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Biochemical consequences of electrical pacing in ischemic-reperfused isolated rat hearts

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Abstract

It is still unclear if performance recovery in postischemic hearts is related to their tissue level of high-energy phosphates before reflow. To test the existence of this link, we monitored performance, metabolism and histological damage in isolated, crystalloid-perfused rat hearts during 20 min of low-flow ischemia (90% coronary flow reduction) and reflow. To prevent interference from different ischemia times and perfusing media compositions, the ischemic ATP level was varied by changing energy demand (electrical pacing at 330 min^{-1}). Under full coronary flow conditions, work output, as well as ATP and phosphocreatine contents were the same in control, spontaneously contracting (n = 23) and paced (n = 21) hearts. During low-flow ischemia, the higher work output ($p < 0.0001$) in paced hearts decreased their tissue content of ATP, phosphocreatine and total adenylates and purines ($p < 0.05$), as opposed to maintained values in control hearts. During reflow, the recovery of mechanical performance and O_2 uptake was 94 ± 5% and 110 ± 9% (p = NS vs. baseline) in controls, vs. 71 ± 5% and 74 ± 6% in paced hearts ($p < 0.004$ vs. baseline). The levels of ATP and total adenylates and purines remained constant in control, but were markedly depressed ($p < 0.05$ vs. baseline) in paced hearts. Phosphocreatine+creatine was the same in both groups. These data, together with the observed lack of creatine kinase leakage and of structural damage, indicate that myocardial recovery during reflow reflects the tissue level of ATP, phosphocreatine and total adenylates and purines during ischemia, regardless of physical cell damage. (Mol Cell Biochem **194**: 245–249, 1999)

Keywords: ATP catabolism, phosphocreatine, reperfusion injury, low-flow ischemia, adenylates

Introduction

It is generally, although not universally, perceived that biochemical factors critically control some of the mechanisms underlying the myocardial dysfunction that follows ischemia-reperfusion. One of these concerns the link between the tissue level of high-energy phosphates, e.g. ATP and phosphocreatine (Pcr), measured in the ischemic myocardium before reflow, and the functional postischemic recovery. This link appears non-existent in some studies [1–4]. In contrast, a causal relationship emerged in other models, where the ischemic level of high-energy phosphates was altered either by changing the length of ischemia [5, 6], or by replenishing hearts with diffusable substrates [7–9]. However, the use of pharmacological agents as well as of different ischemia times might have confounded data, and the basic issue whether myocardial biochemistry may eventually influence recovery in postischemic hearts remains unresolved.

To focus on that issue, we realize that the ischemic ATP level can be modified also by modulating energy demand [10], without altering neither the time duration of ischemia nor the composition of the perfusing medium. In this study, we test the hypothesis that myocardial recovery during reflow reflects the tissue level of ATP, Pcr and their catabolites during ischemia. For this aim, we monitored performance, metabolism and histological damage in isolated, perfused rat hearts during low-flow ischemia and reflow. While the control group is constituted by spontaneously contracting hearts, that underwent downregulation of energy requirements [11], propositus hearts are electrically paced to establish a condition

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of high energy demand, that would decrease the level of high energy phosphates. We show that high demand of energy decreases the myocardial functional recovery presumably by decreasing the intracellular pool of ATP, but not that of Pcr, and that this effect is independent of membrane damage.

Materials and methods

Materials

Hearts from *ad libitum* fed Sprague-Dawley male rats (wt 250–280 g) were Langendorff-perfused [10] with Krebs-Henseleit buffer (2.0 mM free Ca^{2+}, 16.6 mM glucose, pH 7.4, PCO_2 = 43 mmHg, PO_2 = 670 mmHg, 37°C). All hearts (n = 44) were stabilized for 30 min at 15 ml/min flow. A cannula was inserted into the pulmonary artery to collect the venous return and to monitor venous PO_2 by an O_2-sensing electrode (Yellow Springs Inc. model 5300 Oxygen Monitor, Yellow Springs, OH, USA). The balloon volume was adjusted to achieve end-diastolic pressure (EDP) = 10 mmHg and was kept constant throughout. Therefore, subsequent increases of EDP reflect diastolic contracture. Paced hearts (n = 21) were electrically stimulated at heart rate (HR) = 330 beats/min. Electrodes for stimulation were placed on the aortic cannula and on the apex of the ventricle and were connected to a square wave stimulator (Harvard, South Natick, MA, USA, 5 msec pulse duration, 10 V pulse amplitude). At the end of stabilization, 7 control and 6 paced hearts were freeze-clamped with liquid nitrogen cooled clamps for baseline measurements. The remaining hearts underwent 20 min of low-flow ischemia (flow = 1.5 ml/min). At the end of this period, 7 control and 6 paced hearts were freeze-clamped for metabolite measurements. The remaining hearts underwent 20 min of reflow (flow = 15 ml/min), and were finally freeze-clamped for metabolites determination.

Measurements and calculations

Contractility measurements included EDP, HR and left-ventricle developed pressure (LVDP). The product LVDP·HR provides an index of the isovolumic work output. The O_2 uptake (VO_2) was calculated from venous PO_2 and coronary flow. Frozen tissue was extracted with perchloric acid and assayed for Pcr, creatine, ATP, ADP, AMP, adenosine, inosine-5′-monophosphate, inosine, hypoxanthine, xanthine and urate by high-pressure liquid chromatography [12]. The sum of these compounds, except Pcr and creatine, is referred to as total adenine nucleotides and purines (TANP).

Endothelial damage was evaluated at the end of both baseline and reflow in two additional hearts per group. At the selected times, the heart was perfused for 4 min with colloidal carbon black solution (Rotring, Hamburg, Germany, 1/200 v/v in buffer), followed by 2 min of washout with the perfusing medium [13]. The heart was removed from the apparatus, 5 samples of the organ were fixed in 10% paraformaldehyde and stored at –80°C until conventional hematology (hematoxylin-eosin staining) was carried out. The retention of carbon black ink in the interstitium, estimated by optical microscopy on a scale ranging from 1 (no retention) to 4 (maximal retention), is proportional to endothelial cell injury. Grade 4 was arbitrarily assigned to hearts exposed to 20 min of perfusion at PO_2 = 67 mmHg followed by 20 min of reoxygenation as in group A of [11].

Myocyte injury was evaluated in two additional hearts per group at the end of both baseline and reflow by electron microscopy as described [11]. For this evaluation, the heart was freeze-clamped in pre-cooled isopentane. We evaluated the damage to nuclei (margination and clumping of chromatin), mitochondria (enlargement, matrix density, disruption of cristae and occurrence of amorphous material) and myocardial fibers (occurrence and severity of contraction bands), as well as cell edema, on a scale ranging from 1 (no injury) to 4 (maximal injury) [14], where Grade 4 was assigned as above. The damage to membrane was evaluated using the following arbitrary criteria applied to the membrane outline: smooth (grade 1), slightly wrinkled (grade 2), heavily wrinkled (grade 3), irregular and influenced by intracellular structures (grade 4).

To further assess myocyte injury, we assayed creatine kinase activity in the effluent collected for 10 min after reflow in 4 hearts per group. Standard UV methods (CK-NAC, Behring, L'Aquila, Italy) were employed.

Statistics

Data (means ± S.E.) were collected at the end of the periods of baseline, underperfusion and reflow. The recovery was assessed by dividing the value obtained at the end of reflow by that obtained at the end of baseline. Differences were tested by the parametric Student's *t*-test for unpaired observations (significance level, p = 0.05, two-tailed).

Results

Function

Since hearts kept contracting during low-flow ischemia and reflow, all experiments are suitable for analysis. During baseline, HR was higher in paced than in control hearts (Table 1), but LVDP·HR was unchanged, because LVDP decreased proportionally. VO_2 was slightly higher in control than paced hearts (venous PO_2 = 348 ± 22 and 299 ± 13 mmHg, respectively, p = NS). During low-flow ischemia,

LVDP·HR declined in both groups. The venous PO_2 were 100 ± 13 and 137 ± 20 mmHg, leading to almost identical VO_2 values. After reflow, both diastolic contracture and myocardial dysfunction were higher in paced than in control hearts. VO_2 was also more depressed in paced hearts. In contrast, spontaneously contracting hearts could recover almost completely their baseline performance.

Metabolism

During baseline, metabolic data were the same in the two groups, with the exception of higher inosine-5′-monophosphate in paced hearts (Fig. 1). While the ATP and TANP levels remained unchanged throughout ischemia-reperfusion in control hearts, they decreased markedly in paced hearts. The sum Pcr+creatine was unaltered in both groups. In paced hearts, Per first decreased, then overshot above baseline, while remaining unchanged in control hearts. Inosine-5′-monophosphate was higher in paced than control hearts during low-flow ischemia, but at the end of reflow this value was the same in both groups.

Cell damage

No evident sign of cell damage was observed in the two groups: (i) Creatine kinase activity in the effluent collected during the first 5 min of reflow did not exceed 0.03 U/l in both control and paced hearts; (ii) Ultrastructural data indicate only minor damage at the level of mitochondria, nuclei and myofibrils (Table 2); and (iii) Ink retention, index of endothelial damage, was similar in control and paced hearts.

Discussion

In fully perfused hearts, the increase in HR due to pacing offsets the decrease in LVDP, in agreement with [15]: Therefore, LVDP·HR provides a reliable index of the isovolumic work output. Pacing does not alter the tissue high-energy phosphates level, index of energy balance, because the saturating supply of O_2 and substrate [16] allows tight coupling between ADP phosphorylation and ATP dephosphorylation. Although some ATP is likely converted to its catabolites even under baseline conditions, purine salvage and *de novo* purine synthesis compensate the loss of these compounds [17]. The higher level of inosine-5′-monophosphate in paced than control hearts may be due to adrenergic stimulation that enhances AMP conversion to inosine-5′-monophosphate by activating AMP deaminase [18].

During low-flow ischemia in spontaneously contracting hearts, intracellular lactate accumulation downregulates myocardial activity [16]. Thus, the tissue levels of ATP, Pcr and inosine-5′-monophosphate do not change appreciably. Indeed, the favourable perfusion-contraction matching allows balanced energy supply-to-demand ratio despite severe flow reduction. During low-flow ischemia in paced hearts, energy demand increases under conditions of limited supply of O_2 and substrate. This 'unmatches' the energy supply-to-demand balance. The high level of inosine-5′-monophosphate indicates a situation of bioenergetic derangement [10]. Although LVDP·HR does not change with pacing, increased EDP raises the performance level: (LVDP+EDP)·HR, an expression of total work output, is 6.2 ± 0.5 mmHg·10^3/min and 9.9 ± 0.7 mmHg·10^3/min ($p < 0.0001$), respectively, in control and paced hearts. VO_2 is the same in both groups during low-flow

Table 1. Myocardial perfonnance at the end of the various phases in control (spontaneously contracting) and paced hearts

	Heart rate	End-diastolic pressure	Developed pressure	LVDP·HR	O_2 uptake
	beats/min	mmHg	mmHg	mmHg·10^3/min	µmoles/min
Control					
Baseline (n = 23)	270 ± 8	9.5 ± 0.5	150 ± 5	40.6 ± 1.9	6.8 ± 0.5
Low-flow ischemia (n = 16)	178 ± 12#	5.2 ± 0.4#	31 ± 4#	5.2 ± 0.5#	1.2 ± 0.1
Reflow (n = 9)	264 ± 15	7.8 ± 0.6	138 ± 5#	36.7 ± 2.8	7.5 ± 0.9
% Recovery	99 ± 4	79 ± 7	96 ± 2	94 ± 5	110 ± 9
Paced					
Baseline (n = 21)	330 ± 0*	9.6 ± 0.3	113 ± 3*	37.3 ± 1.1	7.8 ± 0.3
Low-flow ischemia (n = 15)	330 ± 0*	13.2 ± 0.5*#	17 ± 1*#	5.5 ± 0.5#	1.1 ± 0.1
Reflow (n = 9)	330 ± 0*	30.2 ± 3.5*#	81 ± 5*#	26.6 ± 1.6*#	5.8 ± 0.4*#
% Recovery	100	292 ± 37	71 ± 5	71 ± 5	74 ± 6

Data obtained at the end of baseline, after 20 min of low-flow ischemia, and after 20 min of reflow (mean ± S.E.). *Significant difference ($p < 0.05$) vs. spontaneously beating hearts; #Significant difference ($p < 0.05$) vs. baseline (Student's *t*-test). LVDP·HR, developed pressure·heart rate.

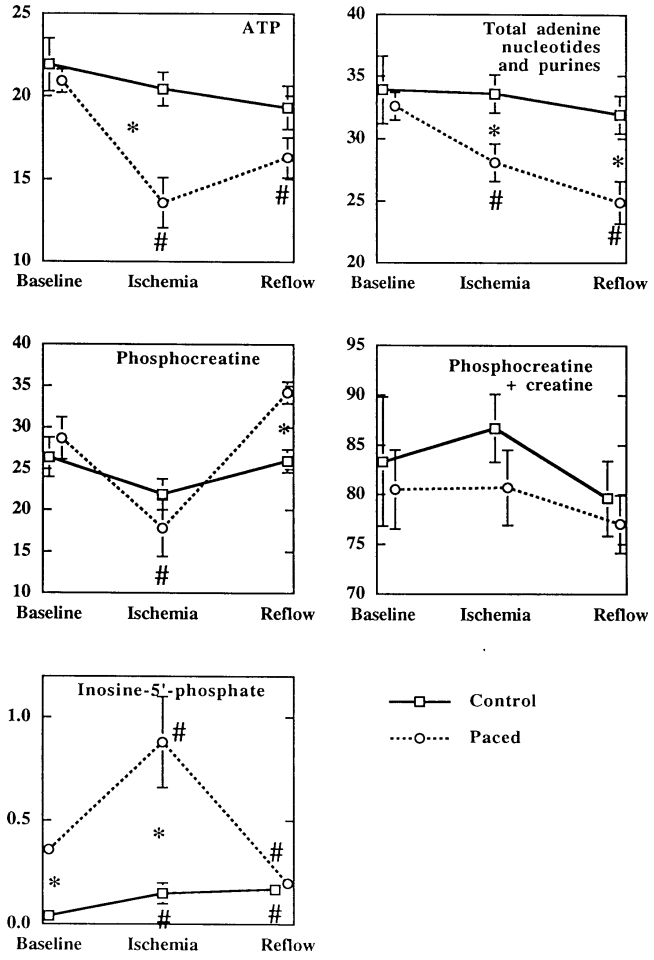

Fig. 1. Tissue levels (μmoles/g dry wt) in spontaneously contracting (control, squares) and paced (circles) hearts, during baseline (n = 7 and 6, respectively), after 20 min of low-flow ischemia (n = 7/6), and after 20 min of reflow (n = 9/8) (mean ± S.E.). *Significant difference ($p < 0.05$) vs. spontaneously contracting hearts; #Significant difference ($p < 0.05$) vs. baseline (Student's *t*-test).

ischemia: Thus, anaerobic glycolysis contributes the extra ATP needed to support the increased work output in paced hearts [16]. As a consequence of impaired energy supply with respect to demand, the ATP and Pcr levels decline more markedly in paced than in control hearts. Enhanced ATP catabolism increases inosine-5′-monophosphate, and decreases TANP. In principle, the latter finding may be attributed to the leak of intracellular compounds secondary to aspecific membrane damage. However, electron and optical microscopy, as well as creatine kinase release, exclude this possibility. Furthermore, constant Pcr+creatine does not support permeability increase, because these compounds do not leak across intact membrane [19, 20]. Therefore, the loss of TANP is attributed to passive diffusion of uncharged ATP catabolites (adenosine, inosine, hypoxanthine, xanthine and urate) across the membrane.

Table 2. Structural and ultrastructural damage at the end of baseline and reflow in spontaneously contracting (control) and paced hearts

	Baseline	Reflow	
		Control	Paced
Ink retention	1	1	1.5
Nucleus	1	1.5	1.5
Mitochondria	1	1	1.5
Myofibrils	1.5	1.5	2
Edema	1.5	2	2
Membrane	1	2	2
Average score	1.2	1.5	1.7

The damage was evaluated on a 1 to 4 scale as explained in the text, grade 4 showing maximal damage (see text for details, n = 2 hearts per group).

Performance recovery during reflow is generally considered as an integrated index of myocardial viability. Although we can not rule out some contribution of the right ventricle, the left ventricle is presumably mostly involved in the observed patterns. Improved recovery in spontaneously contracting with respect to paced hearts reflects their less severe metabolic derangement during ischemia. Although paced hearts are likely able to resynthesize some ATP from its catabolites during reflow, the low level of TANP prevents full restoration of the ATP pool. This compromises performance recovery [7, 21, 22]. The Pcr overshoot during reflow is due to low ADP availability for the creatine-kinase reaction.

In conclusion, high energy demand during ischemia compromises performance recovery during reflow presumably by biochemical mechanisms. Enhanced ATP degradation, secondary to high energy demand, forms uncharged, membrane-diffusable ATP catabolites, that can be washed out by residual flow. This reduces the intracellular ATP pool and compromises recovery during reflow. This mechanism is unrelated to membrane damage and to phenomena associated with the presence of blood. Therefore, some other derangements, that are recognised as part of the ischemia-reperfusion dysfunction in vivo, as for example Ca^{2+} transients changes, free radical injury, and microcirculatory consequences, presumably follow, and do not precede, myocardial metabolism changes [23]. As some of the involved mechanisms are not structurally verifiable, our conclusion is to be checked against further data aimed at demonstrating this particular sequence of events during ischemia-reflow.

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