Increased state 4 mitochondrial respiration and swelling in early post-ischemic reperfusion of rat heart

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Abstract Isolated rat hearts were exposed to 30 min ischemia or to 30 min ischemia followed by 2, 5 or 40 min reperfusion and mitochondria were isolated at these different time points. ADP-stimulated, succinate-dependent respiration rate (state 3) was not significantly changed at the different time points examined. In contrast, state 4 (non-ADP-stimulated) respiration rate was significantly increased after 30 min ischemia, and it increased further during the first post-ischemic reperfusion period. Mitochondrial swelling, as evaluated under conditions of the major controlled ion channels (i.e. permeability transition pore and ATP-dependent mitochondrial K^+ channel) closed, significantly increased in parallel. It is suggested that the inner mitochondrial membrane permeability is increased under exposure of the heart to ischemia and early reperfusion, and that the phenomenon is reversible upon subsequent long periods of reperfusion.

Key words: Mitochondrion; Ischemia and reperfusion; Respiration; Uncoupling; Heart

1. Introduction

Structural, biochemical, and functional abnormalities of mitochondria are important pathogenic factors that affect oxygen-depleted and re-oxygenated myocardial cells (for recent reviews, see [1–3]). Mitochondria not only play a central role in myocardial energy metabolism and calcium homeostasis, but are also responsible for the highest production of reactive oxygen species (ROS) in ischemic and reperfused hearts, and are involved in the mechanisms controlling apoptotic cell death. Several studies have tried to address the role of dysfunctions of mitochondrial respiratory chain complexes during ischemia and reperfusion [4]. The prevalent current idea is that respiration capacity, as assayed under ADP-stimulated conditions (state 3 respiration), diminishes during ischemia, and the decrease is particularly relevant when assayed with NAD-dependent substrates, implying that complex I of the respiratory chain is the most affected enzyme [4]. However, other respiratory complexes, such as complex III and IV, have also been shown to be impaired by ischemia suggesting an alteration of the entire transport chain process [5].

Two important considerations should be made when addressing the effect of ischemia and reperfusion on heart mitochondria. First, early periods of post-ischemic reperfusion appear to be critical for heart recovery [6]. An increased production of ROS by mitochondria and an increased Ca^{2+} concentration in the matrix seem responsible for the pathological opening of the mitochondrial transition pore, with a dramatic loss of nucleotides and cytochrome *c* [3,5]. Nevertheless, the effects of the first few minutes of reperfusion following prolonged ischemia on mitochondrial function have not been definitely stated. Second, mitochondria have been proposed to play a relevant role in myocardial ischemic preconditioning, a phenomenon that protects the heart from ischemic injury and subsequent reperfusion, especially through the activation of an ATP-dependent mitochondrial K^+ channel (mtK^+_{ATP}) [7]. Therefore, the role of mitochondrial respiration as a crucial component of the preconditioning paradigm has been addressed in several studies, but it still warrants further investigation [2].

Since a better understanding of the mechanisms of mitochondrial (dys)function may lead to new perspectives for future research on protection of ischemic-reperfused myocardium, we thought it of interest to measure the mitochondrial swelling, as well as the respiration rate of mitochondria isolated from hearts exposed to ischemia and subsequent reperfusion, using succinate as substrate. In this work we determined the time-dependent changes of mitochondrial respiration under different energizing states during ischemia and subsequent reperfusion, and the intactness of the inner mitochondrial membrane, as evaluated by measuring mitochondrial swelling.

2. Materials and methods

All chemicals, of reagent grade, were purchased from Sigma (St. Louis, MO, USA) and were used without further purification.

2.1. Experimental protocol

The study conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23). Hearts from adult male Sprague–Dawley rats (275–350 g body weight), fed a standard diet, were excised and subjected to 20 min control working perfusion, followed by 30 min zero-flow global ischemia and 2, 5 or 40 min retrograde reperfusion (Fig. 1). During working heart perfusion, the buffer was recirculated (approximate volume: 200 ml); during retrograde perfusion, it was not recirculated. During sustained ischemia, the heart chamber was filled with deoxygenated perfusion buffer, and its temperature was closely monitored and kept at 36.8 ± 0.1°C.

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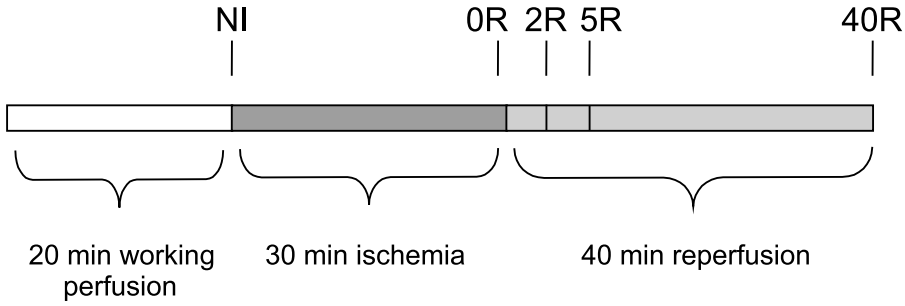

Fig. 1. Schematic diagram of the perfusion protocol. Mitochondria were isolated at each time point indicated: NI (no ischemia), 0R (0 min reperfusion), 2R, 5R, and 40R (2, 5, 40 min reperfusion, respectively).

2.2. Isolation of mitochondria

After ischemia and at the different times of reperfusion, ventricular myocardium was rapidly immersed in an ice-cold 0.18 M KCl, 1 mM EDTA and 10 mM Tris–HCl (pH 7.4) homogenization buffer containing of 0.1% (w/v) fatty acid-free bovine serum albumin and 0.1 mM Mg-ATP. The tissue was minced and homogenized with approximately 5 ml of homogenization buffer per gram of tissue, using a Teflon–glass homogenizer. Mitochondria were isolated by differential centrifugation as previously reported [8]. The protein concentration in the mitochondrial suspension was determined by the Bradford method using bovine albumin fraction V as a standard [9].

2.3. Mitochondrial respiration and swelling

The mitochondrial respiratory function was measured immediately after isolation. Oxygen consumption was determined at 37°C with a Clark oxygen electrode (Strathkelvin Instruments). The incubation chamber (0.15 ml) contained 10 mM Tris–HCl, pH 7.4, 0.25 M sucrose, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 5 mM KCl, 10 mM succinate, 2 μM rotenone, and 0.05 mg of mitochondrial protein. Oxidative phosphorylation was started by the addition of 50 nmol ADP. The following parameters of mitochondrial function were evaluated: state 3 respiration rate: QO_2 (oxygen uptake during ADP phosphorylation, expressed in nmol oxygen/min/mg mitochondrial protein); state 4 respiration rate: QO_2 (oxygen uptake in the absence of exogenous ADP); respiratory control ratio (RCR): ratio of state 3 and state 4 oxygen uptake rates; phosphorylation efficiency: ADP/O ratio (nmol ADP added/ngatoms oxygen consumed). Typically, to complete the respiration assay in each mitochondrial sample, 5 mM dinitrophenol was added, and uncoupled respiration was monitored. Under the latter experimental condition, statistically significant differences between samples were not found.

Mitochondrial swelling was determined in a hypotonic buffer by measuring the decrease in the absorbance at 540 nm, using a Jasco V550 spectrophotometer [10].

2.4. Enzyme activity

ATPase and cytochrome oxidase (COX) activity were measured spectrophotometrically as previously described [11].

2.5. Statistical analysis

Results are expressed as mean ± S.E.M. Differences between groups were evaluated by analysis of variance: Fischer's F-test was first used to compare between-group and within-group variance; if the former was significantly ($P < 0.05$) higher than the latter, individual groups were compared by Student–Newman–Keuls test, as described by Glantz [12].

3. Results

The physiopathological characteristics of the hearts used in our experiments were described in a previous paper [8]. Briefly, the release of both lactate dehydrogenase and creatine kinase increased significantly after prolonged ischemia, and cellular necrosis, as estimated on the basis of tissue staining with triphenyltetrazolium chloride, was around 30% after 30 min ischemia followed by 120 min reperfusion.

The succinate-sustained, ADP-stimulated oxygen consumption capacity of mitochondria isolated from rat heart exposed to 30 min ischemia and 40 min retrograde reperfusion did not change significantly throughout the experiment (Fig. 2), consistent with previous reports [4]. However, at 2 and 5 min reperfusion the mean value was higher than the pre-ischemic level. State 4 respiration rate was significantly increased by ~80% ($n = 6$; $P < 0.01$) following 30 min ischemia, and it increased nearly three-fold after 2 min reperfusion. After 5 min reperfusion, it decreased to the value it had after ischemia, before reperfusion was started (0R). At 40 min reperfusion, state 4 respiration rate was not statistically different from the initial value measured before the induction of ischemia (NI). As a consequence of the low level of coupling following sustained ischemia and at 2 min reperfusion, mitochondria showed both low RCR (2.27 ± 0.32 and 2.01 ± 0.11 at 0R and 2R, respectively) compared with the basal level of 4.49 ± 0.31 ($P < 0.01$, $n = 6$), and a slightly decrease in energy efficiency, with ADP/O scoring 1.40 ± 0.16 and 1.20 ± 0.12, respectively, compared to the basal value of 1.59 ± 0.20, although it did not achieve statistical significance.

To exclude that the observed increase of respiration rate at 2 and 5 min reperfusion was due to differently enriched mitochondrial preparations under the various conditions assayed, COX activity was measured under conditions of maximal activity [11]; this activity was statistically unchanged throughout the experiments (Table 1), which agrees with previous results [13]. In order to protect mitochondria and prevent mtK^+_{ATP} channel opening [14], Mg-ATP was added in the

Table 1
Mitochondrial COX and ATPase activities

	COX (μmol/min/mg)	ATPase (μmol/min/mg)	OS-ATPase (μmol/min/mg)
NI	1.48 ± 0.03	1.63 ± 0.51	1.38 ± 0.10 (85)
0R	1.60 ± 0.09	1.46 ± 0.13*	1.09 ± 0.06* (75)
2R	1.67 ± 0.02	1.18 ± 0.14*	0.86 ± 0.04* (72)
5R	1.61 ± 0.11	1.06 ± 0.11*	0.80 ± 0.05* (76)
40R	1.38 ± 0.18	1.35 ± 0.28*	1.08 ± 0.11* (80)

The results are mean ± S.E.M. of six experiments for each group. In parentheses oligomycin-sensitive ATPase (OS-ATPase) in % of total ATPase activity is shown. *$P < 0.05$ vs. corresponding control basal value (NI).

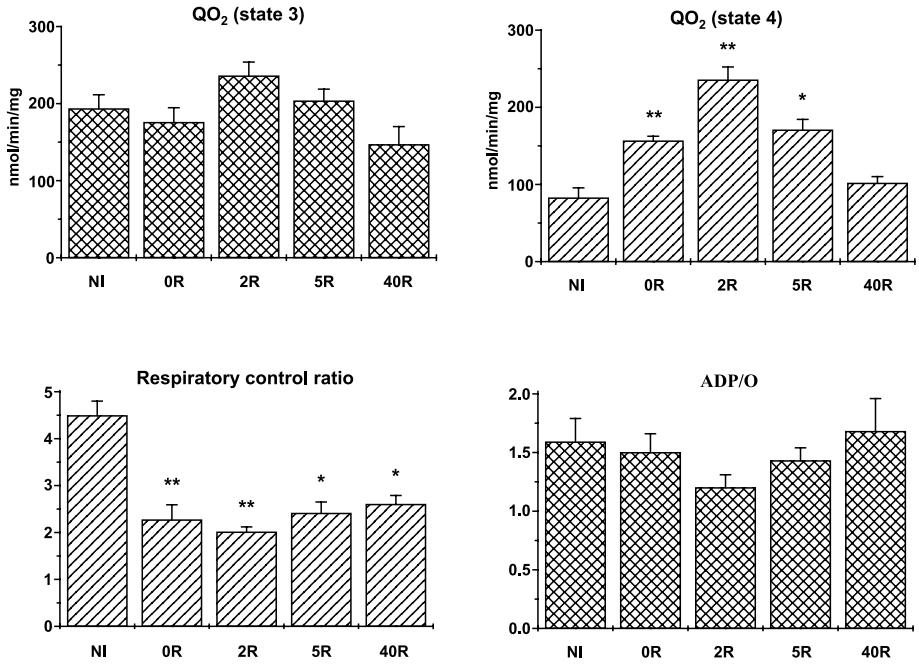

Fig. 2. Mitochondrial respiratory function parameters during ischemia and reperfusion. Means ± S.E.M. of six experiments are shown. No significant differences in state 3 oxygen uptake (state 3 QO_2) and in ADP/O ratio were observed at any time. However, in the early phase of reperfusion, state 4 oxygen uptake (state 4 QO_2) was significantly increased compared to pre-ischemic value (NI) at 0R, 2R (**$P < 0.01$), and 5R (*$P < 0.05$).

buffer used to isolate mitochondria. Addition of either oligomycin (20 μM), a specific F_1F_0-ATPase inhibitor and proton blocking agent through F_0, or EGTA (0.5 mM), a specific Ca^{2+} chelator, had virtually no effect on state 4 respiration rate, whereas cyclosporin A (1 μM) could slightly decrease the rate, but even at 2 min reperfusion its effect was not statistically significant (means ± S.D. were 123.8 ± 12.5 vs. 105.8 ± 14.0 nmol/min/mg in the presence of cyclosporin). Oligomycin-sensitive ATPase activity at 0R, 2R and 5R decreased significantly compared to pre-ischemic baseline (Table 1). This might result from the sum of two opposite effects: uncoupling, due to proton leak through the membrane, which enhances ATP hydrolysis, and inhibition, due to the endogenous protein inhibitor that binds to the enzyme when mitochondria depolarize [15].

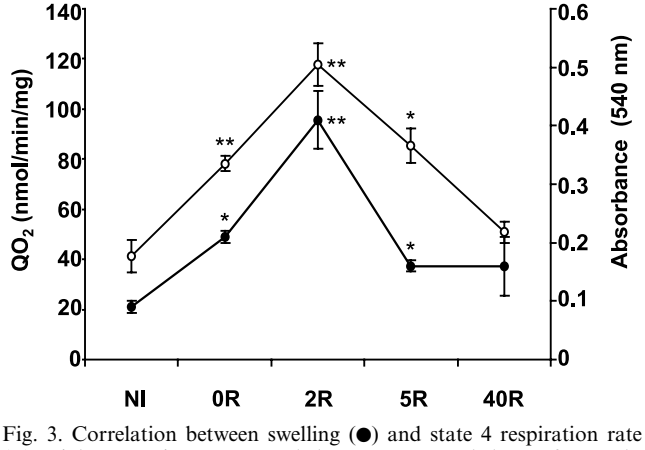

Fig. 3. Correlation between swelling (●) and state 4 respiration rate (○). Light-scattering was recorded at 540 nm, and data refer to absorbance changes 2 min after exposure of mitochondria (0.5 mg/ml) to the hypotonic medium containing 10 mM Tris–HCl, 5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 5 mM KCl, at 25°C. Means ± S.E.M. of six experiments are shown. **$P < 0.01$, *$P < 0.05$ vs. corresponding pre-ischemic value (NI).

To test the mitochondrial membrane integrity and permeability we used the swelling technique, and found that at the various time points it had a parallel increase to that of state 4 respiration rate (Fig. 3).

4. Discussion

In this work we evaluated the mitochondrial respiration and swelling during ischemia and at different times of reperfusion in isolated rat heart mitochondria. For our respiratory measurements we chose succinate as a substrate since its oxidation is a very poorly controlled process. In contrast, the oxidation of NAD-linked substrates is subject to regulation. Moreover, the possible inactivation of matrix NAD-linked dehydrogenases during reperfusion [16] would confuse the interpretation of the respiratory data. Our results indicate that 2 min reperfusion of the heart, following 30 min ischemia, is the time at which the mitochondrial coupling is most affected in the course of the experiment. At 2 min reperfusion, maximum swelling occurred, and respiration rate under non-phosphorylating conditions (state 4) was maximal (RCR was minimal). Three main reasons might be responsible for the increased state 4 respiration rate: (i) uncoupling of respiration from ADP phosphorylation due to proton leak, (ii) cycling phosphorylation of ADP produced by ATPases (F_1F_0-ATPase of broken mitochondria and Ca^{2+}-dependent ATPases present as contaminants of mitochondrial preparations), and (iii) energy-dependent Ca^{2+} cycling into and out of the mitochondria. Since we operated with a low concentration of mitochondria, it is unlikely that contaminating Ca^{2+}-dependent ATPases were present with measurable activities [17], and addition of

oligomycin to the incubation medium could not change state 4 respiration rate, thereby excluding the hypothesis of ADP cycling. Furthermore, measurements carried out in the presence of EGTA, a Ca^{2+} chelator, did not significantly influence the respiration rate. Therefore, our data are consistent with the occurrence of a proton leak at 30 min ischemia and to a greater extent during the following first 2 min of reperfusion. The proton leak might be caused by an alteration in mitochondrial membrane composition and fluidity due to the action of phospholipase A_2, which is activated during ischemic conditions, [18], and to depletion of cardiolipin that follows lipid peroxidation [19,20], and/or a consequence of the unregulated mitochondrial permeability transition pore opening [21]. According to recent reports [22,23], another mechanism might not be ruled out. During early posthypoxic reoxygenation, when both superoxide anions and fatty acids are present in the mitochondrial matrix at high concentration [24,25], to limit ROS overproduction, the cell increases proton conductance through activation of uncoupling proteins. However, the occurrence of increased mitochondrial swelling indicates structural changes in the inner membrane, which possibly occur following loss of ability for ATP synthesis (induced by ischemia) and/or for ROS overproduction (occurring during reperfusion). Although the deterioration of the inner mitochondrial membrane is associated with a low efficiency of oxidative phosphorylation, which in contrast should be high during reperfusion to rapidly restore the loss of ATP caused by ischemia [24], this low efficiency might be as well a protective mechanism for the ischemic and reperfused cardiomyocytes. Since uncoupling decreases $\Delta\overline{\mu}_{H+}$, it facilitates electron flow from reduced dinucleotides and respiratory chain redox centers to oxygen, therefore reducing toxic increased production of superoxide [26–28]. Indeed, this phenomenon concurs with the opening of both the permeability transition pore and the mitochondrial ATP-sensitive potassium channel, important cardioprotective processes in ischemic heart reperfusion, that operate through a mechanism involving depolarization of the inner mitochondrial membrane [29]. In conclusion, concurring with the recent hypothesis of He and Lemasters [21], we showed that, beside the controlled permeability, the unregulated inner mitochondrial membrane permeability of the heart is also increased by sustained ischemia and early reperfusion. Future studies directly measuring mitochondrial membrane potential to demonstrate an increase in mitochondrial membrane permeability, as well as the use of other membrane permeability transition inhibitors are warranted to elucidate the molecular mechanisms underlying the early-reperfusion-induced mitochondrial uncoupling.

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