Alteration of mitochondrial function in a model of chronic ischemia in vivo in rat heart

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1Institut National de la Santé et de la Recherche Médicale U441, Athérosclérose and Institut Fédératif de Recherche 4, 33600 Pessac; 2Laboratory of Bioenergetics, Université Joseph Fourier, Grenoble Cedex 9, France; and Laboratory of Bioenergetics, National Institute of Chemical and Biological Physics, 12618 Tallinn, Estonia

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Boudina, Sihem, Muriel N. Laclau, Liliane Tariosse, Danièle Daret, Gérard Gouverneur, Simone Bonoron-Adèle, Valdur A. Saks, and Pierre Dos Santos. Alteration of mitochondrial function in a model of chronic ischemia in vivo in rat heart. Am J Physiol Heart Circ Physiol 282: H821–H831, 2002; 10.1152/ajpheart.00471.2001.—The aim of this study was to investigate mitochondrial alterations in an animal model of chronic myocardial ischemia in rats obtained by surgical constriction of the left coronary artery. Resting coronary blood flow was measured using the fluorescent microsphere technique. Contractile function, defined by rate-pressure product, and myocardial oxygen consumption were measured in a Langendorff preparation. The mitochondrial function was evaluated on permeabilized skinned fibers. Three weeks after surgery, ischemic hearts showed a significant decrease in coronary blood flow compared with sham. Hemodynamic measurements showed a significant systolic and diastolic dysfunction. Alterations in mitochondrial function in ischemic hearts were mainly characterized by a significant decrease in the maximal velocity and apparent half-saturation constant for ADP, loss of the stimulatory effect of creatine, and a stimulatory effect of exogenous cytochrome c. These functional alterations were supported by structural alterations characterized by mitochondrial clustering and swelling associated with membrane rupture. We conclude that the alterations in systolic function after chronic ischemia are supported by severe modifications of mitochondrial structure and function.

contractile function; energy metabolism; mitochondria

IN A LARGE NUMBER OF PATIENTS affected by chronic coronary artery disease, the magnitude of constriction of the epicardial coronary arteries by atherosclerosis does not correspond to the severity of the clinical manifestations of myocardial dysfunction and failure (5, 28). Similar degrees of coronary stenosis detected angiographically have been found to be associated with variable hemodynamic abnormalities, thus raising questions regarding the significance of these fixed obstructions of the coronary tree in the prediction of the short- and long-term clinical outcomes of ischemic heart disease (5). This apparent inconsistency becomes even greater when anatomic findings are taken into account (5, 27). Similarly, the magnitude of tissue damage represented by multiple focal sites of myocyte loss has been found to be inadequate to explain the marked depression in cardiac performance or the appearance of overt failure (40). Coronary narrowing, involving a reduction in luminal diameter, affects coronary blood flow (CBF), which in turn supports the hypothesis of a decreased oxygen availability. This decrease in blood flow is responsible for an imbalance between energy supply and demand (3). Because energy production in the heart is mainly supported by mitochondrial function, investigations have focused on mitochondrial alterations and energy production during acute ischemia and reperfusion in vitro (17–19). Other works have drawn attention to the interrelations between ventricular contractile performance and the myocardial creatine kinase system (10, 12, 16, 24, 26, 36). However, to our knowledge, the relationships between mitochondrial and energy transfer alterations have never been addressed in vivo in a model of chronic ischemic failure. To study this phenomenon, coronary artery constriction was surgically produced in rats. CBF, contractile reserve, and mitochondrial function were analyzed 3 wk after surgery. The main findings were that ischemia-induced alterations in heart function are explained in part by severe alterations in mitochondrial function, including a decrease or abolition of functional coupling between adenine nucleotide translocase (ANT) and mitochondrial creatine kinase (miCK) and loss of outer mitochondrial membrane integrity. All these alterations may decrease ATP production by mitochondria, energy transfer from mitochondrial matrix to energy-utilizing sites located in the cytosol, and finally affect contractile function.

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MITOCHONDRIAL FUNCTION AND CHRONIC ISCHEMIA

METHODS

Experiments were carried out in male Sprague-Dawley rats weighing 250–350 g (Elevage Janvier; Le Genest St Isle, France). Animals were housed three per cage at constant temperature (22 ± 1°C) in environmental facilities with a 12:12 h light-dark cycle schedule and were given standard laboratory chow and tap water ad libitum. The study was conducted according to the guidelines of “Ministère Français de la Pêche et de l’Agriculture.”

Coronary artery constriction. Male Sprague-Dawley rats weighing 150 g were anesthetized with a mixture of Rompun (0.25 ml/kg) obtained from Bayer (Leverkusen) and Imalgan (2 ml/kg) obtained from Merial (Lyon, France) and heparinized with 1,500 IU of heparin sodium. Rats were intubated and ventilated. A thoracotomy via the fourth left intercostal space was then performed. The left atrium was elevated to expose the left coronary artery. A suture was positioned around the vessel 1 mm from its origin. Subsequently, a probe 300 μm in diameter was held in contact with the vessel before ligation. Finally, the probe was quickly removed to allow expansion of the vessel and avoid complete occlusion. The chest was closed, and the animals were allowed to recover. These ischemic rats were compared with sham-operated control rats, which were subjected to the same procedure except that the ligature around the coronary artery was not tied. All rats were euthanized 3 wk after surgery for analysis.

CBF determination. CBF (expressed in ml·min⁻¹·100 g⁻¹) was determined in separate series, under anesthesia, by the fluorescent microsphere technique (11, 37), 1 h and 3 wk after surgery, in sham-operated and ischemic groups. In each experiment, microspheres with yellow-green (YG), orange (O), and red (R) fluorescent dyes (polystyrene, 15 μm diameter; Molecular Probes) were used. The left ventricle was catheterized via the right carotid artery using a 3-Fr catheter. The position of the cannula tip in the left ventricle was assessed from the pressure waveform. Fluorescently labeled microspheres, suspended in 0.15 M NaCl solution with 0.02–0.05% Tween 20 and 0.02% thimerosal, were sonicated and vortexed before injection in the left ventricle. For each determination, ~3 × 10⁶ microspheres were injected. A reference blood sample was taken from the femoral artery at a rate of 0.5 ml/min to calculate the absolute blood flow rate. Withdrawal of blood started 5 s before injection of the microspheres and was continued for 1 min after microsphere injection. In ischemic hearts, two different areas were examined separately after excision of the hearts: the first in the anterior wall of the left ventricle, close to the ligature (ischemic zone), and the second in the posterior inferior wall of the left ventricle, far from the ligature (nonischemic zone). Each area was divided into two layers of equal thickness: the subendocardium and subepicardium. Samples from the kidney, liver, and lung were also collected. All tissue samples were weighed and then transferred with reference blood samples to 10-ml glass screw-cap test tubes. The samples were digested in 2 N ethanolic KOH with 0.5 ml Tween 80. The fluorescent dyes were then extracted by adding an organic solvent [2-(2-ethoxyethoxy)ethyl acetate]. Fluorescence was determined with an Hitachi F-2000 luminescence spectrophotometer. Resting blood flow values were calculated using the formula: \( Q = \frac{Q_{\text{int}} \cdot \text{Int}}{Q_{\text{tot}} \cdot \text{Int}_{\text{tot}}} \), where \( Q \) and \( Q_{\text{tot}} \) are the blood flow in sample \( i \) and the reference withdrawal speed (equaling 5 ml/min), respectively, and \( \text{Int} \) and \( \text{Int}_{\text{tot}} \) are the fluorescence intensity in sample \( i \) and in the reference blood sample, respectively.

Hemodynamic measurements. Separate groups of sham-operated (\( n = 16 \)) and ischemic rats (\( n = 18 \)) were used 3 wk after surgery to investigate contractile function with the Langendorff technique. Rats were anesthetized with 40 mg of pentobarbital sodium injected intraperitoneally (obtained from Sanofi; Libourne, France) and heparinized with 1,500 IU heparin sodium (obtained from Leo Laboratories; St Quentin, France). The thorax was opened, and the heart was rapidly excised and immediately cooled in iced Krebs buffer and perfused by an aortic cannula delivering warm (37°C) buffer at a constant pressure of 100 mmHg. Hearts were perfused with a modified phosphate-free Krebs-Henseleit solution containing (in mM) 118 NaCl, 5.9 KCl, 1.75 CaCl₂, 1.2 MgSO₄, 0.5 EDTA, 25 NaHCO₃, and 16.7 glucose. The perfusate was gassed with 95% O₂-5% CO₂, which resulted in a Po₂ above 600 mmHg at the level of the aortic cannula and a buffer pH of 7.4. The pulmonary artery was transected to facilitate coronary venous drainage. A polyethylene drain was inserted in the right ventricle to anaerobically collect coronary effluent for myocardial oxygen consumption (MV₀₂) measurements. A left ventricular cannula and a femoral arterial drain was inserted through a left atrial incision to allow thebesian venous drainage. Left ventricular pressure was monitored from a water-filled latex balloon placed through the left atrial appendage and connected to a Statham P23 Db pressure transducer. The volume of the balloon was adjusted to obtain a left ventricular diastolic pressure around 7 mmHg and was kept constant throughout the entire experiment. Cardiac mechanical performance was defined as the product of heart rate and developed pressure (RPP).

The hearts were allowed to stabilize for 20 min before acquisition of baseline RPP and MV₀₂ values. The hearts were then switched to a second buffer containing 1.0 mM CaCl₂. RPP and MV₀₂ were measured after 20 min of stabilization under this condition. The hearts were then switched to a third buffer containing 4 mM CaCl₂. MV₀₂ and RPP were again measured after 20 min of stabilization. This protocol was used to obtain the MV₀₂ versus RPP relationship in each group and to analyze the ability of the heart to respond to calcium-induced inotropic stress. Contractile reserve was defined as the increase in RPP following the transition from 1.75 to 4.0 mM extracellular calcium concentration.

Because of the presence of 0.5 mM EDTA in all the buffers, the actual free calcium concentrations in the different mediums were ~0.5, 1.25, and 3.5 mM.

Permeabilized cardiac fiber preparation. Preparation of permeabilized cardiac fibers, which has been extensively described (33, 38), was used to study mitochondrial function in situ. Scanning electron microscopy has shown that both subsarcolemmal and interfibrillar mitochondria are preserved (29). The fact that this technique samples all of the mitochondria in the fiber is an important advantage, because it has been shown that subsarcolemmal mitochondria undergo a more rapid onset of ischemic damage (22). Furthermore, the maximum respiration rate of the skinned fibers is equal to that expected on the basis of the mitochondrial content in heart tissue and respiration rates determined in vitro under the same experimental conditions (38). Small pieces of cardiac muscle were taken from two different zones, an ischemic and nonischemic zone of the left ventricle, and put into cold solution A (see composition below). All procedures were carried out at 4°C. These samples were rapidly dissected into bundles of fibers. Fibers were incubated for 30 min with shaking in 1.8 ml of solution A in the presence of saponin (50 μg/ml) to selectively destroy the sarcolemma. The bundles were subsequently put into solution B (see composition below) twice for 10 min to wash out adenosine
nucleotides phosphocreatine and saponin. Oxymetric measurements were performed in solution B supplied with pyruvate 10 mM and malate 5 mM. A KCl solution (see composition below) was used to test the integrity of the outer mitochondrial membrane.

**Solutions A and B** were prepared on the basis of the cytoplasmic composition of the muscle cells. 

**Solution A** (in mM) consisted of 2.77 CaK$_2$EGTA, 7.23 K$_2$EGTA, 6.56 MgCl$_2$, 0.5 dithiothreitol, 50 K-methans, 20 imidazole, 20 taurine, 5.3 Na$_2$ATP, and 15 PCR, pH 7.1 was adjusted at 25°C. **Solution B** (in mM) consisted of 2.77 CaK$_2$EGTA, 7.23 K$_2$EGTA (pCa = 7), 1.38 MgCl$_2$, 0.5 dithiothreitol, 50 K-methans, 20 imidazole, 20 taurine, and 3 KH$_2$PO$_4$, pH 7.1 was adjusted at 25°C. KCl solution (in mM) consisted of 125 KCl, 20 HEPES, 10 pyruvate, 5 malate, 3 Mg acetate, 5 KH$_2$PO$_4$, 0.4 EGTA, and 0.3 dithiothreitol. pH 7.1 was adjusted at 25°C, and 2 mg of bovine serum albumin per milliliter were added. In this high KCl concentration condition, the labile compounds of the respiratory chain like cytochrome c dissociates from the inner membrane (38).

**Integrity of the outer mitochondrial membrane assessment.** The integrity of the outer mitochondrial membrane was assessed in a KCl medium, in which endogenous cytochrome c dissociates from the outer surface of the inner mitochondrial membrane but continues to support maximal respiration as long as the outer membrane remains intact (14, 20). First, the initial rate of respiration of skinned cardiac fibers was measured in KCl solution containing substrates and no ADP (state 2). Second, the respiration was stimulated by the addition of ADP at a final concentration of 1 mM, which induced a maximal activation of respiration (state 3). Cytochrome c was added at a final concentration of 8 μM to test the integrity of the outer mitochondrial membrane. Under these experimental conditions, when the outer mitochondrial membrane is intact, endogenous cytochrome c remains in the intermembrane space and maintains a high respiratory activity. In this case, the addition of exogenous cytochrome c has no effect on the respiratory rate. If the outer membrane is damaged, cytochrome c may leave the mitochondrion, and the addition of a high concentration of cytochrome c increases the respiratory rate.

**Determination of ANT-miCK functional coupling.** The respiratory rate of mitochondria in skinned cardiac fibers was measured in solution B supplemented with bovine serum albumin or 2 μM ADP at a final concentration of 1 mM, which induced a maximal activation of respiration (state 3). Cytochrome c was added at a final concentration of 8 μM to test the integrity of the outer mitochondrial membrane. Under these experimental conditions, when the outer mitochondrial membrane is intact, endogenous cytochrome c remains in the intermembrane space and maintains a high respiratory activity. In this case, the addition of exogenous cytochrome c has no effect on the respiratory rate. If the outer membrane is damaged, cytochrome c may leave the mitochondrion, and the addition of a high concentration of cytochrome c increases the respiratory rate.

**Ultrastructural study.** Sham and ischemic hearts were arrested in diastole under a perfusion with a 20% KCl solution. Specimens of 4 mm long and 2 mm wide were taken from the left ventricle of sham hearts and from two different areas of the left ventricle of ischemic hearts: the first close to the ligature (ischemic zone) and the second far from it (non-ischemic zone). All specimens were prepared for ultrastructural examination by fixation in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated with alcohol, and embedded in Epon 812. Ultrathin sections were cut with a Reichert Om U3 ultramicrotome and double stained with uranyl acetate and lead citrate. Each sample was examined with a Philips EM 201 transmission electron microscope.

**Statistical analysis of experimental data.** All data are expressed as means ± SD. A two-way ANOVA for repeated measurements was performed to analyze hemodynamic parameters at different time points in the different series. A single ANOVA was used to investigate respiration parameters. All ANOVA analyses led to highly significant differences. Mean value comparisons were performed by Student’s t-test. RPP versus MV$_{O_2}$ relationships were analyzed by linear regression analysis. Correlation coefficients were compared by normal law on Z transform of r. Comparisons of slopes were obtained by Student’s t-test after controlling the equality of residual variance. A value of P < 0.05 was considered statically significant.

**RESULTS**

**Effects of coronary artery narrowing on heart and body weight.** The surgical procedure and coronary narrowing for a period of 3 wk had no effect on body weight (Table 1). When compared with sham rats, ischemic rats exhibited a 55% increase in heart weight (P < 0.001) with a 25% increase in left ventricular weight (P < 0.001) and a 122% increase in right ventricle weight (P < 0.05) (Table 1). Overall, there was a 45% increase in the heart weight-to-body weight ratio (P < 0.005) in ischemic compared with sham rats.

**Effects of coronary artery narrowing on CBF.** In the effects of coronary artery narrowing on left ventricular function when two different types of microspheres (YG and R or BG and O) were simultaneously injected under resting conditions, there was a very good agreement between CBF measured by YG and R on one hand and BG and O on the other hand in both control and ischemic hearts. Table 2 summarizes the effect of coronary artery stenosis on resting blood flow in the different regions of the left ventricle from both sham and ischemic rats 1 h and 3 wk after the surgical procedure. In the ischemic zone, it can be seen that CBF down-regulation was more marked than in the control areas (significant in all cases).

### Table 1. Effects of coronary constriction on body weight and cardiac weight

<table>
<thead>
<tr>
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<th>Sham Operated</th>
<th>Ischemic</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>BW, g</td>
<td>336 ± 30</td>
<td>360 ± 10</td>
<td>NS</td>
</tr>
<tr>
<td>HW, g</td>
<td>1.10 ± 0.09</td>
<td>1.71 ± 0.23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HW/BW, × 1,000</td>
<td>3.3 ± 0.030</td>
<td>4.8 ± 0.07</td>
<td>&lt;0.005</td>
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Values are means ± SD. BW, body weight; HW, heart weight; NS, not significant.
stream of coronary narrowing was not significantly changed when measured 1 h after ligation. However, when measured 3 wk after the surgical procedure, there was a significant 58% and 70% decrease in CBF measured in the epicardium and endocardium of the ischemic zone, respectively ($P < 0.001$). In the nonischemic zone, we observed a significant decrease in CBF measured 3 wk after surgery in the endocardium only ($P < 0.001$ vs. sham). Overall, these data show a progressive decrease in coronary perfusion after the surgical procedure. This decrease was much more pronounced in the ischemic zone, with the endocardium being more affected than the epicardium.

**Effects on basal left ventricular function.** There was no difference in heart rate between sham-operated and ischemic hearts with values of 258 ± 5 and 263 ± 10 beats/min, respectively. In contrast, left ventricular developed pressure, $dP/dt_{\text{max}}$, and RPP were significantly ($P < 0.001$) lower in ischemic hearts compared with sham (Fig. 1).

**Effects on left ventricular response to calcium-induced inotropic stress.** RPP values were significantly lower in ischemic compared with sham hearts for each extracellular Ca$^{2+}$ concentration ($[\text{Ca}^{2+}]_e$) (Fig. 1A). Moreover, contractile reserve was significantly decreased in ischemic compared with sham hearts with a 42 and 66% increase in RPP, respectively, whereas $[\text{Ca}^{2+}]_e$ was increased from 1.75 to 4.0 mM ($P < 0.001$). Overall, these data point to the alteration of systolic function and contractility of ischemic hearts and their decreased ability to respond to calcium-induced inotropism and contractility of ischemic hearts and their decreased ability to respond to calcium-induced inotropism and contractility. It should be noted that the slope of $\text{MVO}_2$ versus RPP relationship and the intercept were significantly higher in ischemic compared with sham hearts (Fig. 2).

**Effect of coronary artery narrowing on mitochondrial structure.** The functional changes in chronically ischemic hearts described above are related to intracellular alterations, among which the mitochondrial structural deformations are most obvious, as revealed by electron microscopic observations of cardiac tissue (Fig. 3). In the control, in healthy aerobic hearts, the mitochondria were precisely positioned between the myofibrils (Fig. 3A). With respect to sarcomere structure, the mitochondria were almost always positioned between the Z lines at the level of the A band. This very precisely fixed position of mitochondria in cardiac muscle cells has been found to be important for facilitating energy exchanges with myofibrils and sarcoplasmic reticulum, with which mitochondria seem to form functional complexes (30, 35). Figure 3B shows the mitochondrial structure in control cells at higher magnification; both the outer and inner membranes are intact and the mitochondria are firmly attached to the myofibrils. In the nonischemic zone of the chronically ischemic heart, the mitochondria were still firmly attached to the myofibrils, but some clustering did occur (Fig. 3, C and D). Dramatic changes in the mitochondrial structure may be seen, however, in the ischemic zone (Fig. 3, E and F). Mitochondria were always and homogeneously detached from the myofibrils, clustered (Fig. 3E) and often swollen with a clearly broken outer membrane (Fig. 3F). Permeabilized skinned fiber studies of mitochondria in these cells without their isolation showed that the morphological changes, observed microscopically, were closely related to alteration of the respiratory function of mitochondria.

**Effect of coronary artery narrowing on mitochondrial outer membrane barrier function and on maximal respiration rates.** Figure 4A shows the oxygraph recordings of the respiration of skinned cardiac fibers taken from control myocardium, from the nonischemic zone of chronically ischemic hearts, and from the ischemic zone of ischemic hearts of operated animals. The determination of intactness of the outer mitochondrial membrane was carried out in KCl solution (see METHODS) by evaluating the effect of the addition of 8 μM exogenous cytochrome c on the maximal rate of ADP-dependent respiration determined at 1 mM ADP. Only in the case of skinned fibers from ischemic zone did exogenous cytochrome c activate ADP-stimulated respiration, thus showing the defects of outer mitochondrial membrane. When the respiration rates; i.e., basal respiration rate ($V_0$), the rate of ADP-stimulated respiration ($V_{\text{ADP}}$), and the rate of respiration in the presence of exogenous cytochrome c ($V_{\text{ox c}}$), were calculated per milligram of dry weight (Fig. 4B), some decrease was detected in the maximal respiration rate in fibers from the nonischemic zone of the hearts of operated animals (Fig. 4B): maximal respiration rates were 23.2 ± 2.4 and 17 ± 3.5 nmol O$_2$·min$^{-1}$·mg dry wt$^{-1}$ in shams and nonischemic zone of ischemic hearts, respectively ($P < 0.05$) (shaded bars). Both groups of fibers showed a lack of stimulation of maximal respiration by cytochrome c. In contrast, fibers obtained from the ischemic zone of ischemic hearts showed a significant 70% decrease in $V_{\text{max}}$ compared with shams ($P < 0.001$), associated with a 50% stimu-

Table 2. Effects of coronary artery narrowing on resting blood flow in the myocardium

<table>
<thead>
<tr>
<th>Region</th>
<th>Sham 1 h</th>
<th>Sham 3 wk</th>
<th>Ischemic 1 h</th>
<th>Ischemic 3 wk</th>
</tr>
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<tbody>
<tr>
<td>Epicardium</td>
<td>289 ± 70</td>
<td>300 ± 70</td>
<td>303 ± 62</td>
<td>307 ± 98</td>
</tr>
<tr>
<td>Endocardium</td>
<td>339 ± 89</td>
<td>376 ± 77</td>
<td>339 ± 62</td>
<td>263 ± 44‡</td>
</tr>
</tbody>
</table>

Values are means ± SD (in ml·min$^{-1}$·100 g$^{-1}$). IZ, ischemic zone; NIZ, nonischemic zone. *$P < 0.001$ vs. 1 h; †$P < 0.05$ vs. 1 h; ‡$P < 0.001$ vs. sham.
lation of respiration by exogenous cytochrome c. These data reflect ischemia-induced alterations at the level of the outer mitochondrial membrane, leading to a loss of cytochrome c in the ischemic zone of ischemic hearts only, in agreement with the results of morphological observations (see Fig. 3, E and F).

Effect of coronary artery narrowing on regulation of respiration of permeabilized cardiac fibers by ADP and creatine. Figure 5 shows typical examples of double-reciprocal plots of the dependence of ADP-stimulated respiration rates on the concentration of ADP in the presence or absence of creatine. When 20 mM creatine is added to the respiration medium, miCK uses ATP and creatine as substrates to produce phosphocreatine and ADP (31–34, 39). As a result, mitochondrial respiration is not limited by the possibly restricted permeability of the outer mitochondrial membrane for ADP, and functional coupling between ANT and miCK can occur. Addition of creatine had a very pronounced effect in fibers obtained from sham hearts with a more than 83% decrease in $K_{1/2}$ of respiration for ADP (Fig. 5). Overall, these data reflect the restriction of permeability of the outer mitochondrial membrane to ADP (as evidenced by the high value of $K_{1/2}$) and the high efficiency of functional coupling between ANT and miCK (as evidenced by the dramatic decrease in $K_{1/2}$ in the presence of creatine) in fibers obtained from sham hearts. In fibers of sham-operated hearts and from
nonischemic hearts, an analysis of data in the double-reciprocal plots revealed only one population of mitochondria (one straight line in Fig. 5). However, in fibers taken from the ischemic zone of operated animals, there were very often two populations (Fig. 5), which shows the intracellular heterogeneity of mitochondria due to chronic ischemia. This is again in agreement with morphological observations showing the existence of broken areas of the outer mitochondrial membrane.

Fig. 3. Transmission electron microscopic studies of the structural changes in cardiomyocytes in chronic ischemia. A and B: sham (magnification ×12,000 and ×40,000, respectively); C and D: nonischemic zone (magnification ×12,000 and ×40,000, respectively); E and F: ischemic zone (magnification ×12,000 and ×40,000, respectively). Arrow shows broken area of the outer mitochondrial membrane.
in these cells of mitochondria with both intact and broken outer membranes (Fig. 3). To characterize these cells kinetically, the average values for parameters (33) were calculated from experiments described in Fig. 5. Figure 6 contains a summary of these results with statistical analysis obtained from these double reciprocal plots. It can be seen that coronary artery narrowing induced a significant decrease in this $K_{1/2}$ in both nonischemic and ischemic zones of ischemic hearts compared with sham hearts. Moreover, this decrease was significantly more pronounced in the ischemic zone of ischemic hearts than in the nonischemic zone. In fibers obtained from the nonischemic zone of ischemic hearts, the $K_{1/2}$ for ADP was significantly decreased to $169.6 \pm 46.2$ \(\mu\)M compared with sham hearts ($P < 0.001$). Addition of creatine induced a further decrease in $K_{1/2}$ to $81.7 \pm 48.3$ \(\mu\)M. The $K_{1/2}$ was even more decreased in fibers obtained from the ischemic zone of ischemic hearts, thus reflecting the increased permeability of the outer mitochondrial membrane to ADP. Addition of creatine had no effect on this $K_{1/2}$. Overall, these data suggest the increased permeability of the outer mitochondrial membrane to ADP in ischemic hearts together with the impossibility to express functional coupling between ANT and miCK in the ischemic zone of ischemic hearts only. The effect of creatine on the regulation of mitochondrial respiration is summarized in Fig 5B, where it can be seen that the ratio of catalytic efficiency in the presence and/or absence of creatine was dramatically decreased in the

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**Fig. 4.** Oxygraph traces showing effect of cytochrome c (Cyt c) on mitochondrial respiration due to alteration of the mitochondrial outer membrane in situ in chronic ischemia (A) and statistical analysis of changes in the respiration rates (B). A: explanation of the method is given in the text. B: oxygen consumption rates (nmol O$_2$·min$^{-1}$·mg dry wt$^{-1}$) in the presence of only substrate (solid bars), in presence of 1 mM ADP (shaded bars), and in presence of 1 mM ADP and 8 \(\mu\)M Cyt c (open bars). *$P < 0.05$ vs. sham; **$P < 0.001$ vs. sham.
ischemic zone of ischemic hearts compared with sham and the nonischemic zone of ischemic hearts.

DISCUSSION

The results of the present study indicate that a nonocclusive constriction of the left coronary of rat hearts is associated after a 21-day period with alterations in global cardiac performance. This observation is in agreement with other studies performed in the laboratory of Anversa and colleagues on the same model (2, 3, 8, 9). Alterations of heart function were investigated in vivo by this group after various periods of coronary constriction ranging from 5 days to 5 mo. They showed an increase in end-diastolic left ventricular pressure, a decrease in peak left ventricular systolic pressure, a decrease in \(\frac{dP}{dt}_{\text{max}}\) and \(\frac{dP}{dt}_{\text{min}}\), and a decrease in stroke volume (23). All of these observations are consistent with our results obtained on Langendorff-perfused hearts. CBF measured at rest 45 min to 5 days after coronary constriction were not decreased compared with control animals, whereas vasodilation reserve was decreased (8). This observation is consistent with the normal CBF measured 1 h after constriction in our study. However, we observed a decrease in resting CBF 3 wk after surgery, which could induce hibernation in the ischemic zone. Anversa and colleagues attributed these alterations in heart function to the following: 1) extensive left ventricular remodeling associated with foci of reparative fibrosis and myocytolysis in the “ischemic” regions (1); 2) alterations in intracellular calcium homeostasis mainly characterized by an increase in diastolic calcium, a decrease in peak systolic calcium, and a prolonged time to peak systolic calcium (7); 3) a decrease in Ca\(^{2+}\)-myosin ATPase activity with a shift in myosin isoenzymes from V\(_1\) to V\(_3\) (1); and 4) a decrease in \(\beta\)-receptor density (25).

Our hemodynamic data obtained on Langendorff-perfused hearts show an alteration of left ventricular systolic function after 3 wk of coronary artery constriction, as evidenced by the decrease in left ventricular developed pressure rate pressure, RPP, and left ventricular \(\frac{dP}{dt}\) at each level of extracellular calcium concentration. These decreases were particularly pronounced at elevated workloads, when energy production requirements were high and an efficient energy transfer system was operating. Moreover, energy requirements were even higher in ischemic hearts, and MV\(_{\text{O2}}\)/RPP was higher in this group compared with controls. Several mechanisms might explain this increase in the “oxygen cost” of contraction, including remodelling of the left ventricular geometry leading to increased diastolic wall stress (13) and other mechanisms using oxygen in the cell such as the production of oxygen free radicals or increased calcium pumping.

The present work is the first to report alterations in mitochondrial function in this model. The skinned fiber technique has been particularly used to study mito-

**Fig. 5.** Dependence of respiration on ADP concentration. Typical examples of double reciprocal plots in presence (solid symbols) or absence (open symbols) of 20 mM creatine. \(V_{\text{O2}}\) is the ADP-stimulated respiration rate in nmol O\(_2\) (\(V_{\text{O2}} = V_{\text{O2ADP}} - V_{\text{O2state2}}\)).
...cytochrome respiration rate associated with a stimulatory effect of an increase in the permeability of the outer mitochondrial function after zero-flush ischemia that could be detected by acute and low-flush ischemia that that high apparent 

**A** Average values and standard deviations of half-saturation constant ($K_{1/2}$) for ADP in the absence (solid bars) and presence (open bars) of 20 mM creatine. 

**B** Catalytic efficiency of stimulation of respiration by ADP in presence of 20 mM creatine ($V_{\text{max}}/K_{1/2}$), and the catalytic efficiency of stimulation of respiration by ADP in absence of creatine ($V_{\text{max}}/K_{1/2}$). 

**Fig. 6. Control of oxidative phosphorylations by ADP and creatine.** 

**A**: average values and standard deviations of half-saturation constant ($K_{1/2}$) for ADP in the absence (solid bars) and presence (open bars) of 20 mM creatine. 

**B**: catalytic efficiency of stimulation of respiration by ADP in presence of 20 mM creatine ($V_{\text{max}}/K_{1/2}$), and the catalytic efficiency of stimulation of respiration by ADP in absence of creatine ($V_{\text{max}}/K_{1/2}$). 

in which the mitochondrial population also becomes heterogeneous according to biochemical and morphological criteria.

In the normal cardiomyocyte, efficient energy transfer between cytosol and mitochondria depends on two organizational aspects of the mitochondrial isoenzyme of creatine kinase, which catalyses the forward reaction: creatine + MgATP $\rightarrow$ phosphocreatine + MgADP. These two aspects are functional coupling and compartmentation, and both depend strongly on the structure-function of the intermembrane space (IMS). Functional coupling is the result of a close association between miCK and ANT, which is located in the inner mitochondrial membrane. Compartmentation refers to the normal low permeability of the outer membrane to ADP and ATP. In normal cardiac fibers, the low permeability of the outer membrane to ADP is reflected in the high $K_{1/2}$ for ADP-dependent respiration in control hearts, which is $>350$ $\mu$M. Compartmentation is lost after 3 wk of coronary artery narrowing, as reflected by the 3.5 times drop in $K_{1/2}$ (ADP). Functional coupling in normal cardiac fibers is reflected in the profound drop in $K_{1/2}$ (ADP) in the presence of 20 mM creatine, which occurs because creatine stimulates the production of ADP in the IMS, thereby reducing the influence of the outer membrane barrier to ADP. However, it must be noted that this decrease was strongly influenced by the loss of compartmentation and not solely by loss of functional coupling. When nucleotide compartmentation is lost or impaired after coronary artery narrowing, respiration will be controlled by cytosolic ADP concentration, and restriction of ADP diffusion in the cytosol will likely result in a limitation of ATP production, worsening ischemia-induced damage of the cardiomyocyte.

Recent biochemical studies of the regulation of mitochondrial function in muscle cells in situ have shown that high apparent $K_{1/2}$ for exogenous ADP is explained both by decreased permeability of the outer mitochondrial membrane for this substrate and by close structural and functional interaction of mitochondria with other cellular structures, such as sarcomeres and sarcoplasmic reticulum (30, 35). In these intracellular functional complexes the energy transfer is facilitated by the creatine kinase system (35). Some decrease in the apparent $K_{1/2}$ for exogenous ADP in the fibers prepared from the nonischemic zone of operated animal hearts, in the absence of the effects of exogenous cytochrome c on respiration (intact outer mitochondrial membrane), may already be due to some mitochondrial clustering and a change of their position within the cells (Fig. 3, C and D). Similar effects were observed earlier in the case of intracellular clustering of mitochondria in desmin-deficient animals (17–19). These changes may indicate some alterations in cross talk between the mitochondria and energy-consuming systems in the cells in nonischemic zones in hearts of operated animals. However, as expected, mitochondria were dramatically changed in the ischemic zones of the hearts of operated animals. Detachment of mitochondria from myofibrils clearly lead to destruction of func...
tional complexes of mitochondria with sarcomeres called "intracellular energetic units", (30)) and decreased efficiency of mitochondrial-cyttoplasmic cross talk. In addition, the outer membrane of many mitochondria (the intracellular population of which becomes heterogeneous) is broken, probably due to swelling induced by the opening of mitochondrial permeability transition pores (4) due to calcium overloading of the cells. This would explain the dramatic decrease in the ADP-stimulated maximal respiration rate and the lack of the effect of creatine on the respiration of these mitochondria. These changes in mitochondrial function and in the intracellular energy transfer may contribute significantly to the impaired contractile function of chronically ischemic hearts of operated animals. In addition, release of cytochrome c in the ischemic zones may also initiate apoptosis (6, 15).

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