz.19 Force-[Ca\(^{2+}\)] curves were generated by plotting mean force against the mean [Ca\(^{2+}\)], measured during the last 500 ms of stimulation in these contractions. Data points were fitted to the following equation: \(P = P_{\text{max}} [\text{Ca}^{2+}]^{n}/(\text{Ca}_{o}^{2+} + [\text{Ca}^{2+}]^{n})\), where \(P_{\text{max}}\) is the force at saturating [Ca\(^{2+}\)], \(\text{Ca}_{o}^{2+}\) is the [Ca\(^{2+}\)], giving 50% of \(P_{\text{max}}\), and \(n\) describes the steepness of the function. Fatigue was then produced by 250 tetani (50 Hz, 1 s duration) given at 2 s intervals. A subset of fibers were exposed to 2 mmol/L caffeine after 250 tetanic contractions and the fibers were stimulated in the presence of caffeine to produce maximal Ca\(^{2+}\) release from the SR.20 Recovery of force and [Ca\(^{2+}\)] were followed for 30 minutes in those fibers that had not been exposed to caffeine.

Statistics
Data are presented as mean±SEM. Student unpaired t tests were used to establish significant differences between CHF and Sham muscles. A probability value less than 0.05 was considered statistically significant.

Results

Animal Characteristics
Heart weight and the heart to body weight ratio were significantly higher in the CHF than in the Sham group (Table 1). LVEDP was significantly increased at all 3 time points (1, 6, and 17 weeks after myocardial infarction) and increased with time in the CHF group. The systolic pressure was ~20 mm Hg lower in CHF compared with Sham at 1 and 6 weeks after myocardial infarction. Heart rate was not significantly different between the two groups. Taken together, these data are consistent with a marked cardiac dysfunction in the CHF group. Further characteristics of the present rat model of CHF have been reported by us previously.9,11

The weight and water content of soleus and EDL muscles were not significantly different between CHF and Sham groups at any time point (supplemental Table I). Thus, CHF caused no apparent muscle atrophy or edema.

Ca\(^{2+}\) Handling Proteins
Northern and Western blots were performed on soleus and EDL in CHF and Sham at 6 weeks after myocardial infarction (supplemental Figure I). CHF did not significantly alter mRNA or protein levels of RyR, SR Ca\(^{2+}\)-ATPases (SERCA1 and SERCA2), phospholamban (PLB), or Na\(^{+}\)-K\(^{+}\)-ATPase subunits in either soleus or EDL muscles.

It is difficult to detect small changes in protein concentration with Western blots and we therefore also performed functional tests to assess changes in Ca\(^{2+}\)-ATPase, RyR, and Na\(^{+}\)-K\(^{+}\)-ATPase. Thus, the Ca\(^{2+}\)-ATPase content was also assessed by steady state [\(^{32}\)P] incorporation. In the soleus

---

**TABLE 1. Heart Weights and Hemodynamic Data in Sham and CHF Rats**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>CHF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Week</td>
<td>6 Weeks</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HW, mg</td>
<td>814±12 (16)</td>
<td>1028±17 (29)</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>2.95±0.04 (16)</td>
<td>2.69±0.04 (29)</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>5.5±0.6 (17)</td>
<td>2.7±0.3 (29)</td>
</tr>
<tr>
<td>Systolic pressure, mm Hg</td>
<td>119±2 (18)</td>
<td>120±2 (30)</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>406±10 (17)</td>
<td>388±9 (28)</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n). *Significant difference Sham vs CHF (P<0.05).

HW indicates heart weight; BW, body wt; HR, heart rate.
fibers tended to produce a higher maximum force and to be more sensitive to \([\text{Ca}^{2+}]_i\), (Table 2).

Fatigue was induced by 250 repeated tetani and mean tetanic \([\text{Ca}^{2+}]_i\), and force records obtained in the first 5 and final 5 tetani are shown in Figure 2A and B, respectively. In Sham fibers \((n=10)\), tetanic \([\text{Ca}^{2+}]_i\) was \(1.99\pm0.40\ \mu\text{mol/L}\) in the first tetanus and decreased significantly to \(1.10\pm0.21\ \mu\text{mol/L}\) in the last tetanus \((P<0.05)\). Force was also significantly decreased by \(21\pm6\%\ (P<0.05)\). In contrast to the marked decrease in Sham fibers, there was no significant change in tetanic \([\text{Ca}^{2+}]_i\), in CHF fibers \((n=9)\) during fatigue, and tetanic \([\text{Ca}^{2+}]_i\), was \(1.00\pm0.13\ \mu\text{mol/L}\) and \(1.02\pm0.30\ \mu\text{mol/L}\) in the first and last tetanus, respectively. Despite virtually unchanged tetanic \([\text{Ca}^{2+}]_i\), tetanic force was significantly decreased by \(45\pm12\%\) in the last tetanus \((P<0.05)\). The difference in fatigue mechanisms between Sham and CHF fibers is highlighted in Figure 2C, which shows the mean tetanic \([\text{Ca}^{2+}]_i\), and force in the first and last

<table>
<thead>
<tr>
<th>TABLE 2. Hill Parameters for Force-([\text{Ca}^{2+}]_i) Relationship for CHF ((n=7)) and Sham ((n=9)) Fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHF</strong></td>
</tr>
<tr>
<td>(P_{\text{max}}), kN/m²</td>
</tr>
<tr>
<td>([\text{Ca}^{2+}]_{50}), (\mu\text{M})</td>
</tr>
<tr>
<td>(N) (dimensionless)</td>
</tr>
<tr>
<td>Values are mean±SEM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
</tbody>
</table>

Figure 1. Content of \([\text{Ca}^{2+}]_i\)-ATPase \((A)\), \([\text{H}]\)ryanodine binding \((B)\), and \(\text{Na}^+,\text{K}^+\)-ATPase \((C)\) in soleus and EDL muscles from CHF (open symbols) and Sham (filled symbols) rats. Measurements performed 1, 6, and 17 weeks after primary operation as indicated. *Significant difference Sham vs CHF, \(P<0.05\). Values are mean±SEM. (n=6 to 21).

Figure 2. Averaged \([\text{Ca}^{2+}]_i\) (A) and force (B) of isolated soleus fibers records obtained from the first 5 and last 5 tetanic contractions during the fatigue protocol of 250 tetani. Dotted lines are CHF, and solid lines are Sham data. C. Averaged force-\([\text{Ca}^{2+}]_i\) curves obtained under control conditions in Sham and CHF fibers together with mean force-\([\text{Ca}^{2+}]_i\) values of the first and last fatiguing tetanus. Arrows indicate the changes in force-\([\text{Ca}^{2+}]_i\) during fatigue.

Figure 1. Content of \([\text{Ca}^{2+}]_i\)-ATPase \((A)\), \([\text{H}]\)ryanodine binding \((B)\), and \(\text{Na}^+,\text{K}^+\)-ATPase \((C)\) in soleus and EDL muscles from CHF (open symbols) and Sham (filled symbols) rats. Measurements performed 1, 6, and 17 weeks after primary operation as indicated. *Significant difference Sham vs CHF, \(P<0.05\). Values are mean±SEM. (n=6 to 21).

Figure 2. Averaged \([\text{Ca}^{2+}]_i\) (A) and force (B) of isolated soleus fibers records obtained from the first 5 and last 5 tetanic contractions during the fatigue protocol of 250 tetani. Dotted lines are CHF, and solid lines are Sham data. C. Averaged force-\([\text{Ca}^{2+}]_i\) curves obtained under control conditions in Sham and CHF fibers together with mean force-\([\text{Ca}^{2+}]_i\) values of the first and last fatiguing tetanus. Arrows indicate the changes in force-\([\text{Ca}^{2+}]_i\) during fatigue.
tetani together with the average force-[Ca\textsuperscript{2+}] curves of Sham and CHF fibers. In Sham fibers, tetanic [Ca\textsuperscript{2+}], and force followed the force-[Ca\textsuperscript{2+}] curve during fatigue, suggesting that the decline in force was a consequence of reduced SR Ca\textsuperscript{2+} release and thus reduced tetanic [Ca\textsuperscript{2+}]. This was clearly not the case in CHF fibers in which force was markedly reduced during fatigue despite unchanged tetanic [Ca\textsuperscript{2+}]. Thus, by the end of fatigue, the measured force and [Ca\textsuperscript{2+}], were completely off the force-[Ca\textsuperscript{2+}] curve obtained under control conditions. Therefore, in CHF the decline in force during the stimulation may be caused by a reduced myofibrillar Ca\textsuperscript{2+} sensitivity (ie, increased Ca\textsubscript{aq}) or a decreased cross-bridge force generating capacity (ie, decreased P\textsubscript{max}). We attempted to distinguish between these two possibilities by exposing some of the fibers to 2 mmol/L caffeine at the start of fatigue, whereas in CHF fibers tetanic force in the presence of 2 mmol/L caffeine. This resulted in full restoration of the maximum force in the present fibers was 10 Hz, which is very similar to that measured in intact soleus muscles but markedly lower than in single fast-twitch flexor digitorum brevis (FDB) fibers (≈20 Hz).8

### Effects of CHF on Fatigue Development

The force decline during stimulation was more pronounced in soleus fibers from CHF rats than from Sham rats, in accordance with previous in vitro and in vivo findings in whole muscles.7,9,25 Surprisingly the force decrease in CHF soleus fibers was not accompanied by any decrease in tetanic [Ca\textsuperscript{2+}]. This is in contrast to the findings in the Sham soleus fibers as well as our previously reported observations in fast-twitch fibers of both CHF and Sham rats, where there was a clear relationship between the decrease in tetanic force and [Ca\textsuperscript{2+}]. It should be noted that the lack of decline in tetanic [Ca\textsuperscript{2+}], during fatigue does not necessarily mean that there was no decrease in SR Ca\textsuperscript{2+} release. In principle, this could be the result of both a decreased cytosolic Ca\textsuperscript{2+} buffering because of less Ca\textsuperscript{2+} bound to troponin C combined with a decreased SR Ca\textsuperscript{2+} release.26,27

The accelerated force decline during stimulation in the present CHF soleus fibers may be attributed to a decreased Ca\textsuperscript{2+}-sensitivity of the contractile elements or a reduced Ca\textsuperscript{2+} handling between single soleus fibers from CHF and Sham rats under resting conditions. Furthermore, our previous studies revealed only minor changes in contractile function in unfatigued whole soleus muscles of CHF rats.9,22,23 Although we did not perform fiber typing of the single fibers used in the present study, several pieces of evidence indicate that they were slow-twitch type 1 fibers. First, soleus muscles of Wistar rats contain >90% type 1 fibers, and this is not changed in the CHF condition.24,25 Second, the frequency required to get 50% of the maximum force in the present fibers was ≈10 Hz, which is very similar to that measured in intact soleus muscles but markedly lower than in single fast-twitch flexor digitorum brevis (FDB) fibers (≈20 Hz).8

### Discussion

This study measured soleus muscle function at 6 weeks after myocardial infarction and the major results are: (1) In control conditions, CHF did not induce any significant alterations of [Ca\textsuperscript{2+}], and force during contractions of single soleus fibers. (2) Compared with fibers from Sham rats, single soleus fibers from CHF rats showed a reduced fatigue resistance that was not attributable to a decrease in tetanic [Ca\textsuperscript{2+}], but rather to a combination of a decrease in myofibrillar Ca\textsuperscript{2+}-sensitivity and force generating capacity.

### Effects of CHF on Protein Expression and Contraction of Unfatigued Soleus Muscle

The present CHF rat model showed clear signs of severe cardiac dysfunction with major increases in heart weight, heart to body weight ratio, and LVEDP.3 Despite this we observed no significant differences between soleus muscles of CHF and Sham rats regarding muscle weight, water content, or total content of Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, or K\textsuperscript{+}. Moreover, changes in the content of Ca\textsuperscript{2+} handling proteins were modest with small but significant increases observed for SR Ca\textsuperscript{2+}-ATPase and RyR in soleus muscles, which is in line with previous studies on this rat CHF model.22,23 Thus the changes in CHF muscles were predicted to be subtle, and in accordance with this we observed no significant differences in contractile function or intracellular Ca\textsuperscript{2+} handling between single soleus fibers from CHF and Sham rats under resting conditions.

### TABLE 3. [Ca\textsuperscript{2+}] and Contractile Properties During Fatigue of Soleus Fibers From CHF (n=8) and Sham (n=10) Rats

<table>
<thead>
<tr>
<th>Fatigue</th>
<th>First Contraction</th>
<th>Last Contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>CHF</td>
</tr>
<tr>
<td>[Ca\textsuperscript{2+}] decay, ms</td>
<td>119±34</td>
<td>102±24</td>
</tr>
<tr>
<td>50% relaxation time, ms</td>
<td>159±14</td>
<td>149±13</td>
</tr>
<tr>
<td>90% relaxation time, ms</td>
<td>311±30</td>
<td>279±27</td>
</tr>
</tbody>
</table>

Values are mean±SEM. There are no significant differences between CHF and Sham.

**Note:**

- **First Contraction** and **Last Contraction** refer to the initial and final contractions of the soleus fibers, respectively.
- **Fatigue** denotes the period during which the force declined.
- **Sham** and **CHF** indicate the control and heart failure conditions, respectively.

---

**References:**

20. In Sham fibers, 2 mmol/L caffeine increased tetanic force to 93±3% (n=3) at the start of fatigue, whereas in CHF fibers tetanic force in the presence of caffeine was increased to 74±13% (n=5). Thus, in CHF fibers, force could not be restored to pre-fatigue levels despite presumably saturating levels of [Ca\textsuperscript{2+}].

In Sham fibers (n=10), basal [Ca\textsuperscript{2+}], (measured immediately before the first and the last tetani) increased by 75% during activity, from 60±18 mmol/L to 115±48 mmol/L (P<0.05). Basal [Ca\textsuperscript{2+}], showed little change in CHF fibers (n=9) being 50±10 mmol/L and 59±12 mmol/L in the first and last fatiguing tetanus, respectively. There were no significant stimulation-induced differences in the rate of SR Ca\textsuperscript{2+} removal between the two groups as measured by the time taken for [Ca\textsuperscript{2+}], to fall to 50% of its tetanic value (Table 3). Furthermore, in fatigued muscles there were no significant differences between the groups in resting force (see Figure 2) or force relaxation times (Table 3).

Recovery of tetanic force (70 Hz, 1 s duration) was measured at 10 and 30 minutes after the fatigue protocol and showed no significant difference between Sham (n=6) and CHF (n=6) fibers: 89.6±15.4% versus 70.1±13.9% of the original (ie, before the start of stimulation protocol) at 10 minutes and 81.4±12.1% versus 70.4±12.3% at 30 minutes. At 30 minutes recovery we also measured the force production at saturating [Ca\textsuperscript{2+}], by producing a 70 Hz tetanus in the presence of 2 mmol/L caffeine. This resulted in full restoration of the original force in both Sham and CHF fibers (103±3.3% and 100±5.6% respectively of the original).
ability of the cross-bridges to generate force. We have previously distinguished between these two possibilities by constructing force-[Ca$^{2+}$] curves from measurements of force and [Ca$^{2+}$], during the final phase of fatigu ing stimulation.28 Unfortunately this approach could not be used for the present CHF fibers simply because tetanic [Ca$^{2+}$], did not decrease. An alternative method of assessing the maximum cross-bridge force in fatigu ing is to produce tetanic contractions in the presence of 2 mmol/L caffeine that is known to substantially increase [Ca$^{2+}$], in rat soleus muscle fibers.20 We found that although exposure to caffeine at the end of stimulation increased the tetanic force in CHF soleus single fibers by $\approx 10\%$, force was still considerably less than at the start of the stimulation protocol. Thus, the decrease in force was attrib utable to both reduced Ca$^{2+}$-sensitivity of the contractile elements and reduced force generation by the cross-bridges.

In the present study, we followed force recovery for 30 minutes in fibers that had not been exposed to caffeine during the induction of fatigue. At 30 minutes after fatigue, the tetanic force produced in the presence of caffeine was very similar to the tetanic force before the induction of fatigue in both CHF and Sham fibers, which shows that the force decrease during fatigue was fully reversible. Although previous studies have suggested that fatigue under certain conditions can lead to increased degradation of proteins such as troponin I,29–31 the present finding that caffeine could restore force fully to its pre-fatigue value strongly indicate that no significant protein degradation occurred during fatigue under the present conditions.

Decreased mitochondrial capacity and impaired aerobic ATP production have been reported in skeletal muscles in CHF.32 Therefore, CHF muscles might depend more on anaerobic metabolism during intense activity and this will result in larger changes in high energy phosphates, H$^+$ and inorganic phosphate ions (P$_i$), than those seen in Sham muscle. In a careful study in rat skinned fibers, it was demonstrated that increases in H$^+$ and P$_i$ have the most significant effect on cross-bridge force production and myofibrillar Ca$^{2+}$ sensitivity,33 although recent studies indicate that increased P$_i$ has a greater effect than H$^+$ ions on force production in mammalian muscle studied at physiological temperatures.34 Thus, a decreased oxidative capacity may contribute to the accelerated force decrease in our CHF soleus fibers during the induction of fatigue. Another possible consequence of impaired mitochondrial function in CHF muscles is increased oxidative stress caused by increased production of reactive oxygen species (ROS). Increased ROS production has been measured in limb muscles of CHF mice35 and in the diaphragm muscle of CHF rats.36 Recently, it has been demonstrated that muscles in both humans with chronic heart failure and CHF rats have a decreased level of antioxidant enzymes.37,38 Thus, we propose that the faster fatigue development in CHF soleus fibers may be attributable to impaired mitochondrial function resulting in both increased dependence on anaerobic ATP production and an increased ROS production.

Conclusions

Six weeks after coronary artery ligation, basic contractile function and intracellular Ca$^{2+}$ handling were not signifi cantly different between isolated single soleus muscles fibers of CHF and Sham rats, which fit with the limited differences in the content of Ca$^{2+}$ handling proteins. However, CHF soleus fibers fatigue more rapidly than Sham fibers attributable to a marked decrease in myofibrillar Ca$^{2+}$ sensitivity and cross-bridge force production.

Acknowledgments

We are grateful to Annlaug Ødegaard, Line Solberg, and Morten Eriksen for skilful technical assistance.

Sources of Funding

This study was supported by grants from Anders Jahre’s Fund for Promotion of Science, The Norwegian Council of Cardiovascular Diseases, Eastern Norway Regional Health Authority, The Swedish Research Council (Projects 10842 & 14453), The Swedish National Center for Sports Research, and funds at the Karolinska Institutet.

Disclosures

None.

References


Sources of Funding

This study was supported by grants from Anders Jahre’s Fund for Promotion of Science, The Norwegian Council of Cardiovascular Diseases, Eastern Norway Regional Health Authority, The Swedish Research Council (Projects 10842 & 14453), The Swedish National Center for Sports Research, and funds at the Karolinska Institutet.


Effects of Congestive Heart Failure on Ca$^{2+}$ Handling in Skeletal Muscle During Fatigue
Per Kristian Lunde, Ole M. Sejersted, Hanne-Mari Schiøtz Thorud, Theis Tønnessen, Unni Lie Henriksen, Geir Christensen, Håkan Westerblad and Joseph Bruton

_Circ Res._ 2006;98:1514-1519; originally published online May 11, 2006;
doi: 10.1161/01.RES.0000226529.66545.e5
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/98/12/1514

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2006/05/11/01.RES.0000226529.66545.e5.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
Online data supplement

**Materials and Methods**

**Experimental model**

Two groups of rats have been used in this study. For single fiber measurements rats weighing about 300 g were intubated and operated under isoflurane anaesthesia (Forene, Abott Scandinavia AB, Solna, Sweden, 2-2.5% in 30/70% O\textsubscript{2}/N\textsubscript{2}O). The rest of the rats (weighing about 265 g) were operated under halotane anaesthesia (Fluothane, Zeneca Pharma, Chesire, UK; 1-2% in 30/70% O\textsubscript{2}/N\textsubscript{2}O). As described in earlier work from this laboratory, a limit of LVEDP at 15 mmHg were used to select for animals with CHF. This limit discriminates well between rats with CHF and rats with myocardial infarction, but no CHF.\textsuperscript{1,2} The CHF rats have increased lung weight\textsuperscript{3} and increased myocardial expression of ANF.\textsuperscript{4} After determination of LVEDP the rats were sacrificed and for [Ca\textsuperscript{2+}]\textsubscript{i} and force measurements the soleus muscle was dissected free and kept in solution containing (mM) NaCl 121, KCl 5, MgCl\textsubscript{2} 0.5, Na\textsubscript{2}HPO\textsubscript{4} 0.4, CaCl\textsubscript{2} 1.8, EDTA 0.1, NaHCO\textsubscript{3} 24, glucose 5.5 and foetal calf serum (0.2 %, Gibco), bubbled with 95% O\textsubscript{2}/5% CO\textsubscript{2} (pH 7.4). Soleus and EDL from the other group of rats were dissected free and immediately frozen in liquid nitrogen. The heart was removed and weighed. During the period between the primary and secondary operation the rats were kept in standard cages and given free access to water and food. The night and day cycle was 12 h/12 h. About 85 % of the rats were alive 2 days after the operation and a total of 80 % of the rats lived until they were sacrificed.

**Protein immunoblot analysis**

Muscle membrane proteins were isolated as previously described.\textsuperscript{5} In short, frozen muscle samples from the six week group (10-20 mg) were homogenised in a buffer containing 210...
mM sucrose, 30 mM HEPES (pH 7.4), 2 mM EGTA, 40 mM NaCl and 2 mM PMSF with a Polytron homogeniser at full speed for 2 x 15 s. The homogenate was mixed with a KCl/Na\(^+\)-pyrophosphate buffer ([final]: 500 mM/25 mM) and incubated on ice for 15 min. All membranes were recovered in a pellet after centrifugation at 200,000 g for 75 min.

For test of antibody specificity 50 µg membrane protein was loaded on to a 8 % SDS/polyacrylamid gel and electrophoretical separated by 150 V for 1.5 h at 4 °C. Proteins were transferred from the gel to polyvinylidene difluoride (PVDF) membrane by applying 100 V for 2 h at 4 °C and the membrane was treated as described later.

For quantification of the different proteins, 0.5, 1 and 2 µg of the membrane preparation were loaded onto a PVDF filter membrane by the use of a slot blot filtration manifold, type Minifold II (Schleicher & Schuell GmbH, Dassel, Germany). PVDF membranes were blocked by incubating for 1 h at room temperature or overnight at 4 °C in 10 % non-fat dry milk in Tris buffered saline, pH 7.5 with 0.1 % Tween-20 (TBS-T) or in 3 % IgG free bovine serum albumin (BSA) in TBS-T overnight for the membranes used for the determination of α1, α2 and β1 subunit of the Na\(^+\),K\(^+\)-ATPase. The membranes were then incubated for 1 h at room temperature with the primary antibodies [anti-SERCA1 (MA3-912), anti-SERCA2 (MA3-919), anti-RyR (MA3-925), anti-Na\(^+\),K\(^+\)-ATPase (α1 subunit) (MA3-929) anti-Na\(^+\),K\(^+\)-ATPase (α3 subunit) (MA3-915) all from Affinity Bioreagents, CO, USA and anti-PLB (#05-205), anti-Na\(^+\),K\(^+\)-ATPase (α2 subunit) (#06-168), anti-Na\(^+\),K\(^+\)-ATPase (β1 subunit) (#06-170) all from Upstate Biotechnology, NY, USA] diluted in blocking solution at following dilutions: SERCA1: 1:2500, SERCA2: 1:1000, RyR: 1:5000, PLB: 1:2000, Na\(^+\),K\(^+\)-ATPase (α1 subunit): 1:3000, Na\(^+\),K\(^+\)-ATPase (α2 subunit): 1:1000, Na\(^+\),K\(^+\)-ATPase
(α3 subunit): 1:1000 and Na⁺,K⁺-ATPase (β1 subunit): 1:1000. After 3 washes with TBS-T the PVDF membranes were incubated for 1 h with anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (NA-931 or NA-934, respectively, Amersham, Oakville, Ontario, Canada) in TBS-T. The PVDF membranes were washed 5 times with TBS-T, and the immunoreactivity was detected by the enhanced chemiluminescence method (RPN3103H, Amersham, Oakville, Ontario, Canada). The signal intensity of the bands was quantified with the ImageQuant software (Molecular Dynamics Pty Ltd., Queensland, Australia). A linear relationship between the signal intensities and the amount of protein on the membrane indicated that the membrane was not overloaded. Between the detection of each antibody the primary and secondary antibody were removed by incubation in a stripping buffer containing 100 mM 2-mercaptoethanol and 2 % SDS in 62.5 mM Tris-HCl for 30 min at 50 °C. As control tissue we used rat kidney (#12-146) and rat brain (#12-144) microsomal membrane protein preparation from Upstate Biotechnology, NY, USA.

**mRNA quantification**

Extraction of PolyA⁺mRNA from around 250 mg muscle from the six week group was done with oligo(dT)-conjugated paramagnetic beads (Dynal A/S, Oslo, Norway). PolyA⁺mRNA was denatured in 60 % formamide (v/v) and 7.2 % formaldehyde (v/v), size fractionated on a formaldehyde-agarose gel (1 %) with 20 mM Na-phosphate (pH7.0) using 10 µg polyA⁺mRNA per lane and transferred to a nylon filter membrane (Nytran 0.2 µm) by capillary blotting. When the membranes should be used for determination of PLB-, SERCA1-, SERCA2a- and RyR1-mRNA, the RNA was UV-cross-linked to the nylon membrane and prehybridised for at least 2 hours at 42 °C with salmon sperm DNA before hybridisation was carried out at 42 °C overnight with random primed (³²PαdCTP and dATP)
cDNA probes. For the determination of mRNA for α1, α2 and β1 subunit of the Na⁺,K⁺-
ATPase the membranes were prehybridised for 16-20 hours and hybridised for 3 days. The
probes used were a 1000 bp EcoRI insert of phospholamban (PLB) cDNA (pRCPLB1), a
EcoRI/XhoI fragment (585 bp) of SERCA2a cDNA (pRH39) (both probes kindly provided
by K. Schwartz, Paris, France), a EcoRI insert of RyR1 cDNA (CRC105) (kindly provided
by A. Marks, NY), a EcoRI/Sall fragment (600 bp) of SERCA1 cDNA (kindly provided by
M. Thelen, Amsterdam, Netherlands), EcoRI/HindIII fragments of α1 (332 bp) and α2 (344
bp) subunit of Na⁺,K⁺-ATPase (both kindly provided by D. Charlemagne, Paris, France) and
a EcoRI insert (1.2 kb) of Na⁺,K⁺-ATPase β1 subunit cDNA (kindly provide by R. Levenson,
Pennsylvania, USA). Following hybridisation, blots were either washed sequentially with 2
x SSC/0.1 % SDS at room temperature for 5 x 5 min and with 0.1 x SSC/0.1 % SDS at 60 °C
for 2 x 15 min (PLB, SERCA1, SERCA2a, RyR1) or washed 4 times with 0.1 x SSC/0.1 %
SDS in 15 min at 35 °C (α1, α2 and β1 subunit of the Na⁺,K⁺-ATPase). Radioactivity on the
membrane was recorded and scanned in a Phosphorimager and a densitometric analysis was
carried out with ImageQuant software (both from Molecular Dynamics Pty Ltd., Queensland,
Australia). For a more accurate quantification 0.5, 1 and 2 µg polyA⁺mRNA was directly
applied to nylon membrane by the use of a slot-blot manifold (Minifold II,
Schleicher&Schuell GmbH, Dassel, Germany), and hybridised with the same probes as
above. The obtained mRNA signal was normalised to the signal obtained by hybridisation
with an end labelled oligo(dT)18 probe (Eurogentec, Seraing, Belgium).

Statistics

Data are presented as means ± SEM. Weights, water-, electrolyte-, RyR-, Ca²⁺-ATPase- and
Na⁺,K⁺-ATPase-content were analysed by multivariate analysis of variance (MANOVA) with
“duration of CHF” and “CHF” as effect variables, followed by Student Newman-Keuls test (Statistica, StatSoft Inc., Tulsa, OK, USA) when appropriate. Comparison between groups for Western and Northern blot results were done by Student’s unpaired t-test. A $P$ value less than 0.05 was considered statistically significant.


**Figure legends**

Figure 1: Northern blot (A) of mRNA from soleus and EDL from CHF and Sham operated rats. mRNA content in soleus (B) and EDL (C) were normalised to the Oligo-dT signal and presented as percentage of Sham values. PLB mRNA could not be detected in EDL. Western blot (D) of protein from soleus and EDL from CHF and Sham operated rats. Protein content in soleus (E) and EDL (F) are presented as percentage of Sham values. CHF: filled bars, Sham: open bars. Values are mean ± SEM. (n = 6). (NaK: Na⁺K⁺-ATPase).

Figure 2: Total electrolyte (A; Ca, B; Mg and C; K) content in soleus () and EDL () from CHF (open symbols) and Sham (filled symbols) operated rats one, six and 17 weeks after primary operation. There were no significant differences between Sham and CHF at any time points in either soleus or EDL. Overall Ca, Mg and K content were all significantly higher in EDL compared to soleus (55, 33, 35 %). During the 17-weeks period there was a significant fall in Ca-content in the soleus muscle, whereas this ion was well maintained in EDL. Mg and K increased significantly in soleus during the experimental period, but this was not seen in EDL. Values are mean ± SEM. (n = 5 – 14).
Table 1, Data supplement

Table 1: Weights and water contents of soleus and EDL muscles of Sham and CHF rats.

<table>
<thead>
<tr>
<th></th>
<th>Sham 1 week</th>
<th>Sham 6 weeks</th>
<th>Sham 17 weeks</th>
<th>CHF 1 week</th>
<th>CHF 6 weeks</th>
<th>CHF 17 weeks</th>
<th>treatment</th>
<th>CHF treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>277.0±3.8 (18)</td>
<td>393.9±7.4 (37)</td>
<td>260.0±5.1 (13)</td>
<td>379.4±10.7 (28)</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus Muscle weight (mg)</td>
<td>84.0±2.9 (14)</td>
<td>118.0±2.7 (20)</td>
<td>142.0±4.5 (10)</td>
<td>115.0±3.2 (14)</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water content (g/100g dw)</td>
<td>336.0±6.4 (11)</td>
<td>336.0±2.4 (13)</td>
<td>382.0±11.2 (10)</td>
<td>338.0±4.6 (7)</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL Muscle weight (mg)</td>
<td>89.0±2.4 (6)</td>
<td>125.0±3.6 (19)</td>
<td>156.0±3.7 (10)</td>
<td>121.0±3.4 (14)</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water content (g/100g dw)</td>
<td>314.0±5.1 (15)</td>
<td>318.0±4.9 (9)</td>
<td>354.0±7.9 (10)</td>
<td>294.0±5.1(4)</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. (n).
Figure 1, Data supplement

A

<table>
<thead>
<tr>
<th></th>
<th>Soleus</th>
<th>EDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serca1</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>Serca2</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>NaK α1</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>NaK α2</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td>NaK α3</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
<tr>
<td>NaK β1</td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>GAPDH</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
</tr>
</tbody>
</table>

B (Soleus)

![](image15)

C (EDL)

![](image16)

D

<table>
<thead>
<tr>
<th></th>
<th>EDL</th>
<th>Soleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serca1</td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
</tr>
<tr>
<td>Serca2</td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
</tr>
<tr>
<td>NaK α1</td>
<td><img src="image21" alt="Image" /></td>
<td><img src="image22" alt="Image" /></td>
</tr>
<tr>
<td>NaK α2</td>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
</tr>
<tr>
<td>NaK α3</td>
<td><img src="image25" alt="Image" /></td>
<td><img src="image26" alt="Image" /></td>
</tr>
<tr>
<td>NaK β1</td>
<td><img src="image27" alt="Image" /></td>
<td><img src="image28" alt="Image" /></td>
</tr>
</tbody>
</table>

E (Soleus)

![](image29)

F (EDL)

![](image30)
Figure 2, Data supplement

**A**

Calcium (mmol/kg w.w.)

- EDL
- Soleus

**B**

Magnesium (mmol/kg w.w.)

- EDL
- Soleus

**C**

Potassium (mmol/kg w.w.)

- EDL
- Soleus