Creatine kinase-deficient hearts exhibit increased susceptibility to ischemia-reperfusion injury and impaired calcium homeostasis

Matthias Spindler, Klaus Meyer, Hinrik Strömér, Andrea Leupold, Ernst Boehm, Helga Wagner, and Stefan Neubauer

1Department of Cardiology, Medizinische Universitätsklinik Würzburg, 97080 Würzburg, Germany; and 2Department of Cardiovascular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, United Kingdom

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Spindler, Matthias, Klaus Meyer, Hinrik Strömér, Andrea Leupold, Ernst Boehm, Helga Wagner, and Stefan Neubauer. Creatine kinase-deficient hearts exhibit increased susceptibility to ischemia-reperfusion injury and impaired calcium homeostasis. Am J Physiol Heart Circ Physiol 287: H1039–H1045, 2004. First published April 22, 2004; 10.1152/ajpheart.01016.2003.—The creatine kinase (CK) system is involved in the rapid transport of high-energy phosphates from the mitochondria to the sites of maximal energy requirements such as myofibrils and sarcosommal ion pumps. Hearts of mice with a combined knockout of cytosolic M-CK and mitochondrial CK (M/Mito-CK⁻/⁻) show unchanged basal left ventricular (LV) performance but reduced myocardial high-energy phosphate concentrations. Moreover, skeletal muscle from M/Mito-CK⁻/⁻ mice demonstrates altered Ca²⁺ homeostasis. Our hypothesis was that in CK-deficient hearts, a cardiac phenotype can be unmasked during acute stress conditions and that susceptibility to ischemia-reperfusion injury is increased because of altered Ca²⁺ homeostasis. We simultaneously studied LV performance and myocardial Ca²⁺ metabolism in isolated, perfused hearts of M/Mito-CK⁻/⁻ (n = 6) and wild-type (WT, n = 8) mice during baseline, 20 min of no-flow ischemia, and recovery. Whereas LV performance was not different during baseline conditions; however, M/Mito-CK⁻/⁻ mice showed a greater increase in diastolic Ca²⁺ concentration ([Ca²⁺]d) during ischemia (237 ± 54% vs. 167 ± 25% of basal [Ca²⁺]d) compared with WT mice. In conclusion, CK-deficient hearts show an increased susceptibility of LV performance and Ca²⁺ homeostasis to ischemic injury, associated with a blunted posts ischemic recovery. This demonstrates a key function of an intact CK system for maintenance of Ca²⁺ homeostasis and LV mechanics under metabolic stress conditions.

The creatine kinase (CK) system comprises a family of mitochondrial (Mito-CK) and cytosolic (MM-, MB-, and BB-CK) isoenzymes that are critically involved in intracellular energy homeostasis. The primary role of CK is to catalyze the reversible transfer of a high-energy phosphoryl group between ATP and phosphocreatine (PCr; PCr + ADP + H⁺ ↔ ATP + creatine). The functional and physical coupling of certain members of the CK isoenzyme family to the sites of energy production and utilization has underscored the integrated properties of this important enzyme system in excitable tissue, particularly in muscle cells (26). MM-CK, for example, is present in membrane vesicles of the sarcoplasmic reticulum (SR) isolated from skeletal muscle (15), suggesting that an efficient and fast energy replenishing system is necessary for optimal functioning of SR Ca²⁺-ATPase. This view is further supported by studies in transgenic mice in which Mito-CK and cytosolic M-CK are ablated, showing not only altered contractile properties in skeletal and diaphragm muscle but also impaired Ca²⁺ release and reuptake dynamics in myotubes of skeletal muscle (19, 20, 27). Although a direct link between the missing CK system and alterations in cellular Ca²⁺ homeostasis was postulated, studies of myocardial Ca²⁺ metabolism in CK-deficient mice have not been reported so far. Because the CK system in cardiac muscle differs from that in skeletal muscle in several important aspects, it is difficult to predict how Ca²⁺ handling in heart muscle will be affected by the loss of CK.

On one hand, in addition to the upregulation of existing alternative energy generation systems such as glycolysis or the adenylate cyclase system, additional adaptational changes might have occurred in cardiac muscle of CK knockout mice that would be integrated into an already complex metabolic network. In fact, major adaptations, including shortened intracellular diffusion distances, were recently reported in hearts with combined knockout of M-CK and Mito-CK (M/Mito-CK⁻/⁻) (7). On the other hand, it is likely that functional consequences of CK knockout will differ between resting conditions, where energy supply and demand is balanced, and conditions of severe energy supply-demand mismatch in the sense that compensatory changes might be sufficient to enable normal cardiac performance at baseline but fail to maintain myocardial performance during substantial metabolic stress as induced by ischemia-reperfusion.

The goal of the present study was therefore twofold: The first aim was to characterize Ca²⁺ homeostasis on a beat-to-beat basis under physiological conditions in isolated, perfused CK-deficient hearts. The aequorin bioluminescence technique was adapted to test the hypothesis that the near complete loss of the CK system leads to direct alterations in myocardial Ca²⁺ homeostasis. The second aim was to test whether a cardiac phenotype can be unmasked by ischemia-induced metabolic stress. Because ischemia leads to imbalance of myocardial energy homeostasis, we tested whether CK-deficient hearts show increased susceptibility to ischemia-reperfusion injury and whether this is accompanied by alterations in Ca²⁺ homeostasis.
MATERIALS AND METHODS

Animals and biochemical assays. This investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85-23, Revised 1996). M/Mito-C/-/- mice were obtained from Dr. Bé Wieringa (University of Nijmegen, Nijmegen, The Netherlands) (20). Mice 20–30 wk of age equally distributed among both sexes were studied. CK-deficient mice had a mixed C57BL/6–129/Sv background, and wild-type mice were C57BL/6. There was no difference between wild-type and M/Mito-C/-/- mice regarding heart weight or heart weight-to-body weight ratios. The genotype of each mouse was verified by confirming the complete ablation of the cytosolic and mitochondrial CK isoenzymes except BB-CK. Total CK activity (CK \( V_{\text{max}} \)) and the proportion of this activity attributable to each isoenzyme of CK were measured by methods described previously (14); all values are expressed as millimol per second at 37°C.

Isolated, perfused heart preparation. Hearts of wild-type and M/Mito-C/-/- mice were isolated and perfused in a Langendorff preparation as described previously (18). Retrograde perfusion via the aorta was carried out at a constant coronary perfusion pressure of 75 mmHg. Modified Krebs-Henseleit buffer containing (in mM) 118 NaCl, 5.3 KCl, 2.0 CaCl\(_2\), 1.2 MgSO\(_4\), 0.5 EDTA, and 25 NaHCO\(_3\), with 10 glucose and 0.5 pyruvate as substrates, was used as described previously (18). All hearts were electrically paced with platinum pacemaker wires at 7 Hz. Cardiac temperature was monitored with a temperature probe attached to the right ventricle. Special care was taken to ensure isothermal conditions (37.0 ± 0.5°C) during the ischemia protocol with a temperature-regulated organ bath.

Measurement of isovolumic contractile performance. A water-filled balloon custom made of polyvinyl chloride film was connected to a pressure transducer (Statham P23 Db, Gould Instruments) for continuous recording of left ventricular (LV) pressure and heart rate. The size of the balloon was carefully matched to the size of the LV, and the balloon was large enough that a negligible pressure (<1.0 mmHg) resulted when the balloon alone was inflated up to the maximum volume used. The balloon was inflated to set LV end-diastolic pressure (LVEDP) between 6 and 8 mmHg for all hearts, and the balloon volume was then held constant. These perfusion modalities yielded a stable isovolumic LV performance with changes in LV developed pressure (LVDP) of <5% over 60 min of control perfusion.

Contractile performance data were digitized online at a sampling rate of 1 kHz by a 12-bit analog-to-digital converter and analyzed offline. LVDP, LVEDP, time to peak pressure (\( T_{\text{pv}} \)), and maximum and minimum rates of contraction and relaxation were derived from the pressure tracing.

Aequorin loading. This is the first report on measurements of Ca\(^{2+} \) transients with aequorin in perfused mouse hearts at 37°C, and the protocol is therefore described in detail. After an initial perfusion period of 5–10 min at 30°C with a Ca\(^{2+} \) concentration ([Ca\(^{2+} \)] ) in the coronary perfusate of 1.0 mM, aequorin was microinjected with a technique adapted from the rat whole heart preparation (22) and modified for the intact mouse heart. Specifically, 3–5 μl of an aequorin-containing solution (1 μg/ml) were injected with a glass micropipette into the interstitium of the inferoapical region of the LV. Care was taken to avoid excess injection, which could produce dissection or an isolated pocket of aequorin within the tissue. Thereafter, the heart was positioned in an organ bath with the aequorin-loaded area of the LV directly toward the cathode of a photomultiplier and submerged in Krebs-Henseleit solution. Five to ten minutes after aequorin loading, [Ca\(^{2+} \)] in the coronary perfusate was elevated in a stepwise fashion up to 2.0 mM, the temperature was slowly increased to 37°C, and the hearts were finally paced at 7 Hz. Preliminary experiments with different aequorin loading procedures including different perfusate [Ca\(^{2+} \)] values and temperatures showed that this approach yielded the highest success rate for stable Ca\(^{2+} \) transients at 37°C.

To reduce signal-to-noise ratios, 10–100 light cycles were averaged. As described for the analysis of the mechanical parameters, the wave-averaged light signals were analyzed for peak systolic (L\( \text{sys} \)) and end-diastolic light (L\( \text{dia} \)), time to peak light (\( T_{\text{pv}} \)), time constant of exponential light signal decay (\( T_{\text{1/2}} \)), with use of the variable asymptote method, and time to 50% decrease of light signal (\( T_{\text{50}} \)). Because myocardial tissue of the isolated, perfused hearts was used for biochemical analysis at the end of the experimental protocol, the method of fractional luminescence for quantitative calibration of the Ca\(^{2+} \) signal including Triton X lysis could not be applied in the present study. Therefore, in addition to the characterization of the time course parameters of the Ca\(^{2+} \) light transients (\( \Delta L \)) are reported with the precischemic amplitude of the Ca\(^{2+} \) light transients as a normalization reference. The amount of aequorin consumed throughout the experiment was taken into account as previously described (22).

Experimental protocol. The experimental protocol was started 5–10 min after steady-state levels of all mechanical parameters were reached. Fifteen minutes of baseline perfusion were followed by 20 min of no-flow ischemia. During ischemia, time to contracture (TTC) was defined as the time from the onset of ischemia to the time when pressure exceeded 10 mmHg, and VOMC refers to the value of maximal contracture during ischemia. After 20 min of ischemia, flow was recommenced and hearts were reperfused for 30 min. After 15 and 30 min of reperfusion, aequorin light and functional parameters were again measured.

Statistical analysis. All data are expressed as means ± SE. Comparisons among groups were made by repeated-measures ANOVA. When significant effects were observed, paired and unpaired Student’s t-tests were used as appropriate. Statistical analyses were performed with the use of Statview (Brainpower, Calabasas, CA), and values of \( P < 0.05 \) were considered statistically significant.

RESULTS

Cardiac function during baseline, ischemia, and reperfusion. Under identical loading conditions during baseline perfusion, no differences in LV functional parameters in wild-type and M/Mito-C/-/- mice were detectable (Table 1). At a constant perfusion pressure of 75 mmHg, coronary flow per gram of LV weight was 0.025 ± 0.002 ml·min\(^{-1}\)·mg\(^{-1}\) for wild-type hearts compared with 0.020 ± 0.002 ml·min\(^{-1}\)·mg\(^{-1}\).

Table 1. Left ventricular mechanical function of isolated, perfused hearts of wild-type and M/Mito-C/-/- mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>M/Mito-C/-/-</th>
</tr>
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<tbody>
<tr>
<td>Wild Type</td>
<td>M/Mito-C/-/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( n = 8 )</td>
<td>( n = 6 )</td>
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<tr>
<td>Preischemia</td>
<td></td>
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</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>7±1</td>
<td>8±1</td>
</tr>
<tr>
<td>LVDP, mmHg</td>
<td>83±1</td>
<td>87±2</td>
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<tr>
<td>( +dP/dt/LVDP ), s(^{-1} )</td>
<td>38±1</td>
<td>42±4</td>
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<tr>
<td>( -dP/dt/LVDP ), s(^{-1} )</td>
<td>32±1</td>
<td>32±3</td>
</tr>
<tr>
<td>During 20 min of no-flow ischemia</td>
<td></td>
<td></td>
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<tr>
<td>TTC, s</td>
<td>678±54</td>
<td>408±72*</td>
</tr>
<tr>
<td>VOMC, mmHg</td>
<td>35±2</td>
<td>55±2*</td>
</tr>
<tr>
<td>After 30 min of reperfusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>10±2</td>
<td>22±3†</td>
</tr>
<tr>
<td>LVDP, mmHg</td>
<td>48±2†</td>
<td>52±3†</td>
</tr>
<tr>
<td>( +dP/dt/LVDP ), s(^{-1} )</td>
<td>74±4†</td>
<td>72±6†</td>
</tr>
<tr>
<td>( -dP/dt/LVDP ), s(^{-1} )</td>
<td>57±2†</td>
<td>51±3†</td>
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Values are means ± SE. LVEDP, left ventricular (LV) end-diastolic pressure; LVDP, LV developed pressure; TTC, time to ischemic contracture (>10 mmHg); VOMC, value of maximum contracture; dP/dt, first derivative of LV pressure. For details, see MATERIALS AND METHODS. * \( P < 0.05 \) M/Mito-C/-/- vs. wild-type hearts, † \( P < 0.05 \) vs. preischemia.
for M/Mito-CK\(^{-/-}\) hearts [not significant (NS)] and contractile performance was stable, with variations in LVDP of <5% during the 30-min baseline perfusion period.

After the initiation of no-flow ischemia, LVDP declined to 0 within <2 min in wild-type and M/Mito-CK\(^{-/-}\) hearts (Figs. 1 and 2). However, ischemic contracture developed significantly earlier (408 ± 72 vs. 678 ± 54 s; \(P < 0.05\)) and the maximum extent of contracture was significantly greater (55 ± 2 vs. 35 ± 2 mmHg; \(P < 0.05\)) in CK-deficient hearts compared with wild-type hearts. On reperfusion, after a transient drop in the first 2 min, LVEDP increased further in both groups. This increase was significantly greater in M/Mito-CK\(^{-/-}\) (83 ± 2 mmHg) than wild-type (42 ± 2 mmHg) hearts. In the subsequent 20 min of reperfusion, LVEDP slowly declined and LV systolic pressure increased gradually. However, posts ischemic recovery of LVDP was significantly delayed in CK-deficient hearts (Fig. 2). For example, after 10 min of reperfusion, LVDP was 31 ± 6 mmHg in wild-type hearts and 13 ± 4 mmHg in M/Mito-CK\(^{-/-}\) hearts (\(P < 0.05\)). Similarly, recovery of diastolic function (LVEDP) at the end of the reperfusion period was significantly impaired in CK-deficient hearts. Whereas LVEDP completely returned to baseline levels in wild-type hearts (10 ± 2 mmHg; not significant vs. baseline), LVEDP in M/Mito-CK\(^{-/-}\) hearts at the end of reperfusion was significantly higher (22 ± 3 mmHg; \(P < 0.05\)) compared with baseline levels as well as compared with wild-type hearts (Fig. 2).

**Fig. 1.** Original strip-chart recording of aequorin light signal, isolated left ventricular (LV) pressure (LVP), and coronary perfusion pressure (cPP) during baseline perfusion, ischemia, and reperfusion in a wild-type (A) and a creatine kinase (CK)-deficient (M/Mito-CK\(^{-/-}\); B) heart. After onset of ischemia, LV developed pressure (LVDP) decays rapidly to zero and subsequently ischemic contracture develops.
Intracellular Ca\(^{2+}\) homeostasis in CK-deficient hearts. Representative examples of an aequorin light signal during baseline perfusion conditions of an isolated, perfused wild-type heart and a M/Mito-CK\(^{-/-}\) heart are shown in Fig. 3. The timing parameters of the light signals, including \(T_{PL}\) and positive first derivative of light signal as indexes of Ca\(^{2+}\) release, but also \(T_{50L}\), \(\tau_c\), and negative first derivative of light signal with time, as indexes of Ca\(^{2+}\) resequestration into the SR, were found to be similar for wild-type and M/Mito-CK\(^{-/-}\) hearts (Table 2). This indicates that myocardial Ca\(^{2+}\) handling in terms of Ca\(^{2+}\) release and reuptake in CK-deficient hearts is unaltered during baseline perfusion conditions.

Within the first 10–15 min after the initiation of ischemia, intracellular resting [Ca\(^{2+}\)] increased, as indicated by an increase in the aequorin light signal normalized to preischemic values (Figs. 1 and 4). This ischemic Ca\(^{2+}\) overload occurred earlier and was more pronounced in M/Mito-CK\(^{-/-}\) than in wild-type hearts. Because of the relatively large variability in the Ca\(^{2+}\) signal during ischemia, this difference was not statistically significant until later in the ischemic period (244 ± 51% vs. 162 ± 23% of basal [Ca\(^{2+}\)]).

On early reperfusion, intracellular [Ca\(^{2+}\)] rapidly increased (~3-fold), reaching its peak within the first minute of reperfusion. After 15 min of reperfusion, [Ca\(^{2+}\)] fell below preischemic values, but no differences between the groups were detectable, suggesting that the observed difference in postischemic LV function was not caused by prolonged or increased availability of Ca\(^{2+}\) to activate the myofilaments.

DISCUSSION

Transgenic mice with a combined knockout of cytosolic M-CK and Mito-CK (M/Mito-CK\(^{-/-}\)) provide an opportunity for a better understanding of the importance of the specific CK
isoenzymes in different muscle types. On the basis of the functional and physical association of the CK isoenzymes with subcellular sites of ATP production and hydrolysis, it has been proposed that, in addition to its role as an energy buffer, the CK isoenzyme system acts as an energy transport (shuttle) system linking sites of energy production (mitochondria) to sites of maximal energy requirement (ATPases). However, because heart muscle has in total only 25% of the CK activity of skeletal muscle, it had been hypothesized that heart muscle relies on an unaltered CK system as an energy buffer and transport system predominantly during situations of acute and very severe energy demand-supply mismatches. Although isovolumic LV performance in CK-deficient mice at low- and moderate-workload conditions was unaltered in this experimental preparation are currently lacking. This is mainly due to the fact that it is difficult to stress an isolated, perfused mouse heart above in vivo baseline levels (17). Finally, although a direct link between the missing CK system and alterations in cellular Ca\(^{2+}\) homeostasis was postulated, results of studies of myocardial Ca\(^{2+}\) dynamics at moderate workload. A key function of an intact CK system for maintenance of Ca\(^{2+}\) homeostasis under metabolic stress conditions.

**Ca\(^{2+}\) handling in CK-deficient hearts under baseline conditions.** To our knowledge, this is the first study to report results of successful Ca\(^{2+}\) measurements in intact, beating hearts of CK-deficient mice. Using the aequorin light technique, we were able to show that under baseline conditions intracellular Ca\(^{2+}\) handling was similar in CK-deficient and wild-type hearts. It is important to point out that special care was taken to perform these measurements in an intact, beating heart setup at a physiological temperature and with, compared with other small rodent hearts, high stimulation frequencies. Hampton et al. (5) were the first to demonstrate that quantitative and qualitative Ca\(^{2+}\) changes can be successfully measured with the aequorin bioluminescence method in isolated mouse hearts. However, a complex aequorin loading protocol was necessary, and, more importantly, the experiments were performed at a reduced temperature of 30°C (5). This non-physiological temperature precludes the comparison of those results with the results of many other experiments, e.g., using \(^{31}\)P NMR spectroscopy or \(\text{O}_2\) consumption, acquired under more physiological conditions. Given the fact that thermody-

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**Table 2. Time-course parameters of aequorin light signals from isolated, perfused hearts of wild-type and M/Mito-CK\(^{-/-}\) mice at baseline perfusion conditions**

<table>
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<th>Wild Type (n = 8)</th>
<th>M/Mito-CK(^{-/-}) (n = 6)</th>
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<tbody>
<tr>
<td><strong>(T_{PL}) (ms)</strong></td>
<td>27.5±1.0</td>
<td>29.3±4.0</td>
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<tr>
<td><strong>(T_{D1}) (ms)</strong></td>
<td>31.0±3.0</td>
<td>27.3±3.9</td>
</tr>
<tr>
<td><strong>(\tau_L), ms</strong></td>
<td>59.8±12.0</td>
<td>44.6±2.6</td>
</tr>
<tr>
<td><strong>(+dL/dr) (s(^{-1}))</strong></td>
<td>79.7±2.5</td>
<td>76.5±5.9</td>
</tr>
<tr>
<td><strong>−dL/dr) (s(^{-1}))</strong></td>
<td>40.3±6.7</td>
<td>43.3±3.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. M/Mito-CK\(^{-/-}\), mitochondrial creatine kinase-deficient mice; \(T_{PL}\), time to peak light; \(T_{D1}\), time to 50% decrease of light signal; \(\tau_L\), time constant of monoexponential decay of light signal; \(dL/dr\), first derivative of the light signal. For details see MATERIALS AND METHODS.
namic limitations might be ultimately responsible for the differences in Ca\(^{2+}\) release and uptake in CK-deficient hearts, it is essential to ensure physiological experimental conditions. Finally, not only the extent but also the reproducibility of any ischemia-reperfusion injury is critically dependent on the precise and reliable maintenance of a physiological temperature.

In support of our finding of unchanged baseline Ca\(^{2+}\) handling, Boehm et al. (1) demonstrated in saponin-skinned muscle fibers of CK-deficient mice that SR function in terms of Ca\(^{2+}\) uptake is unaltered as long as sufficient glycolytic intermediates are present. Interestingly, in a recent report Crozatier et al. (2) showed that caffeine-induced Ca\(^{2+}\) release kinetics are significantly altered in skinned fibers of CK-deficient hearts whereas the kinetics of the Ca\(^{2+}\) transients were completely normal in isolated cardiomyocytes, suggesting that the presence of functioning mitochondria is a prerequisite for normal Ca\(^{2+}\) homeostasis in CK-deficient hearts. Different results were obtained in fura 2-loaded myotubes of CK-mutant skeletal muscle: whereas basal intracellular Ca\(^{2+}\) levels under standard conditions were similar to those in wild-type skeletal muscle, Ca\(^{2+}\) response on depolarization and Ca\(^{2+}\) uptake were significantly impaired. Interestingly, SR Ca\(^{2+}\)-ATPase expression in skeletal muscle of CK-deficient mice was not altered (19). Thus if SR Ca\(^{2+}\)-ATPase plays a role in the altered Ca\(^{2+}\) dynamics, this most likely occurs through structural redistribution or a functional deficit rather than a decrease in overall content.

Thus the published data and the current results suggest that skeletal muscle, with its higher total CK activity and lower mitochondrial density and therefore lower capacity for aerobic ATP generation, is more dependent on an unaltered CK system to maintain normal Ca\(^{2+}\) homeostasis than heart muscle.

**Increased susceptibility of CK-deficient hearts to ischemia-reperfusion.** A central finding of the present study is that hearts lacking an efficient high-energy phosphate transport system are more susceptible to a challenge of severe energy supply-demand mismatch than wild-type hearts. It was shown previously that, although myocardial energetics are significantly altered in CK-deficient hearts, isovolumic contractile performance at moderate-workload conditions (comparable to the baseline perfusion conditions in the present study) was unaltered (17). This is in agreement with previous studies showing normal LV function at low workload in isolated rat hearts, even when CK activity was chemically inhibited to <1% of control (24). Only when extracellular [Ca\(^{2+}\)] was increased to increase workload in these rat hearts was an impairment in contractile reserve observed. In contrast, increasing extracellular [Ca\(^{2+}\)] in CK-ablated mouse hearts was not only well tolerated but also led to comparable increases in rate-pressure product or O\(_2\) consumption (17). As discussed by MacGowan et al. (13), it is reasonable to assume that Ca\(^{2+}\)-induced inotropy is not an adequate challenge to maximize ATP utilization to achieve a situation of severe energy supply-demand mismatch. In contrast, prolonged myocardial ischemia imposes a metabolic stress that is a much more severe challenge for the heart than increasing force production by increasing extracellular [Ca\(^{2+}\)]. Accordingly, 10–20 min after the onset of ischemia, a gradual rise in diastolic tension, i.e., ischemic contracture, occurs. We demonstrated that in CK-deficient hearts the ischemic contracture occurred significantly earlier and was more pronounced. Despite extensive research (9, 21), the underlying mechanism(s) of the development of ischemic contracture in general is not completely understood. Two basic mechanisms have been proposed. First, the rise in tension can be caused by an increase in intracellular [Ca\(^{2+}\)], leading to Ca\(^{2+}\)-activated cross-bridge cycling, or second, cross bridges may lock in the rigor state as a result of a decrease in myocardial ATP and PCr levels or increased ADP levels (6, 23, 25). A number of recent experimental studies argue in favor of the second hypothesis. Eberli et al. (3) were able to show that when Ca\(^{2+}\) availability was experimentally altered during ischemia, there was no alteration in LV diastolic pressure, suggesting that ischemic diastolic dysfunction is not directly mediated by extra Ca\(^{2+}\)-activated tension. Similarly, using NMR spectroscopy for direct measurement of high-energy phosphate and [Ca\(^{2+}\)] during a prolonged period of ischemia, Kuretsune and Marban (10) demonstrated that a fall in ATP coincides closely with the onset of contracture, whereas the profound intracellular acidosis and P\(_i\) accumulation during ischemia render the myofilaments insensitive to Ca\(^{2+}\).

What, therefore, is the relative contribution of these two mechanisms in causing ischemic contracture in the CK-deficient hearts? We showed previously by NMR spectroscopy (17) as well as by HPLC analysis of CK-deficient hearts (16) that, despite the fact that the concentration of ATP in isolated well-perfused hearts was indistinguishable from that in wild-type hearts (9.6 ± 0.1 vs. 9.3 ± 0.2 mM), high- and low-energy phosphate levels at baseline (P\(_{CR}\) −40%, ADP +170%, P\(_{i}\) +20%) were found to be significantly altered. Moreover, increasing cardiac workload leads to a significantly greater decrease in P\(_{CR}\) (−50%) and a greater accumulation of ADP (−95%), leading to larger decreases in free energy release from ATP hydrolysis (\(\Delta G_{ATP}\) −3.6 kJ/mol) compared with wild-type hearts (17). It is therefore reasonable to assume that during a period of more severe energy supply-demand mismatch as induced by ischemia energetic alterations are even more pronounced. Accordingly, it is hypothesized that the earlier and more pronounced occurrence of ischemic contracture in CK-deficient hearts is induced by an earlier fall in P\(_{CR}\) and, more importantly, an earlier increase in myofibrillar ADP-to-ATP ratio, leading to a drop in \(\Delta G_{ATP}\). This in turn would facilitate the unrestrained entry of Ca\(^{2+}\) into the myocardial cells (resulting from a combination of increased Ca\(^{2+}\) influx, decreased Ca\(^{2+}\) efflux, and decreased Ca\(^{2+}\) reuptake by the SR), explaining the greater increase in diastolic [Ca\(^{2+}\)] during the ischemic period. Because the minimal energy requirements of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase are higher than those of myosin ATPase, alterations in Ca\(^{2+}\) homeostasis would be predicted to occur before the development of rigor cross bridges (8). However, more importantly, because the myofilaments during the ischemic period are rather insensitive to Ca\(^{2+}\) because of profound intracellular acidosis (9), the pattern and time course of the ischemic contracture are dissociated and/or independent from the changes in intracellular [Ca\(^{2+}\)].

The significantly greater increase in intracellular [Ca\(^{2+}\)] in CK-deficient hearts at the end of the ischemic period, however, has a significant impact on the subsequent reperfusion injury, known to be mediated by the transient Ca\(^{2+}\) overload within the first minutes of reperfusion (12). During the course of reperfusion, systolic and diastolic LV performance remained significantly impaired in both groups of hearts despite lower...
resting [Ca^{2+}+]_{i} during reperfusion. A possible mechanism is the decreased Ca^{2+} responsiveness of the postischemic myofila-
ments rather than the failure of activator Ca^{2+} delivery (4, 11).

However, direct experimental evidence, for example, by 31P
NMR spectroscopy, demonstrating that thermodynamic limi-
tations (ΔAG_{ATP}) are responsible for the observed difference in
the ischemic response in CK-deficient mouse hearts currently
escapes detection because of the very limited temporal resolu-
tion of NMR spectroscopy in isolated mouse hearts (8 min in
our hands), which exceeds the time difference in LVEDP
increase between wild-type and CK-deficient hearts in the first
minutes of ischemia.

In conclusion, the present study demonstrates that, during
moderate workload, loss of an efficient CK system in trans-
genic CK-deficient hearts is well compensated by adaptational
mechanisms. However, during more pronounced mismatches
in energy supply and demand, such as induced by ischemia-
reperfusion injury, significant alterations in LV performance and
Ca^{2+} homeostasis become unmasked, providing further
evidence for a key function of an intact CK system for
maintenance of calcium homeostasis under metabolic stress
conditions.

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ISCHEMIA-REPERFUSION AND Ca^{2+} HOMEOSTASIS IN CK-KO HEARTS

H1045