Subcellular Creatine Kinase Alterations
Implications in Heart Failure


Abstract—We have tested the hypothesis that decreased functioning of creatine kinase (CK) at sites of energy production and utilization may contribute to alterations in energy fluxes and calcium homeostasis in congestive heart failure (CHF). Heart failure was induced by aortic banding in 3-week-old rats. Myofilaments, sarcoplasmic reticulum (SR), mitochondrial functions, and CK compartmentation were studied in situ using selective membrane permeabilization of left ventricular fibers with detergents (saponin for mitochondria and SR and Triton X-100 for myofibrils). Seven months after surgery, animals were in CHF. A decrease in total CK activity could be accounted for by a 4-fold decrease in activity and content (Western blots) of mitochondrial CK and a 30% decrease in M isoform of CK (MM-CK) activity. In myofibrils, maximal force, crossbridge kinetics, and α-myosin heavy-chain expression decreased, whereas calcium sensitivity of tension development remained unaltered. Myofibrillar CK efficacy was unchanged. Calcium uptake capacites of SR were estimated from the surface of caffeine-induced tension transient (SCa) after loading with different substrates. In CHF, SCa decreased by 23%, and phosphocreatine was 2 times less efficient in enhancing calcium uptake. Oxidative capacities of the failing myocardium measured as oxygen consumption per gram of fiber dry weight decreased by 28%. Moreover, the control of respiration by creatine, ADP, and AMP was severely impaired. Our observations provide evidence that alterations in CK compartmentation may contribute to alterations of energy fluxes and calcium homeostasis in CHF. (Circ Res. 1999;85:68-76.)

Key Words: mitochondrial respiration ■ myofibril ■ compartmentation ■ sarcoplasmic reticulum ■ skinned fiber

The mechanisms underlying the decline in cardiac pump function in heart failure are incompletely understood. They lead to a gradual increase in left ventricular (LV) end diastolic pressure and a decrease in systolic pressure. In the past decade attention has been focused on the alterations of the various steps in excitation-contraction coupling and intracellular calcium homeostasis, whereas the possible involvement of a mismatch in energy supply and demand has received less attention. It has been shown recently that in human heart failure, there is a generalized alteration of the creatine kinase (CK) system with a decrease in total enzyme activity and content (Western blots) of mitochondrial CK and a 30% decrease in M isoform of CK (MM-CK) activity. Moreover, in patients with dilated cardiomyopathy, the phosphocreatine (PCr)/ATP ratio, governed by CK activity, may be a predictor of both total and cardiovascular mortality. However, the precise cellular mechanisms by which altered CK may compromise energy fluxes and contractility are not well understood.

CK is an important enzyme involved in energy maintenance and energy transfer in muscle and brain cells. It catalyzes the reversible transfer of a phosphate moiety between ATP and creatine. Four different isoforms of CK are expressed in a tissue-specific and developmentally regulated manner. A major part of muscle CK exists as dimers composed of 2 subunits, M and B, giving 3 isoenzymes, MM, BB, and MB. In addition, there is a fourth isoenzyme in the mitochondria (sarcomeric mitochondrial CK [mi-CK]), which differs biochemically and immunochemically from the cytosolic forms and can form both octameric and dimeric structures. In muscle cells, reactions involved in ATP generation and utilization are not governed by stochastic events, but rather occur within structural and functional entities and are spatially and temporarily coordinated. CK isoenzymes are not evenly distributed in the cardiac cell, but the CK system constitutes an example of a compartmentalized metabolic pathway. MM-CK has been found in myofibrils and described as a structural protein of the M band participating in the connections between myosin filaments inside muscle fibers. This bound MM-CK is functionally coupled to the myosin ATPase and can provide enough energy to sustain maximal force and normal kinetics of contraction (for review,
see Reference 10 and references therein). Similarly, MM-CK is strongly bound to sarcoplasmic reticulum (SR) membranes, in which it is functionally coupled to the Ca\(^{2+}\)-ATPase, and ensures efficient energy provision of the SR by the local regeneration of ATP.\(^{1,11-14}\) mi-CK is found on the outer surface of the inner mitochondrial membrane, in the vicinity of the ATP-ADP carrier, so that ATP generated by oxidative phosphorylation, after transport through the inner mitochondrial membrane, is transphosphorylated to PCr (for review, see References 15 and 16 and references therein). The sites of energy production and energy utilization are integrated through near-equilibrium reactions of CK in the cytosol, promoting sequential equilibration that results in almost instantaneous transfer of phosphoryl groups and metabolic signal.\(^{15,16}\) It is conceivable that altered compartmentation of the CK isoenzymes in congestive heart failure (CHF) may impair the functioning of intracellular compartments, thus compromising integration between energy production and utilization.

The goal of this study was to investigate the role of bound CK isozymes on the intrinsic functional properties of the 3 main intracellular cardiac compartments involved in excitation-contraction coupling (myofibrils and SR) and energy production (mitochondria) and their alterations in heart failure. Using an animal model of prolonged chronic heart failure induced by aortic banding, we examined (1) whether the function of these cellular compartments is altered in heart failure, (2) whether alterations in localized CK isozymes may alter the regulation and function of these subcellular compartments, and (3) how these alterations may contribute to the pathophysiology of heart failure.

Materials and Methods

Rat Model of CHF

Male Wistar rats were randomly assigned to 1 of 2 groups, the CHF or the sham-operated group.

After induction of anesthesia with intraperitoneal injection of pentobarbital (60 mg/kg), aortic stenosis was created in weaned male rats (60 to 70 g) by placing a stainless steel hemoclip of 0.6-mm ID on the ascending aorta via a thoracic incision (CHF group).\(^{17}\) Age-matched controls underwent the same procedure without placement of the clip (sham group). All rats were fed a normal chow diet or the sham-operated group.

Morphological and Biochemical Studies

Collagen was quantified using a histological method. Cryosections of left ventricle were exposed to hematoxylin and then to acid fuchsin according to the van Gieson method. Three or four areas of each transverse section representative of the left ventricle were magnified, and video images of the section were digitized (Visioblab 200 image-processing system, Biocom). Frozen tissue samples were weighed; homogenized in ice-cold buffer (50 mg/mL) containing (in mmol/L) HEPES 5 (pH 8.7), EGTA 1, DTT 1, and MgCl\(_2\) 5, and 0.1% Triton X-100; and incubated for 60 minutes at 0°C to ensure complete enzyme extraction. The total activities of adenylate kinase (AK), CK, pyruvate kinase (PK), phosphoglycerate kinase (PGK), and lactate dehydrogenase (LDH) were assayed (30°C, pH 7.5) using coupled enzyme systems as previously described.\(^{18,19}\) CK isoenzymes were separated using agarose (1%) gel electrophoresis performed at 200 V for 90 minutes; individual isoenzymes were resolved by incubating the gels with a coupled enzyme system.\(^{19}\) Internal standards of commercial MM-CK were run in parallel with tissue samples to ensure linearity in isoenzyme quantification. To avoid saturation of the various CK isozyme signals, 3 dilutions were run for each sample. Isoenzyme bands were visualized by the fluorescence of NADPH. The LDH isozyme profile was determined using agarose gel electrophoresis (Sigma LDH reagent kit) at 200 V for 90 minutes. Gels were quantified using an image-analysis system (Bio-Rad). Activity of AK and LDH isoenzymes was quantified by multiplying each percentage by total activity, as determined spectrophotometrically. Determination of citrate synthase activity was performed according to Frere.\(^{20}\) Protein concentration was determined with the Lowry method, using BSA as a standard.

Native myosin was extracted from frozen tissues according to d’Albis et al.\(^{21}\) Myosin isozymes were separated by PAGE. Gel running buffer consisted of 20 mmol/L sodium pyrophosphate (pH 8.5), 10% glycerol, 0.01% 2-mercaptoethanol, and 2 mmol/L MgCl\(_2\). Cylindrical (6×0.5-cm) gels contained 4% polyacrylamide (3.88% acrylamide and 0.12% N,N’-methylene-bis(acrylamide). Between 1 and 5 µg of myosin was loaded on each gel. Electrophoresis was carried out at a constant voltage of 90 V, for 22 hours, between 2°C and 4°C.\(^{22}\)

Western Blot Analysis

Protein extracts (1 and 4 µg) from sham and CHF hearts and from 1 control and 1 mi-CK knockout mouse\(^ {23}\) were separated by PAGE. Gel running buffer consisted of 20 mmol/L sodium pyrophosphate (pH 8.5), 10% glycerol, 0.01% 2-mercaptoethanol, and 2 mmol/L MgCl\(_2\). The flow per gram wet weight (ww) was kept constant in both series (8 mL/min per gram ww). Hearts were stimulated at 240 bpm. A balloon, inserted in the left ventricle, was progressively inflated to reach the plateau of the pressure/volume relationship (the volume required was 240 µL in CHF and 260 µL in sham). LV developed pressure (LVP) and LV end diastolic pressure (LVEDP) were analyzed online. The “arterial” PO\(_2\) (Pao\(_2\)) just above the aorta and “venous” PO\(_2\) (Pvo\(_2\)) in the pulmonary artery were measured online through 2 flow cells, Clark electrodes, and an oxymeter (Strathkelvin Institute). The oxygen consumption (Pao\(_2\)-Pvo\(_2\)) was expressed in mmol O\(_2\)/min per gram ww (QO\(_2\)).
Briefly, thin fiber bundles (100 to 250 μm in diameter) were excised from the endocardial wall of the left ventricle and incubated for 30 minutes at 4°C in solution S (see below) containing 50 μg/mL saponin to permeabilize the sarcoplemma. Respiratory rates were determined using a Clark electrode (Strathkelvin Instruments) in an oxygraphic cell containing 3 mL of solution R (see below) at 22°C with continuous stirring. The solubility of oxygen was ~230 mmol of O2/mL in buffer after standard calibration in water. After measurements, the fiber bundles were carefully removed and dried. Respiration rate were expressed as μmol O2/min per gram dry weight.

Solutions S and R contained (in mM/L) EGTA–calcium-EGTA buffer 10 (free Ca2+ concentration, 100 mM/L), MgCl2 1, taurine 20, DTT 0.5, imidazole 20, and ionic strength 160 (potassium methanesulphonate).2–4 In addition, solution S (pH 7.1) contained 5 mM/L MgATP and 15 mM/L PCr, and solution R (pH 7.1) contained (in mM/L) glutamate 5, malate 2, and phosphate 3, and 2 mM/L fatty acid–free BSA. Functional activity of mi-CK in skinned fibers was assessed by the determination of ADP kinetic parameters in the presence (20 mM/L) or absence of caffeine. The ADP-stimulated respiration (VADP) above basal oxygen consumption (Vo) was plotted as a function of [ADP]. The apparent Michaelis-Menten constants (Km, Vmax) for ADP and VADP were calculated using a nonlinear fitting of the Michaelis-Menten equation. Maximal respiration rate (Vmax) was VADP+Vo. Acceptor control ratio was calculated as Vmax/Vo. The functional activity of AK was evaluated by the percentage increase in the respiration rate after addition of 2 mM/L AMP in the presence of 0.2 mM/L ATP (VADP/Vo). One to three determinations were made for each animal.

**Mechanical Experiments**

Muscle fiber bundles were dissected from papillary muscles of the left ventricle in a zero-Ca2+ Krebs solution (pH 7.4) and incubated for 1 hour in a relaxing solution (pCa 9; see solutions below) containing 1% Triton X-100 to solubilize the membranes. Bundles were mounted between a vibrator and a force transducer (model AE 801, SensoNor Microelectronics) as previously described.18 Sarcomere length was measured by laser diffraction and adjusted to 2.1 to 2.2 μm. Muscles were immersed in 2.5-mL chambers continuously stirred at high speed (22°C). Solutions were prepared according to Fabiato2–4 as previously described and contained (in mM/L): EGTA 10, imidazole 30 (pH 7.1), NaCl 30.6, MgCl2 3.16, and DTT 0.3, with acetate as anion. Ionic strength was adjusted to 160 (potassium methanesulphonate). Two to three determinations were made for each animal.

After emptying the SR by brief application of caffeine (5 mM/L), loading was carried out in strongly buffered (10 mM/L EGTA) loading solutions at pCa 6.25. Each fiber was randomly loaded for 10, 30, 60, 180, and 420 seconds in the presence of either MgATP+PcR (control load) or ATP alone (ATP load). To assess efficacy of glycolysis in supporting calcium uptake, SR was loaded for 420 seconds in the presence of (in mM/L) MgADP 1+NAAD 4+glyceroldehyde 3-P+P 2, (glycolytic load), which allows activation of the 2 ATP-generating steps of glycolysis, PGK and PK. The caffeine-induced tension transient was used to calculate the time course of free [Ca2+] close to myofilibrils during the release, taking the pCa/tension dependence as internal calibration, as previously described.14 For this purpose, the pCa/tension relationship in the presence of 5 mM/L caffeine was obtained at the end of each experiment, and tensions were normalized with respect to the maximal calcium-activated tension. The data from each fiber were fitted to the Hill equation, and the [Ca2+] at each step of the tension-time integral was recalculated using Labview software (National Instruments Corp) to obtain [Ca2+] × time integrals (S0), which were taken to evaluate the SR Ca2+ loading capacity.

**Statistical Analysis**

The data are expressed as mean±SEM. A Student t test was used to determine the statistical difference of means between the sham and CHF groups. A paired t test was used to compare tension and calcium-time integral obtained in different substrates within each group. Sc values were analyzed by ANOVA using a 2 (load)×2 (condition: sham or CHF)×5 (time) factorial design with repeated measures. When a significant F ratio was found, a Newman-Keuls test was used to detect differences between groups. Values of P<0.05 were considered significant.

**Results**

**Anatomical and Functional Data**

Anatomical data are summarized in Table 1. CHF animals showed decreased body weight (−29%) and increased heart weight (−90%). Expressed per body length, heart weight (+118%), left (+91%) and right (+117%) ventricular weights, and lung (+100%) weight were all significantly increased. Moreover, obvious clinical evidence of cardiac decompensation (brown and “nutmeg” liver, ascites, pleural effusions, enlarged atria, leathery and dyspnea, or left atrial thrombi) was observed in all CHF rats.

Experiments with isolated perfused hearts confirmed that CHF hearts exhibit systolic and diastolic abnormalities that characterize heart failure (Table 1). LVDP was not different between the 2 groups but decreased by 39% when normalized per grams of left ventricle. LVEDP was significantly higher in CHF than in sham rats. This was associated with a 28% decrease in VO2.

**Biochemical Data**

Enzyme activities are presented in Table 2. The total protein content of CHF myocardium was slightly lower and interstitial collagen was higher in CHF myocardium, evidencing signs of edema and increased fibrosis with broad variations from heart to heart. Enzyme activities were thus reported per mg protein using the Lowry method, which minimizes the contribution of aromatic amino acid residues abundant in collagen.28 The activity of AK, a phosphotransfer enzyme, was unchanged in CHF. LDH activity was also unchanged in CHF myocardium, but the proportion of the cardiac aerobic subunit (H-LDH) was significantly decreased. PK and PGK, the 2 steps of glycolytically produced ATP, were decreased as
well in CHF. Citrate synthase activity, a marker of mitochondrial capacities or mitochondrial mass, was decreased by 33%.

**CK System**

CK activity and isoenzyme profile, whether expressed as IU/mg protein or as percentage of total CK activity, were greatly affected in CHF rats (Table 3). Total CK activity was reduced by 45%, with great differences among the 4 isoenzymes. Although the relative proportion of the B isofrom of CK (BB-CK) increased in CHF myocardium (+136%), the activity was maintained, as was the activity of the B-subunit (BB-CK+/1⁄2MB). Activity of MM-CK and the heterodimer of B and M CK (MB-CK) exhibited a 30% to 40% decrease. The most dramatic change was an 83% decrease in the mi-CK isoform. This decrease exceeded the overall decrease in mitochondrial activity, since the mi-CK to citrate synthase ratio exhibited a 4-fold decrease. Western blot analysis of mitochondrial CK protein (Figure 1 and Table 3) showed that this decrease could be entirely accounted for by a decrease in

**TABLE 1. Characterization of CHF Rats Compared With Sham**

<table>
<thead>
<tr>
<th>Anatomical data</th>
<th>Sham (n=15)</th>
<th>CHF (n=13)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic banding duration, months</td>
<td>7.4±0.1</td>
<td>7.3±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>677±17</td>
<td>480±19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart weight (HW), g</td>
<td>1.8±0.1</td>
<td>3.4±0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Right ventricle weight (RVW), g</td>
<td>0.31±0.02</td>
<td>0.59±0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Left ventricle weight (LVW), g</td>
<td>1.2±0.1</td>
<td>2.0±0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lung weight (LuW), g†</td>
<td>1.7±0.07</td>
<td>3.3±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver weight (LW), g†</td>
<td>20±1</td>
<td>16±1</td>
<td>0.002</td>
</tr>
<tr>
<td>Tibia length (TL), cm†</td>
<td>4.67±0.03</td>
<td>4.58±0.04</td>
<td>NS</td>
</tr>
<tr>
<td>HW/LW, g/cm†</td>
<td>0.34±0.02</td>
<td>0.74±0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RW/LW, g/cm†</td>
<td>0.060±0.005</td>
<td>0.130±0.009</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LW/LW, g/cm†</td>
<td>0.23±0.01</td>
<td>0.44±0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lw/LW, g/cm†</td>
<td>0.36±0.02</td>
<td>0.72±0.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LiW/LT, g/cm†</td>
<td>4.4±0.2</td>
<td>3.4±0.2</td>
<td>0.003</td>
</tr>
</tbody>
</table>

| Functional data                           |             |            |
|-------------------------------------------|-------------|------------|-----|
| LVDP, mm Hg                               | 180±6       | 167±7      | NS  |
| LVDP/g, mm Hg/g                           | 116±6       | 71±4       | <0.001|
| LVDP, mm Hg                               | 10±2        | 62±18      | 0.03|
| QO2, µmol O2/min per gram, ww             | 8.5±0.4     | 6.1±0.2    | 0.003|

n indicates number of animals.
*Compared with sham.
†n=11 for sham.

**TABLE 2. Enzymatic Activities in Left Ventricle of CHF Rats Compared With Sham**

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Sham (n=6)</th>
<th>CHF (n=6)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, g protein/g ww</td>
<td>0.086±0.005</td>
<td>0.072±0.004</td>
<td>0.05</td>
</tr>
<tr>
<td>Collagen, % of CSA</td>
<td>1.4±0.2</td>
<td>4.7±1.3</td>
<td>0.05</td>
</tr>
<tr>
<td>CS, IU/mg protein</td>
<td>1.73±0.07</td>
<td>1.17±0.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AK, IU/mg protein</td>
<td>2.72±0.38</td>
<td>2.46±0.21</td>
<td>NS</td>
</tr>
<tr>
<td>LDH, IU/mg protein</td>
<td>6.21±0.25</td>
<td>5.89±0.43</td>
<td>NS</td>
</tr>
<tr>
<td>H-subunit, %†</td>
<td>70±1</td>
<td>64±2</td>
<td>0.007</td>
</tr>
<tr>
<td>PK, IU/mg protein</td>
<td>6.13±0.82</td>
<td>3.48±0.25</td>
<td>0.02</td>
</tr>
<tr>
<td>PKG, IU/mg protein</td>
<td>3.06±0.26</td>
<td>2.35±0.16</td>
<td>0.05</td>
</tr>
</tbody>
</table>

CSA indicates cross-sectional area; CS, citrate synthase.
*Compared with sham.
†H-subunit=H4+1⁄2H2M+1⁄4H4M2+1⁄4HM3, where H4, H2M, H4M2, and HM3 are homotetramers and heterotetramers of LDH.

**TABLE 3. CK System in Left Ventricle of CHF Rats Compared With Sham**

<table>
<thead>
<tr>
<th>CK System</th>
<th>Sham (n=6)</th>
<th>CHF (n=6)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CK, IU/mg protein</td>
<td>9.1±0.5</td>
<td>5.0±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mi-CK, IU/mg protein</td>
<td>2.8±0.3</td>
<td>0.49±0.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>% of total CK</td>
<td>31±3</td>
<td>10±1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MM-CK, IU/mg protein</td>
<td>4.3±0.3</td>
<td>3.1±0.2</td>
<td>0.01</td>
</tr>
<tr>
<td>% of total CK</td>
<td>47±3</td>
<td>62±3</td>
<td>0.009</td>
</tr>
<tr>
<td>MB-CK, IU/mg protein</td>
<td>1.7±0.2</td>
<td>1.0±0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>% of total CK</td>
<td>19±2</td>
<td>19±3</td>
<td>NS</td>
</tr>
<tr>
<td>BB-CK, IU/mg protein</td>
<td>0.3±0.1</td>
<td>0.4±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>% of total CK</td>
<td>5.6±0.7</td>
<td>8.5±1.8</td>
<td>0.04</td>
</tr>
<tr>
<td>B-CK subunit, IU/mg protein†</td>
<td>1.2±0.1</td>
<td>0.9±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>mi-CK/CS</td>
<td>1.6±0.2</td>
<td>0.43±0.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mi-CK protein, au</td>
<td>5.9±0.5</td>
<td>1.4±0.3</td>
<td>0.001</td>
</tr>
<tr>
<td>mi-CK specific activity, IU/au</td>
<td>0.5±0.1</td>
<td>0.4±0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

CS indicates citrate synthase; IU, µmol/min; au, arbitrary units.
*Compared with sham.
†B-CK subunit=BB+1⁄2MB.

Figure 1. Mitochondrial CK content in left ventricle of CHF and sham rats. Representative Western blot analysis of sham and CHF rat LV homogenates probed with mi-CK antibody. Tissues were homogenized in low-ionic strength pH 8.7 buffer, and 1 or 4 µg of proteins per lane were electrophoresed on 12% SDS-polyacrylamide gels. To check antibody specificity, ventricular homogenates of 1 control mouse (mi-CK+/−) and 1 mi-CK−/− knockout mouse (mi-CK−/−) were added. After blotting, membranes were incubated with primary antibody against mi-CKs for 2 hours. After incubating the membranes with horseradish peroxidase–labeled secondary antibody, they were revealed with ECL chemiluminescent substrate. Light emission was detected by autoradiography and quantified using image analysis. Only 1 band of the appropriate size was observed (43 kDa). A clear decrease in mi-CKs proteins is seen in CHF rat hearts. No band was observed for the homogenate of the mi-CK−/− mouse.
mito-CK protein content with no change in the enzyme specific activity.

Mitochondrial Function and CK

To establish the functional consequences of the decrease in mi-CK on mitochondrial regulation, oxygen consumption of permeabilized preparations from control (sham) and experimental (CHF) rats was recorded as a function of ADP concentration (Figure 2) in the absence or in the presence of creatine. As ADP concentration increased, respiration rate reached a plateau that was significantly lower in failing than in control hearts. The addition of creatine shifted the relationship toward lower ADP concentrations, both in sham and CHF, although to a lesser extent in the latter. The relationship between respiration rate and ADP concentration could be reasonably fitted by the Michaelis-Menten equation. The averaged results are presented in Table 4. Maximal and basal respiration rates showed a \( \approx 30\% \) reduction, which suggests a decrease in oxidative capacities in CHF. As the acceptor control ratio was high and preserved in CHF, this decrease suggested a decreased amount of mitochondria without changes in the oxidation-phosphorylation coupling. Indeed, reduced respiration correlated to reduced mitochondrial content assessed by citrate synthase activity. In the absence of creatine, the \( K_m \) for ADP was significantly higher in CHF than in sham. After addition of creatine, the decrease in \( K_m \) for ADP (CK efficacy) was less in CHF (2.3 times) than in sham (4 times). The stimulation of respiration by AMP in the presence of ATP was 70% in sham and only 45% in CHF \((P=0.02)\), which shows an impairment of mitochondrial AK efficacy as well.

It thus appeared that both the maximal capacities and the regulation of mitochondria were altered in CHF.

SR Function and CK

The function of the SR was studied in saponin-skinned fibers loaded with \( \text{Ca}^{2+} \) for different periods of time and emptied by the application of 5 mmol/L caffeine. The tension transients were used to estimate calcium uptake and release properties of the SR of sham and CHF myocardium. Because the calcium sensitivity of tension development in the presence of caffeine was higher in CHF \((pC_{a_{0}}=5.824 \pm 0.012)\) than in sham \((5.776 \pm 0.012, P=0.017)\), the \( [\text{Ca}^{2+}] \times \text{time integral} \) \((S_{Ca})\) was recalculated from tension transients by using the calcium sensitivity of tension development for each fiber to minimize any difference caused by myofilament properties. \( S_{Ca} \) reflects the amount of calcium released by the SR and reaching myofilaments. \( S_{Ca} \) was plotted as a function of the

**Figure 2.** Regulation of mitochondrial respiration by ADP and creatine in LV of CHF and sham rats. ADP dependence of mitochondrial respiration in representative saponin-skinned ventricular fibers from sham \((M)\) and CHF \((E)\) rats in the absence \((A)\) and in the presence \((B)\) of creatine is shown. Oxygen consumption rates \((V_{O_2})\) were measured using a Clark electrode with glutamate and malate as substrates and were plotted as a function of ADP concentration. Data were fitted using a Michaelis-Menten equation to obtain \( K_m \) and \( V_{max} \) (maximum ADP-stimulated respiration) values (dotted and solid lines, respectively). \( V_{ADP} \) was lower in CHF than in sham. The presence of creatine resulted in an increased affinity of mitochondria for ADP, with no change in \( V_{ADP} \). However, creatine was less effective in CHF than in sham.

### TABLE 4. Mitochondrial Function in Saponin-Treated LV Fibers of CHF Rats Compared With Sham

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>CHF</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_0 ), ( \mu \text{mol O}_2/\text{min per gram dry weight} )</td>
<td>4.4±0.3 ((n=20))</td>
<td>3.1±0.2 ((n=22))</td>
<td>0.003</td>
</tr>
<tr>
<td>( V_{max} ), ( \mu \text{mol O}_2/\text{min per gram dry weight} )</td>
<td>32±2 ((n=20))</td>
<td>23±1 ((n=22))</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acceptor control ratio</td>
<td>7.9±0.5 ((n=20))</td>
<td>8.1±0.6 ((n=22))</td>
<td>NS</td>
</tr>
<tr>
<td>( K_m ) for ADP, ( \mu \text{mol/L} )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without creatine ((n=10))</td>
<td>204±12</td>
<td>275±27</td>
<td>0.03</td>
</tr>
<tr>
<td>With creatine ((n=11))</td>
<td>54±5</td>
<td>128±18</td>
<td>0.002</td>
</tr>
<tr>
<td>CK efficacy ((n=6))</td>
<td>3.9±0.5</td>
<td>2.3±0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>AK efficacy, %</td>
<td>70±8 ((n=10))</td>
<td>45±5 ((n=11))</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\( n \) indicates number of experiments. Acceptor control ratio = \( V_{max}/V_0 \). CK efficacy refers to the ratio of \( K_m \) for ADP in the absence and in the presence of creatine; AK efficacy, increase in respiration (in %) by addition of 2 mmol/L AMP in the presence of 200 \( \mu \text{mol/L ATP} \).

*Compared with sham.
loading time in sham (Figure 3A) and CHF (Figure 3B). When MgATP was the only substrate, $S_{Ca}$ increased slightly with time. There was no difference between sham and CHF. When PCr was added in sham, $S_{Ca}$ increased sharply, reaching a 3.2 times higher value for a 420-second load than with MgATP alone. In CHF (Figure 3B), for a 420-second load, $S_{Ca}$ was 23% lower than in sham in the presence of both MgATP alone and was not significantly affected in CHF (Figure 3C). For a 420-second load, PCr increased $S_{Ca}$ 3.2 times in sham but only 2.4 times in CHF because of rapid rephosphorylation of ADP by bound CK in CHF, SR was loaded in the presence of glycolytic substrates (4 mmol/L NAD, 6 mmol/L glyceraldehyde 3-P with 1 mmol/L MgADP, 4 mmol/L NAD$^+$, and 2 mmol/L P$_1$). A 420-second load resulted in a $S_{Ca}$ reaching 107±6% of that in the presence of ATP alone and was not significantly affected in CHF (103±9%) (data not shown). This finding also shows that PGK and possibly PK are present and active close to SR substrates (41.0±4.1 μmol/L×seconds in sham versus 31.7±3.2 μmol/L×seconds in CHF, $P=0.01$). It appeared, therefore, that the addition of PCr enhanced calcium uptake to a much lower extent in CHF (Figure 3C). For a 420-second load, PCr increased $S_{Ca}$ 3.2 times in sham but only 2.4 times in CHF ($P=0.004$) (CK efficacy). To investigate whether ATP supplied by bound glycolytic enzymes could provide energy for calcium uptake and whether this was altered in CHF, SR was loaded in the presence of glycolytic substrates (6 mmol/L glyceraldehyde 3-P with 1 mmol/L MgADP, 4 mmol/L NAD$^+$, and 2 mmol/L P$_1$). A 420-second load resulted in a $S_{Ca}$ reaching 107±6% of that in the presence of ATP alone and was not significantly affected in CHF (103±9%) (data not shown). This finding also shows that PGK and possibly PK are present and active close to SR ATPase in skinned fibers. It thus appeared that one main alteration in SR function in CHF was the inability of bound ATPase in skinned fibers. It thus appeared that one main alteration in SR function in CHF was the inability of bound

**Discussion**

This study examined whether CK system alterations may contribute to altered energy fluxes and contractile dysfunction in heart failure. We investigated the intrinsic functional properties of 3 intracellular compartments involved in energy production (mitochondria) and excitation-contraction coupling (myofibrils and SR). Next, we examined whether alterations in the functional coupling between CK isoenzymes and local ATP and ADP concentrations can compromise these subcellular functions. The results show the following: (1) MM and mi-CK isoenzymes are primarily affected in CHF, with mitochondrial CK exhibiting a dramatic decrease in activity and protein content; (2) the oxidative capacity of cardiac fibers and the control of mitochondrial respiration by phosphate acceptors (creatine, ADP, and AMP) were depressed; (3) SR calcium uptake capacities were preserved when ATP was the substrate, whereas the ability of bound CK to stimulate SR calcium uptake was depressed; and (4) the ability to develop force was decreased in CHF, but calcium sensitivity and myofibrillar CK ability to relax rigor tension were preserved. These results show that local disturbances in energy fluxes may compromise the fine tuning between energy supply and utilization, as well as excitation-contraction coupling.
Alterations in CK System in Heart Failure

This study shows that, as in human heart, CK activity is largely depressed in severe CHF in rats, and that this decrease mainly affects mitochondrial CK activity and MM-CK. AK (another phosphotransfer enzyme) and LDH activities were not affected, which shows that the CK alterations were rather specific. Moreover, we show that the 4-fold decrease in mi-CK activity was entirely accounted for by a decrease in the mi-CK protein content that largely exceeded the decrease in mitochondrial mass. Failure to observe consistent alterations in CK activity or enzymes in animal models of heart failure has thus far not been explained by the presence of caffeine, as was previously observed in compensated hypertension.37,38 The decrease in the rate constant of calcium uptake brought about by addition of PCr in the presence of caffeine, as was previously observed in compensated hypertrophy,37,38 The decrease in the rate constant of tension changes reflects the decrease in the relative amount of the fast myosin isoenzyme generally observed in pressure overload in rat heart.39 However, in CHF, despite a 30% decrease in total activity, MM-CK bound to myofibrils was still able to induce a 54-fold decrease in the ATP_50 for relaxation of rigor tension (Table 5). The stability of myofibrillar CK could be due to the fact that MM-CK is an integral part of the myofibrillar structure, forming intermyosin bridges in the M line.9 On the other hand, it could be argued that the more economic contractile behavior of pressure-overloaded myocardium due to decreasing amount of the fast myosin isoform could be adequately supplied by a lowered amount of MM-CK. It can be concluded that despite altered myofibrillar function, the myofibrillar CK efficacy is preserved in CHF.

Mitochondrial Function and CK

Few data are available concerning the oxidative capacities of the failing heart. Skinned fiber studies of mitochondrial respiration offer the unique opportunity to assess intrinsic oxidative capacities in muscles with nonlimiting amounts of substrates and oxygen. In CHF rats, we observed a 30% decrease in both basal and maximal respiration rates paralleled by a 33% decrease in citrate synthase activity, which suggests a decrease in the amount of mitochondria with preserved coupling between oxidation and phosphorylation. This was accompanied by a decrease in the relative proportion of the cardiac form of LDH and decreased glycolytic ATP-regenerating steps. Decreased oxidative capacities may explain the lower oxygen consumption rate observed in isolated hearts. As the heart utilizes 80% to 90% of its maximum oxidative capacities during exercise,41 decreased aerobic capacities could by themselves limit the ATP synthesis and contractile capacities of the failing heart and contribute to the limited exercise capacity in heart failure.

Cardiac mitochondria maintained within the cellular architecture exhibit regulatory properties that greatly differ from isolated mitochondria.16 This is characterized by a low sensitivity of mitochondrial respiration for extramitochondrial ADP, whereas creatine addition decreases the apparent K_m for ADP because of functional coupling between oxidative phosphorylation, translocase, and mi-CK.16 Such a coupling has also been described for mitochondrial AK.42 Regulation of mitochondrial respiration by mitochondrial kinases provides a specialized system by which different cytosolic metabolic pathways linked to cytosolic kinases are connected to mitochondrial respiration.43–45 These phosphotransfer systems may provide an efficient integration between energy utilization and production within oxidative tissues. In situ mitochondria from CHF rats appeared 26% less sensitive to ADP, 58% less sensitive to creatine, and 36% less sensitive to AMP (Table 4) than mitochondria from sham, showing a loss of regulation of oxidative phosphorylation by phosphate acceptors. This suggests a generalized loss of integration between cytosolic signals and mitochondria in CHF, which could be responsible for altered energy fluxes and incapacity of the failing myocardium to adapt energy production to energy utilization and to mobilize contractile reserve.6

SR Function and CK in Heart Failure

A crucial alteration in SR function was that the increase in calcium uptake brought about by addition of PCr in the
presence of ATP was lower in CHF than in sham (Figure 3). This is the first demonstration of a possible alteration in SR function by decreased efficacy of bound CK. The decreased efficacy may be attributed to decreased amount of bound MM-CK in SR or disorganization of SR architecture, leading to looser coupling between Ca\(^{2+}\)-ATPase and CK.

It is generally assumed that a defect in the capacity of the SR to accumulate calcium participates in the pathophysiology of heart failure. Changes in expression of the different proteins involved in calcium uptake and calcium release by the SR has received much attention, and there is evidence that Ca\(^{2+}\) ATPase is down-regulated in the CHF model used in this study.\(^{17}\) However, efficiency of the calcium pump also depends on adequate energy supply and effective withdrawal of the end products of ATP hydrolysis. Indeed, ATP and ADP exert a kinetic (through affinity and inhibition constants) as well as thermodynamic (through free energy of ATP hydrolysis) control on calcium uptake. A functional coupling between SR Ca\(^{2+}\) ATPase and bound CK allows the maintenance of a high ATP/ADP ratio in the close vicinity of the catalytic site, an adequate energy supply, and relief of product inhibition on the ATPase.\(^{11–13}\) Thus markedly enhancing the calcium uptake capacity of the SR.\(^{14}\) Implication of MM-CK in kinetics and amplitude of the calcium transient was recently underscored in skeletal muscle of mice deficient in MM-CK\(^{46}\) and in CK-inhibited rat hearts.\(^{47}\)

**CK System and Heart Failure**

The pathogenesis of heart failure is as yet not clearly defined, and the two main hypotheses so far put forward are (1) inadequate calcium uptake and release and (2) mismatch between energy production and utilization. However, these 2 cellular events are closely interrelated. Calcium withdrawal depends on efficient energy-driven calcium pumps and, secondarily, sodium pumps, whereas calcium concentration determines energy expenditure through cellular ATPases. Each of these steps depends on the subcellular architecture and protein organization. Increasing evidence also supports the proposal that localized control of energy fluxes influences calcium homeostasis and contractility. Disturbances in one of these finely controlled cellular processes will make the myocyte enter a vicious cycle of energy mismatch and calcium dysregulation, especially in periods of increased workload.

An alteration in energy metabolism in CHF was demonstrated by (1) decreased oxidative capacity, (2) altered glycolytic pathway, (3) altered expression of the CK system, (4) altered regulation of mitochondrial respiration by phosphate acceptors, and (5) decreased control of localized energy fluxes by bound CKs in mitochondria and SR. Moreover, whole-organ enzymology of the CK system, studied by nuclear magnetic resonance spectroscopy, showed that substrates of the CK reaction were altered in heart failure and that the capacity for ATP resynthesis was 83\% lower in failing than in control myocardium.\(^{48}\)

In normal heart, the fine integration between energy utilization and energy production is produced by functional coupling in which a locally increased ADP is quickly rephosphorylated and the amplified signal is transmitted through the cytosolic energy transfer systems (CK and AK) to mitochondria, where it can stimulate respiration.\(^{16,45}\) In CHF, this fine regulation is impaired, leading to mismatch between synthesis and utilization of energy, and the decreased PCr/ATP ratio\(^{7}\) most probably reflects the chronic imbalance between ATP supply and demand. Decreased efficacy of SR-bound CK to control adenine nucleotides in the vicinity of SR Ca\(^{2+}\) ATPase could contribute to the slowing of calcium uptake and twitch relaxation and to increased diastolic calcium concentration in CHF.\(^{49–51}\)

**Acknowledgments**

R.V.-C. is supported by Centre National de la Recherche Scientifique. A. Kaasik was supported by a Federation of European Biochemical Societies grant. This work was supported by a Programme de Recherche en Santé grant from INSERM. The help of Dr D. Charlemagne with Western blot analysis is warmly acknowledged. The authors thank R. Fischmeister for continuous support, P. Lechène for engineering assistance, and F. Lefebvre for technical assistance.

**References**

17. Feldman AM, Weinberg EO, Ray PE, Lorell BH. Selective changes in cardiac gene expression during compensated hypertrophy and the tran-


25. Saks VA, Khuchua ZA, Kuznetsov AV, Vekslar VI, Sharov VG. Heart mitochondria in physiological salt solution: not ionic strength but salt composition is important for association of creatine kinase with the inner membrane surface. *Biochim Biophys Res Comm.* 1986;139:1262–1271.


Subcellular Creatine Kinase Alterations: Implications in Heart Failure

Circ Res. 1999;85:68-76
doi: 10.1161/01.RES.85.1.68

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 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/85/1/68

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/