Alteration of the Cytosolic-Mitochondrial Distribution of High-Energy Phosphates During Global Myocardial Ischemia May Contribute to Early Contractile Failure

Ursula Rauch, Karsten Schulze, Bernhard Witzenbichler, Heinz P. Schultheiss

Abstract  Depending on its duration, temporary myocardial ischemia leads to a disturbance of myocardial function before irreversible cellular necrosis is developed. Mechanical, electrical, and metabolic disturbances were suggested to be possible mechanisms accounting for the altered mechanical performance in ischemic hearts. To further investigate the alteration of myocardial energy metabolism on the subcellular level, we determined, by means of nonaqueous fractionation, the cytosolic-mitochondrial distribution of high-energy phosphates and other metabolites (ATP, ADP, phosphocreatine, creatine, and inorganic phosphate) in ischemic (zero-flow) guinea pig hearts after isolated perfused working heart preparation. Additional experiments using 31P nuclear magnetic resonance spectroscopy were performed to determine pH, and [Mg2+]i changes during global ischemia. The total ATP content of myocardial tissue dropped only slowly to 76% of control ATP at 10 minutes and to 51% at 30 minutes and reached almost zero at 60 minutes of ischemia. However, striking differences were observed on the subcellular level: While cytosolic phosphocreatine was almost completely consumed after 3 minutes of ischemia (from 19.1±1.6 to 3.3±0.5 mmol/L), ATP concentration in the cytosol decreased within 30 minutes from 8.4±0.6 to only 5.4±0.9 mmol/L. Mitochondrial ATP was rapidly and linearly reduced to 60% after 5 minutes of ischemia and was nearly unmeasurable after a further 20 minutes. Thus, in contrast to the breakdown of phosphocreatine in cytosol, the only slight alteration of cytosolic ATP reveals a reduction in cytosolic ATP utilization. Moreover, the unaffected cytosolic-mitochondrial difference in the phosphorylation potential of ATP demonstrates the intact function of the ADP/ATP carrier during early ischemia. These results might indicate a disturbance of the functional coupling between carrier and phosphocreatine kinase (phosphocreatine shuttle), which could be of importance for the early contractile failure in myocardial ischemia. (Circ Res. 1994;75:760-769.)

Key Words  • myocardial ischemia • early contractile failure • compartmentation of high-energy phosphates • ADP/ATP carrier • phosphocreatine shuttle

Myocardial ischemia leads to contractile failure before apparent cell damage and necrosis.1 The contractile function of hearts subjected to global ischemia declines rapidly during the first few minutes after the onset of ischemia.2-3 The mechanism of this contractile dysfunction has not yet been fully explained. Several hypotheses concerning mechanical, electrical, and metabolic alterations have been discussed, such as (1) a decrease of perfusion pressure in the vascular system leading to reduced myofibril length and a decline in pressure development,2-5 (2) depression and shortening of the action potential6-8 as well as reduced Ca2+ release from the sarcoplasmic reticulum,8,9 affecting the excitation-contraction coupling of the heart, (3) accumulation of inorganic phosphate10,11 and protons,12 which interfere with myofilaments and other important proteins, and (4) depletion of high-energy phosphates and changes of the free energy of ATP hydrolysis.13,14 Our present work refers to the latter of these hypotheses, considering energy metabolism during ischemia. We observed changes in the compartmentation of high-energy phosphates that occur during depletion of oxygen and substrates.

Although the mitochondrial ATP production diminishes in acute ischemia because of a lack of oxygen supply, several investigators have shown that the ATP content of the myocardial tissue remains nearly constant over the first minutes of ischemia, whereas the phosphocreatine (CP) pool decreases rapidly.1,2,15 Disturbances in energy utilization16,17 and failure in energy transport processes have been proposed to help understand these observations.

Mainly two compartments of the myocyte have to be considered in energy metabolism: the mitochondrial matrix space in which ATP is produced by oxidative phosphorylation and the cytosolic space where it is dephosphorylated by the energy-consuming reactions. Because of the impermeability of the inner mitochondrial membrane for these hydrophilic adenine nucleotides, a well-characterized ADP/ATP carrier imports cytosolic ATP into the mitochondrial compartment in a countercurrent with newly synthesized mitochondrial ATP. A reduced function of the ADP/ATP carrier has been suggested to contribute to the altered mechanical performance during ischemia.18 The present study attempts to clarify the impact of ischemia on the mitochondrial energy status with special focus on the func-
tion of the ADP/ATP carrier. No methods are available for direct determination of the function of the ADP/ATP carrier in vivo. Thus, its transport activity was assessed by measuring compartmental changes in high-energy phosphate concentration and phosphorylation potentials.

Experiments were performed on working guinea pig hearts, a well-characterized model to study myocardial hypoxia and ischemia. After early and advanced periods of zero-flow ischemia up to 60 minutes, subcellular high-energy phosphate contents in the myocardial tissue were measured by nonaqueous fractionation. The phosphorylation potentials of ATP in mitochondria and cytosol were calculated from these metabolic data.

Materials and Methods

Substrate, enzymes, and coenzymes for enzymatic analyses were purchased from Boehringer Mannheim and from Sigma Chemical Co. Protein was calibrated with 100% purified bovine serum albumin from Behringwerke AG. All chemicals used for high-performance liquid chromatography (HPLC) were from E. Merck and from Pierce Chemical Co.

Working Heart Preparation and Perfusion

The hearts of guinea pigs with a body weight of 200 to 300 g were isolated and perfused as described in detail by Becker et al. and Bürger et al. Briefly, the thorax was opened, and the heart was rapidly excised through median thoracotomy and immersed in an ice-cold physiological NaCl solution. The aorta was dissected free from surrounding tissue and connected to the perfusion apparatus. Langendorff retrograde perfusion was introduced with a modified Krebs-Henseleit buffer containing (mmol/L) NaCl 127, KCl 4.7, NaHCO3 24.9, CaCl2 1.25, MgSO4 0.6, KH2PO4 1.2, pyruvate 0.3, and glucose 5.5, along with 5 U/L insulin. The nonrecirculating perfusion medium was calibrated to pH 7.4 with 94.4% O2/5.6% CO2 at 37°C. These conditions were provided to ensure adequate substrate and oxygen availability to the myocardium.

After a stabilization period of 20 minutes, perfusate was applied to the left ventricle via the cannulated left auricle. All other arterial veins were ligated except the coronary sinus, which was drained through the pulmonary artery.

Atrial filling pressure and aortic pressure were continuously recorded by Statham P23DB strain gauges (Gould). Heart rate was taken from the phasic pressure signals with a heart-rate module (HRM type 669, Hugo Sachs Elektronik). External heart work was calculated from the pressure-volume work of the left ventricle and acceleration work during ejection. Two Clarke-type electrodes (Bachofer) were used to determine the myocardial oxygen consumption, which was derived from the difference of oxygen tension between aortic perfusate and coronary effluent.

Experimental Protocol

Retrograde Langendorff perfusion of the isolated guinea pig hearts was maintained for an equilibration period of 20 minutes to obtain a steady state of substrate and oxygen consumption. Perfusion was then continued in the antegrade “working modes” with a preload of 12 mm Hg and an afterload of 60 mm Hg. After ~15 minutes, hemodynamic parameters, coronary flow, and oxygen consumption were recorded. After 30 minutes of the heart in the working state, global ischemia was applied to the heart by clamping atrial and aortic cannu-

las. During ischemic periods of 0.5, 3, 5, 10, 20, 30, and 60 minutes, the temperature of the myocardium was kept at a constant level by immersing it in nonoxygenated buffer at 37°C. The ischemic phase was stopped by clamping the myo-

cardium between aluminum blocks that had been precooled in liquid nitrogen.

All experiments were made under steady-state conditions. Control measurements without ischemia (n = 7) were performed under stable hemodynamic conditions after 2 hours of the heart in working state. Further investigations were carried out to determine the concentrations of intracellular adenine nucleotides (ATP and ADP), CP, and other substances.

Nonaqueous Fractionation of Myocardium

The frozen myocardial tissue was crushed in a mortar containing liquid nitrogen. It was then lyophilized at −40°C and 0.001 mm Hg for 3 days. During the lyophilization process, all dissolved metabolites, such as proteins, enzymes, high-energy phosphates, and other substances, were fixed onto the membranes of their respective cellular compartments. The myocardial membranes of the freeze-dried tissue were broken up into a homogenate of small membrane fragments by sonicating the tissue for 4 minutes at 5-second intervals (Sonifier 250 Branson, Sonic Power Co). Purification of the homogenate was achieved by successive filtration through columns filled with glass beads of different diameters (1.0 to 0.5 mm).

Because of the higher density of the cytosolic versus the mitochondrial membrane particles, compartmental enrichment of the purified tissue homogenate was achieved by heptane/carbon tetrachloride density gradient centrifugation for 4 hours at 16,000 g (Sorvall RC-5B refrigerated superspeed centrifuge with swing-out rotor, Du Pont Instruments). The gradient yielded eight fractions containing different proportions of cytosolic and mitochondrial proteins. The fractions were dried in a desiccator. In a first aliquot, the activities of the cytosolic marker enzyme phosphoglycerate kinase (PGK), the mitochondrial marker enzyme citrate synthase (CS), and the total protein content were analyzed. The concentrations of high-energy phosphates, creatine, and inorganic phosphate were determined in a second aliquot. The protein content was obtained as described by Lowry et al. by using an Eppendorf filter photometer model 940 from Kontron Instruments. The measurements of the activities of PGK and CS and the determination of creatine and inorganic phosphate were performed by enzymatic analyses. All enzymatic tests were performed with a dual-beam dual-wavelength spectrophotometer (Sigma ZFP 22, Biochem) by using 366 nm as the measuring wavelength and 405 nm as the reference wavelength. Adenine nucleotide and CP concentrations were measured with HPLC.

Measurement of Adenine Nucleotides and CP by HPLC

Adenine nucleotides (ATP and ADP) were separated from each other by using reverse-phase ion-pairing HPLC as described previously by Hammer et al., whereas CP content was determined according to Juengling and Kammermeier. First, the aliquots of the heart tissue had to be prepared for HPLC use, which included deproteinization and extraction of the different constituents into a solvent. Therefore, perchloric acid (0.6N) was mixed with each sample and, after centrifugation, was neutralized with ice-cold triocylamine and Freon (1:4 mixture). Between each preparation step, all aliquots were kept on ice to avoid the loss of a large number of high-energy phosphates. The aqueous extract was then used for adenine nucleotide and CP determination by reverse-phase ion-pairing HPLC.

A Kontron HPLC system consisting of two pumps, a 430 UV detector with variable wavelengths, a refrigerated automatic injector system (autosampler 460), and a computer data system for solvent delivery programming and peak area integration was available. Separations were carried out with an OSD Hypersil reversed-phase column (Hewlett Packard).

All preparations were made with highest purified water (HPLC grade). For adenine nucleotide analysis, two different
aqueous buffers were used as mobile phase: buffer A, containing 30 mmol/L KH_2PO_4 and 7.5 mmol/L tetrabutylammonium phosphate at pH 5.45, and buffer B, consisting of 50% acetonitril and 50% KH_2PO_4 (30 mmol/L) at pH 7.0. The aqueous eluate for CP measurement contained 0.2% KH_2PO_4 and 0.3% tetrabutylammonium phosphate at pH 3.0. All solvents were filtered through a 0.2-μm Millipore membrane and degassed with helium before being applied to the HPLC column. The detector wavelengths were set at 254 and 210 nm for adenine nucleotide and CP separation, respectively.

The metabolite concentrations of each aliquot were quantified by comparison of peak area with that of commercially available external standard. Standard curves were linear for all concentrations examined.

**Calculation of Intracellular Concentrations**

The total metabolite content (M_{tot}) of each aliquot obtained from the density gradient consists of mitochondrial and cytosolic portions (M_{mit} and M_{cyt}, respectively). The distribution of marker enzymes CS and PGK in each fraction correlates with the membrane and substrate content of the respective cellular compartment. Since the relation between marker enzyme and compartmental metabolite content remains constant (values of M_{mit}/PGK and M_{cyt}/CS are constant), calculations of mitochondrial as well as cytosolic substrate concentrations can be obtained by linear regression by using the following formulas:

(1) \[ M_{tot} = M_{cyt} + M_{mit} \]

(2) \[ M_{mit}/PGK = a \]

(3) \[ M_{cyt}/CS = b \]

Combination and transformation of Equations 1 through 3 leads to

(4) \[ M_{mit}/CS = a(PGK/CS) + b \]

For the determination of the metabolite concentrations, cytosolic and mitochondrial compartments were referred to the protein content of the corresponding compartment. Calculation factors of 5.82 μL water per milligram cytosolic protein and 1.8 μL water per milligram mitochondrial protein were used to obtain the subcellular concentrations.

**Calculation of K_{CPK} and Phosphorylation Potential**

Because most of the cytosolic ADP is bound to the contractile filaments of the heart muscle, free cytosolic ADP had to be calculated from the creatine phosphokinase reaction:

(5) \[ [ATP]/[ADP]=\frac{[CP]×[H^+][\text{creatine}]}{K_{CPK}} \]

The equilibrium constant for the creatine kinase reaction (K_{CPK}) changes as a function of pH and free intracellular Mg^{2+} concentration as shown by Lawson and Veech and van der Meer et al. The following empirical equation for the simultaneous pH and Mg^{2+} dependence of K_{CPK} was established by Bünger et al.

(6) \[ [H^+]_{K_{CPK}} = \text{antilog}(7.52-0.97\times pH+3.12[Mg^{2+}]_o^{1/2}) \]

The combination of 5 and 6 leads to

(7) \[ [ATP]/[ADP]=[CP][\text{creatine}] \times \text{antilog}(7.52-0.97\times pH+3.12[Mg^{2+}]_o^{1/2}) \]

This formula allows the determination of the free intracellular Mg^{2+} concentration and any near-physiological pH values.

The phosphorylation potential of ATP (\Delta G_{ATP}) was calculated from the free ATP-to-ADP ratio for both mitochondrial and cytosolic compartments:

(8) \[ \Delta G_{ATP} = \Delta G^o + RT \times \ln([ATP]/[ADP]×[\text{phosphate}]) \]

where \Delta G^o equals −31.9 kJ/mol under conditions prevailing intracellularly. R is Faraday’s constant, and T is the absolute temperature in degrees kelvin.

**Determination of Cytosolic pH and Intracellular Free Mg^{2+} by 31P Nuclear Magnetic Resonance Spectroscopy**

Additional experiments using 31P nuclear magnetic resonance (NMR) spectroscopy were accomplished to investigate the alterations of pH, and free Mg^{2+} concentration during ischemia in order to calculate K_{CPK}. Therefore, five guinea pig hearts, isolated and perfused as described above, were placed into a 20-mm NMR glass tube (Wilmad). The 31P NMR scans were performed in a Bruker wide-bore vertical magnet (9.4-T, 7.0-cm bore diameter) at a phosphorus resonance frequency of 162 MHz. Each scan represented the sum of 20 (ischemia minutes 1 through 10) and 98 single-pulse acquisitions (pre-ischemia, ischemia minutes 20 and 30). Acquisition parameters were as follows: size: 2000 (+2000 zero fill); sweep width: 6000 Hz, pulse width: 170 microseconds; interpulse delay, 2.8 seconds; and line broadening, 5 Hz. The intracellular phosphate content was estimated from the pH-dependent split of the phosphorus resonance into two partially merged peaks representing the extracellular and the intracellular or cytoplasmatic fraction of inorganic phosphorus in the magnetic field. Nevertheless, the spectral resolution was high enough to allow an accurate separation of both peaks by application of a Lorentzian line-fitting routine. To determine pH, the equation for the chemical shift of inorganic intracellular phosphate as a function of pH was used:

(9) \[ \delta = \text{pH} - 6.79 - \log(\delta - 5.75)/(3.25 - \delta) \]

where δ is defined as the chemical shift (in parts per million) of the resonance of intracellular inorganic phosphorus relative to CP.

The intracellular free Mg^{2+} concentration was estimated from the difference of the chemical shift of α and β resonances of ATP (δ, in parts per million) as a function of free Mg^{2+} and pH:

(10) \[ [Mg^{2+}]_{\text{free}} = K_{D_{\text{MgATP}}} \frac{\delta_{\text{ATP}} - \delta_{\text{MgATP}}}{\delta - \delta_{\text{MgATP}}} - 1 \]

with K_{D_{\text{MgATP}}, \delta_{\text{ATP}},} and \delta_{\text{MgATP}} determined from calibration solutions of an ionic strength of 0.18 mmol/L and a temperature of 37°C. The Mg^{2+} calibration was done in the physiologically and pathophysiological relevant pH range of 6.0 to 7.8 at an ATP concentration of 5 mmol/L. For the dissociation constant of the Mg-ATP complex (K_{D_{\text{MgATP}}}), a value of 38 μmol/L (at 37°C) was used. The pH-dependent end points of the calibration curve (δ_{\text{ATP}} and δ_{\text{MgATP}}) were derived from the formula recently developed by Mosher et al. δ_{\text{MgATP}} is the maximum value of δ. The largest chemical shift difference of α and β resonances of ATP is observed in the Mg^{2+}-free calibration solution. δ_{\text{MgATP}} is the minimum value of δ occurring at a pH-dependent Mg^{2+} concentration.

The concentration values for Mg^{2+} were obtained by dividing the metabolite amount by the wet weight of the heart and by the intracellular space, which was 0.37 mL per gram wet mass. In this manner, the alteration of pH, and free Mg^{2+} concentration was determined during a period of 60-minute ischemia applied to guinea pig hearts according our experimental protocol (see above).

**Statistical Evaluation**

Student’s t test for unpaired samples was used to analyze the data statistically. Values are given as mean±SEM of n inde-
TABLE 1. Subcellular Metabolite Concentrations in Ischemic Isolated Perfused Guinea Pig Hearts

<table>
<thead>
<tr>
<th>Subcellular Metabolite Concentration, mmol/L</th>
<th>CP</th>
<th>ATP</th>
<th>ADP</th>
<th>Creatine</th>
<th>Phosphate</th>
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<td>Mitochondria</td>
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CP indicates phosphocreatine. Values are mean±SEM. Seven control animals and 24 test animals were used for the experiments; 3 test animals were subjected to each ischemic period of 0.5, 1, 3, 5, 10, 20, 30, and 60 minutes. Cytosolic and mitochondrial concentrations of high-energy phosphates, creatine, and inorganic phosphate were obtained by fractionation of myocardial tissue through density gradient centrifugation in nonaqueous media using known subcellular water contents for the cytosolic and the mitochondrial space.29,30

Results

Ischemic periods of 0.5, 1, 3, 5, 10, 20, 30, and 60 minutes were applied to hearts at 37°C before freeze clamping. Effects of ischemia on energy metabolism were determined by nonaqueous fractionation of these frozen hearts, in particular the cytosolic and mitochondrial metabolite distribution. All results obtained from ischemic hearts were compared with the data of control animals, which were not subjected to ischemia.

A rapid loss of cytosolic CP from 19.1±1.6 to 11.7±1.3 mmol/L (P<.01), reaching 61% of control values, was observed over the first 30 seconds of ischemia. After a further 3-minute period, values of 3.3±0.5 mmol/L (P<.001) were obtained representing 17% of the normal CP level (Table 1). It is a well-known fact that high concentrations of CP are found in the cytosolic space, whereas the mitochondria do not contain noticeable CP. Thus, the observed fall of the total CP content during early ischemia takes place only in the cytosolic compartment, and the mitochondrial CP remains near zero (0.2±1.2 mmol/L) during the entire period of ischemia. The total creatine content increased continuously during the rapid CP breakdown (Fig 1), whereby creatine molecules accumulated in both compartments, reaching nearly threefold normal creatine concentrations. Since mitochondria normally contain only small amounts of creatine, it must be pointed out that during reduced oxygen availability, as in ischemia, the creatine levels start to increase even in the mitochondrial space (Table 1).

In contrast to the rapid decline of CP, the total ATP pool of the myocardial tissue dropped only slowly to 76% of control ATP at 10 minutes and to 51% at 30 minutes. Fig 1 shows the decrease of the whole-tissue ATP and, adversely, the accumulation of the whole-tissue ADP and inorganic phosphate during 60-minute ischemia. Although the total ATP content diminished quite slowly, striking differences in ATP decrease between the mitochondrial and cytosolic compartments were observed (Table 1). The mitochondrial ATP content was rapidly and linearly reduced to 60% after 5 minutes of ischemia, and almost no ATP (<10%) was measurable after 20 minutes. In contrast, the cytosolic ATP decline from 8.4±0.6 to 7.6±0.8 mmol/L was very slow and first appeared after a 10-minute period of ischemia. The resulting rise in ADP concentration was highest for mitochondria after 5 minutes, whereby ADP
levels rose nearly threefold from 2.1±0.2 to 5.1±0.9 mmol/L (P<.001), as compared with normal mitochondrial ADP concentrations. In contrast to the sudden increase of mitochondrial ADP, a moderate and advanced elevation of ADP from 1.3±0.2 to 1.9±0.1 mmol/L (P<.01) was observed after 5 minutes in the cytosolic compartment. These findings might indicate that during early ischemia, ADP molecules generated within the cytosol are transported to the mitochondrial space, resulting in an accumulation of ADP in mitochondria.

During the breakdown of high-energy phosphates, especially of CP and ATP, the total inorganic phosphate pool increased rapidly within 60 minutes of ischemia, reaching more than fourfold preischemic levels (Fig 1). The subcellular amount of inorganic phosphate changed during the first 5 minutes from 11.6±1.4 mmol/L in mitochondria and 7.3±1.0 mmol/L in cytosol to 33.6±1.2 and 20.5±1.7 mmol/L (P<.01), respectively, and remained nearly constant for a further 55 minutes. Thus, phosphate molecules are able to reach the inside of the mitochondrial space during short times of oxygen depletion. Table 1 lists the subcellular metabolite concentrations for the cytosolic and mitochondrial compartment over a 60-minute period of ischemia.

Since most of the ADP molecules in myocardial muscle are bound to proteins, the free ADP concentration cannot be estimated from the total ADP content of the tissue. It has to be calculated from the pH- and Mg$^{2+}$-dependent mass action ratio of the creatine kinase reaction (K$_{CPK}$) as mentioned above. For this reason, $^{31}$P NMR spectroscopy was used to obtain the alterations of pH$_i$ and free Mg$^{2+}$ concentration during ischemia. pH$_i$ revealed a significant decline from 7.12±0.02 to 6.81±0.03 within the first 5 minutes, reaching a value of 6.07±0.07 after 30 minutes of ischemia. The intracellular free Mg$^{2+}$ concentration was adversely affected. A slight increase from 0.34±0.03 to 0.49±0.07 mmol/L after a period of 5 minutes and a further increase to 1.68±0.24 mmol/L after 30 minutes of ischemia were monitored. Since it was not possible to detect $\alpha$ and $\beta$ resonances of ATP after a full hour of...
ischemia, the determination of the intracellular free Mg²⁺ was limited to the first 30 minutes. Fig 2 demonstrates the time course of pH, and free Mg²⁺ concentrations during ischemia. In addition, the creatine kinase equilibrium constant and cytosolic free ATP-to-ADP ratio were recalculated for the different ischemic periods by using the measured pH and Mg²⁺ values according to Equations 6 and 7 (see above).

The changes of the ADP pool were opposite those of ATP; the free ATP-to-ADP ratios dropped very quickly from 178±20 in the cytosol and 4.4±0.5 in the mitochondria to 27±16 and 1.1±0.2 (P<.01), respectively, during the first 3 minutes of ischemia. For the mitochondrial space, values near zero were reached after 20 minutes of ischemia (Table 2). Furthermore, the determination of free ATP-to-ADP allows the calculation of the phosphorylation potential (ΔG) for both compartments. The cytosolic ΔG of 57.9±1.0 kJ/mol ATP obtained for control hearts diminished to 50.2±0.8 kJ/mol ATP (P<.05) within 5 minutes. Since an evaluation of the cytosolic ΔG value could only be obtained for the first 5 minutes of ischemia, these calculations were omitted for more extended ischemic periods (see “Discussion”). The mitochondrial ΔG value declined from 47.5±1.2 to 40.0±0.8 kJ/mol ATP (P<.05) during the first 5 minutes and dropped another 5 kJ/mol ATP after a further 20 minutes of ischemia. Table 2 also lists the mitochondrial-cytosolic difference of ΔGₜₚ that is

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Cytosol</th>
<th>Mitochondria</th>
<th>Cytosol-Mitochondria</th>
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<tr>
<td></td>
<td>ATP/ADP&lt;sub&gt;free&lt;/sub&gt;</td>
<td>ΔG</td>
<td>ATP/ADP</td>
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<tr>
<td>Before ischemia</td>
<td>178±20</td>
<td>57.9±1.0</td>
<td>4.4±0.5</td>
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<td>0.5</td>
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<td>52.9±0.8</td>
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<td>27±16</td>
<td>50.5±0.9</td>
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<td>25±18</td>
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ATP/ADP<sub>free</sub> indicates the free ATP-to-ADP ratio; ΔG, phosphorylation potential. Values are mean±SEM. ATP/ADP<sub>free</sub> and ΔG values were determined in the cytosol and mitochondrial space during ischemia. ATP/ADP values were calculated from the mass action ratio of the creatine kinase reaction using Equation 6 (see text). ΔG values of ATP are given in kilojoules per mole ATP. The cytosolic-mitochondrial values for ΔG demonstrate the amount of the cytosolic-mitochondrial potential difference over the mitochondrial membrane, which is an important parameter for the assessment of the activity of the ADP/ATP carrier in the inner mitochondrial membrane. Before ischemia represents the mean value of control animals (n=7) at 30 minutes in working hearts before ischemia is induced. All other points indicate the mean±SEM of 3 test animals subjected to ischemia.
≈10 kJ/mol ATP and does not change within the first 5 minutes of ischemia.

**Discussion**

**Subcellular Metabolite Alterations in Ischemic Guinea Pig Hearts**

The total CP pool declined rapidly to 10% of preischemic levels within the first 3 minutes of ischemia, whereas the breakdown of total ATP of the myocardial tissue was much slower. After 60 minutes of ischemia, 20% of the preischemic ATP pool was still detected. During the rapid degradation of CP, creatine was accumulated in both cytosolic and the mitochondrial compartments, reaching threefold normal concentrations (Table 1). The flux of creatine into the mitochondrial matrix was previously described by Altschuld et al.37 The presence of an elevated level of mitochondrial creatine might indicate permeability changes of the mitochondrial membrane induced by oxygen depletion.

In addition, large amounts of inorganic phosphate were liberated from the breakdown of CP and ATP, leading to an approximately fourfold increase in the inorganic phosphate concentration of both compartments. Hütter et al38 showed the rise of inorganic phosphate in the intramitochondrial space during oxygen depletion and proposed a mitochondrial ATP–inorganic phosphate. The ability of phosphate to retain calcium within the mitochondria as calcium phosphate precipitate39,40 might affect the availability of Ca2+ for contraction.10 Therefore, both cytosolic and mitochondrial metabolism were affected by the high increase in inorganic phosphate and creatine concentrations.

A rapid initial reduction of the mitochondrial ATP concentration was observed during early ischemia, whereas the cytosolic ATP concentration remained nearly constant within the first 10 minutes. This rapid decline of ATP and the accumulation of ADP in mitochondria during early ischemia could be explained by a substantially unchanged activity of the nucleotide translocator delivering the rest of the mitochondrial ATP to the cytosol by counterexchange with ADP after cessation of oxidative phosphorylation. However, the significant disappearance of mitochondrial ATP within the first minutes of oxygen depletion might not be mediated only by the 1:1 ATP-ADP exchange via the adenine translocator or by simple degradation of high-energy phosphates; different mechanisms have been postulated for the pathway of mitochondrial ATP loss, all dependent on the property of inorganic phosphate being an easily permeable anion. Meisner and Klingenberg41 demonstrated that a specific ATP efflux driven by inorganic phosphate uptake occurred unidirectionally from mitochondrial to cytosolic space by the way of the adenine translocator without counterexchange for ADP. Other investigators proposed an ATP–inorganic phosphate exchanging protein.38,42 However, the relevance of these mechanisms for mitochondrial ATP loss during oxygen depletion in hearts is still unknown. Nevertheless, the significant shift of ATP from the mitochondrial to the cytosolic space and vice versa for ADP and inorganic phosphate leads to marked changes in compartmentation of high-energy phosphates, an effect that has not yet been reported for ischemic hearts.

**pH- and Mg2+-Dependent Calculation of KCPK During Ischemia**

Since most of the cytosolic ADP is bound to the contractile filaments of the heart muscle, free cytosolic ADP had to be calculated from the pH- and Mg2+-dependent creatine kinase reaction. The time course of pH, and free inorganic Mg2+ concentration was determined by 31P NMR spectroscopy during ischemia. The pH, revealed a significant steady decline over a 30-minute period, consistent with previous reports.12,43,44 The free Mg2+ concentration was elevated from the preischemic value of 0.34±0.03 to 1.68±0.24 mmol/L after 30 minutes of ischemia. Murphy et al45 demonstrated a similar increase in the amount of Mg2+ during the first 15 minutes of oxygen depletion. Changes in pH may modulate the free intracellular Mg2+ concentration, probably by affecting cytosolic Mg2+ binding. In this manner, both decreased pH and elevated Mg2+ concentration have been proposed to affect cellular function, especially the myocardial contractility. However, it is well known that KCPK changes as a function of pH and free Mg2+ concentration.29,30 Bünger’s empirical formula28 was used to recalculate KCPK in view of the ischemic-induced changes in pH and Mg2+ concentration. Because a valid application of this equation is justified only for near physiological pH values, KCPK estimation was limited to the first 5 minutes of oxygen depletion. Thereafter, the pH dropped, critically impeding further calculations of cytosolic free ATP-to-ADP ratios and phosphorylation potentials. Furthermore, it must be assumed that the mitochondrial creatine kinase operates far from its equilibrium, especially under normal conditions of oxygen supply and energy demand.47 However, Saks et al48 demonstrated that the mass action ratio of creatine kinase approached its equilibrium value within seconds when oxidative phosphorylation was inhibited. If this is the case, then usual equilibrium calculations can be performed at least from 30 seconds of ischemia on, whereas they might be erroneous in nonischemic hearts. Our data indicate that this error probably does not play a major role. Free ATP-to-ADP ratios and ΔG values in nonischemic control hearts as calculated from the creatine kinase reaction fit well into the expected range when the data from 30 seconds to 5 minutes of ischemia are extrapolated.

In addition, the cytosolic phosphorylation potential of ATP was determined according to the definition of Gibb’s free energy change49 that is also pH and Mg2+ dependent. Derived from Alberty’s contour diagrams, changes in pH from 7.2 to 6.6 and in magnesium concentration from 100 to 1000 μmol/L reduce the amount of Gibb’s free energy change <6%.49 This very small shift in ΔG was not considered to be of significance and thus not taken into account in further calculations.

**Role of the ADP/ATP Carrier During Ischemia**

The nucleotide translocator yields elevated ATP-to-ADP ratios and phosphorylation potentials for the cytosolic compartment and lower ratios and potentials for the mitochondrial space. Our nonischemic control hearts demonstrate this typical distribution of energy state. Marked declines in the cytosolic and mitochondrial ATP-to-ADP ratio and ΔGATP were obtained dur-
ing early ischemia as shown in Table 2. The rapid decrease in the free ATP-to-ADP ratio and phosphorylation potential has previously been reported by Kammermeier and colleagues,\(^{4,55}\) and Gibbs,\(^{57}\) who suggested that free energy change of ATP hydrolysis rather than ATP depletion seemed to account for myocardial dysfunction during early hypoxic failure. Thereby, a steep diminution of contractile performance was obtained after the free energy change was reduced to a critical point of \(\approx 80\%\) normal energy levels. Our investigation supports the implication that changes in the contractile function are far more related to the rapid decline in the phosphorylation potential than to the availability of ATP itself.

The mitochondrial-cytosolic difference of \(DG_{\text{ATP}}\) reflects the activity of the ADP/ATP carrier.\(^{51}\) Our results do not reveal a significant change of the cytosolic-mitochondrial difference of \(DG_{\text{ATP}}\) \((\Delta G_{\text{cyt-mito}})\) within the first 5 minutes of ischemia \((\Delta G_{\text{cyt-mito}}=10.5\pm1.0 \text{kJ/mmol ATP before ischemia and } \Delta G_{\text{cyt-mito}}=10.2\pm0.9 \text{kJ/mmol ATP after 5 minutes of ischemia}).\) Cytosolic and mitochondrial \(DG_{\text{ATP}}\) values were reduced in parallel. Obviously, the capacity of the nucleotide transfer is high enough to guarantee sufficient transmembrane nucleotide transport, at least during early ischemia.

Several authors suggested that large increases of fatty acyl coenzyme A esters, occurring in \(O_2\)-deficient hearts during necrotic decline, lead to an inhibition of the ADP/ATP carrier.\(^{52,53}\) The binding of fatty acyl coenzyme A esters to the nucleotide translocase was speculated to contribute to the transition from reversible to irreversible ischemic damage.\(^{54}\) But most of these investigations were in vitro studies on isolated mitochondria taken from rat hearts after advanced periods of ischemia. High contents of fatty acyl coenzyme A are not necessarily consistent with a decrease in the function of the ADP/ATP carrier.\(^{55}\) Also in agreement with our findings were the results of Asimakis and Conti,\(^{50}\) who pointed out that the dysfunction associated with myocardial ischemia cannot be attributed to a decline in the adenine nucleotide translocase activity and that a loss of adenine nucleotides from the mitochondrial matrix space did not affect the integrity and the function of the translocator protein.

In summary, it cannot be excluded that advanced ischemia may affect the activity of the ADP/ATP carrier. During early periods of ischemia, however, a sufficient nucleotide exchange was observed, thus a disturbance in nucleotide transport mechanism caused by the ADP/ATP carrier does not account for early contractile dysfunction.

**Early Contractile Failure**

Although a marked decrease in ATP content was recorded for the mitochondrial space during the first minute, this decline cannot be responsible for the contractile failure of the myocardium due to the high cytosolic ATP concentration. Under normal energy conditions, ATP is used in the cytosolic compartment by contractile proteins that are involved in the force-generating processes. Thereby, only a relatively small ATP concentration is required to saturate the substrate binding sites of the energy-consuming proteins.\(^{57}\) Furthermore, lack of ATP as substrate for the contractile proteins would be associated with rigor of the myo-

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**Fig 3. Diagram showing creatine (Cr)-phosphocreatine (CrP) shuttle, an important energy transfer system in the myocardial cell.** Pi indicates inorganic phosphate; Cr, creatine kinase; myo, myocardial; ANT, adenine nucleotide translocase (ADP/ATP carrier); and e and i, extracellular and intracellular, respectively.

Fibrils. Koresnune and Marban\(^{58}\) recently demonstrated that the initiation of rigor contracture correlates closely with the fall in ATP concentration to \(<10\%\) of control levels such that prolonged periods of oxygen depletion are required to obtain such a pronounced ATP decrease below the rigor threshold. Nevertheless, a decrease in myocardial contractility can be observed during the first few seconds and minutes of ischemia before rigor development.\(^{59}\) All these arguments lead to the conclusion that the mechanism of early contractile failure cannot be explained by the alteration in ATP content during oxygen depletion.

Accordingly, a significant decrease of CP was monitored within the first 30 seconds of ischemia. Thus, it seems more likely that the rapid decline in CP concentration, as observed here and elsewhere,\(^{44}\) may correlate with early contractile failure. Schaefer et al\(^{59}\) recently showed that changes in the CP-to-PO\(_4\) ratio are closely associated with alterations in myocardial function during graded regional ischemia. Although a parallel diminution of CP content and contractile force can be demonstrated, this relation does not necessarily imply causality. CP declines as quickly as inorganic phosphate and protons accumulate. Thus, a possible effect on myocardial function induced by PO\(_4\) and H\(^+\) accumulation may also contribute to early contractile failure.

**CP Shuttle**

Whereas the CP content was rapidly used for energy-demanding processes, no significant reduction of cytosolic ATP concentration was found during the first 10 minutes of ischemia, although CP was no longer available. A disturbance in ATP utilization during ischemia can be proposed to explain these observations. The CP shuttle represents the relation between CP and ATP, and this must be taken into account.

Fig 3 shows a scheme of the CP shuttle. Energy-producing and energy-utilizing processes are linked by this shuttle mechanism, in which CP mediates the transfer of energy from mitochondria to cytosolic myofibrils. ATP, under normal oxygen supply produced in the mitochondrial matrix via oxidative phosphorylation, is delivered by the ADP/ATP carrier from the mitochondrial to the cytosolic space, where it is available for the rephosphorylation of creatine to CP via mitochondrial creatine kinase. CP diffuses through the cytosolic compartment to the contractile filaments, where it is converted back to creatine by the cytosolic creatine kinase.
kinase isoenzyme, thereby regenerating ATP, which is then hydrolyzed by the contractile protein ATPase to induce contraction. Normally, the rate of mitochondrial ATP synthesis is greatly increased when ADP is generated via mitochondrial creatine kinase and is delivered by the ADP/ATP carrier to the mitochondrial space for rephosphorylation, supporting ATP coupling, and isoenzyme supporting by coupling, thus strengthening the role of the PC shuttle as an important energy transfer mechanism in the cardiac muscle. It was previously suggested that disturbances associated with oxygen depletion affect the functional relations of creatine kinase and the ADP/ATP carrier at the mitochondrial level. Furthermore, evidence was given by Vial et al. that the mitochondrial creatine kinase can dissociate from or reassociate with the mitochondrial membrane depending on the height of the intracellular phosphate concentration and the decrease in pH. Such a rapid decline in pH and a fourfold increase in inorganic phosphate could be demonstrated for ischemic guinea pig hearts.

In conclusion, our results indicate a disturbance of ATP utilization in the cytosolic compartment that may play a role in the impairment of mechanical performance in ischemia. The function of the ADP/ATP carrier per se seems not to be altered during the first 5 minutes of ischemia. However, the rapid decrease of cytosolic CP on one hand and the only slight loss of cytosolic ATP on the other suggests that during myocardial ischemia the functional coupling between the ADP/ATP carrier and phosphocreatine kinase is altered. An imbalance between the PC shuttle and the ADP/ATP carrier might reduce the PC supply during early ischemia. This could be a substantial contribution for the processes leading to early ischemic contractile failure.

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