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Original Contribution

Complex I syndrome in myocardial stunning and the effect of adenosine

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ABSTRACT

Isolated rabbit hearts were exposed to ischemia (I; 15 min) and reperfusion (R; 5–30 min) in a model of stunned myocardium. I/R decreased left-ventricle O_2 consumption (46%) and malate–glutamate-supported mitochondrial state 3 respiration (32%). Activity of complex I was 28% lower after I/R. The pattern observed for the decline in complex I activity was also observed for the reduction in mitochondrial nitric oxide synthase (mtNOS) biochemical (28%) and functional (50%) activities, in accordance with the reported physical and functional interactions between complex I and mtNOS. Malate–glutamate-supported state 4 H_2O_2 production was increased by 78% after I/R. Rabbit heart Mn-SOD concentration in the mitochondrial matrix (7.4 ± 0.7 μM) was not modified by I/R. Mitochondrial phospholipid oxidation products were increased by 42%, whereas protein oxidation was only slightly increased. I/R produced a marked (70%) enhancement in tyrosine nitration of the mitochondrial proteins. Adenosine attenuated postischemic ventricular dysfunction and protected the heart from the declines in O_2 consumption and in complex I and mtNOS activities and from the enhancement of mitochondrial phospholipid oxidation. Rabbit myocardial stunning is associated with a condition of dysfunctional mitochondria named "complex I syndrome." The beneficial effect of adenosine could be attributed to a better regulation of intracellular cardiomyocyte Ca^{2+} concentration.

Conventionally, ischemic heart disease and heart ischemia/reperfusion (I/R) include a number of entities that have been grouped in accordance with their physiopathology and time course. A sustained ischemia (more than 20 min) leads to myocardial infarction with cellular death and necrosis, whereas a transient ischemia (less than 20 min) produces a reversible ventricular postischemic dysfunction also known as "stunned myocardium" [1,2]. Myocardial stunning is a postischemic systolic and diastolic mechanical dysfunction with the absence of irreversible injury, i.e., necrosis, and with preserved contractile reserve [1–3]. Although myocardial stunning is a fully reversible phenomenon, contractile dysfunction persists for hours or even days. Myocardial stunning is observed in patients who undergo myocardial revascularization surgery, thrombolytic drug therapies, angioplasty, cardiac transplant, stable/unstable angina, and exercise-induced ischemia [1,3].

Two main hypotheses, not mutually exclusive, explain the molecular mechanisms that lead to mechanical dysfunction observed in heart stunning: (a) a transient Ca^{2+} overload and (b) an increased production of oxygen and nitrogen reactive species. Coronary flow interruption promotes a fall in the intracellular pH that activates the Na^+/H^+ exchanger (NHE). The NHE activation increases the intracellular Na^+ concentration and establishes the enhancement of the intracellular Ca^{2+} concentration through the Na^+/Ca^{2+} exchanger [4,5]. Although an early myocardial reperfusion is undoubtedly the most effective approach in the treatment of myocardial ischemia and hypoxia, reoxygenation encompasses an enhancement in superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) production [6–9]. In cardiac cells, mitochondria are the major source of $O_2^{\bullet -}$ and H_2O_2 [10]. Isolated cardiomyocytes subjected to hypoxia show increased mitochondrial reactive oxygen species production [11]. During hypoxia, the absence of O_2 determines a maximal reduction state of mitochondrial respiratory chain components, and upon reoxygenation the excess of electrons leads to an increased $O_2^{\bullet -}$ and H_2O_2 production [8]. Heart mitochondria were early recognized by Boveris and Chance [12] as an active source of H_2O_2 in a process that depends on the redox state of the respiratory chain and on the mitochondrial metabolic state. Superoxide anion is the stoichiometric precursor of H_2O_2 and is generated through the oxidation by O_2 of the intermediate semiquinones ($UQH^{\bullet}$ and $FMNH^{\bullet}$) of the redox pairs ubiquinol/ubiquinone and $FMNH_2$/FMN, components of the NADH dehydrogenase [10]. In addition, cardiac nitric oxide (NO), regulated by the Ca^{2+} levels of the contraction and relaxation cycles and produced by

Abbreviations: I/R, ischemia/reperfusion; mtNOS, mitochondrial nitric oxide synthase; LV, left ventricle.

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heart nitric oxide synthases (NOSs), is essential for heart homeostasis and mechanical activity [13]. It is worth noting that NO acts in signaling through cGMP-dependent pathways and in the regulation of mitochondrial respiration through a cGMP-independent way. At submicromolar concentrations, NO exhibits two main effects on the mitochondrial respiratory chain: the competitive inhibition of cytochrome oxidase [14,15] and the stimulation of $O_2^{\bullet-}$ production through inhibition of electron transfer at complex III [16]. Moreover, NO and $O_2^{\bullet-}$ react to yield peroxynitrite ($ONOO^-$), which is able to nitrate protein tyrosine residues and inactivate mitochondrial proteins.

Taking into account that reactive oxygen and nitrogen species are probably involved in myocardial stunning, the aim of this work was to study left-ventricle tissue O_2 uptake and mitochondrial function in isolated perfused rabbit heart subjected to brief global ischemia in a model of stunned myocardium. Of note, the xanthine oxidase activity in rabbits and humans is low (<1 nmol urate/min . g tissue) [17], suggesting that rabbit heart I/R is an adequate experimental model for extrapolating the results to human ischemic syndromes. In addition, considering the observed cardioprotective action of adenosine [3], the effect of this drug was studied.

Materials and methods

Chemicals

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-nitric oxide synthase antibodies (anti-nNOS—H299) and anti-nitrotyrosine antibodies (clone 1A6) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Millipore (Billerica, MA, USA), respectively. Other reagents were of analytical grade.

Animals

New Zealand male rabbits (1.8–2.0 kg) were housed in separate cages in an environmentally controlled facility at 25 °C. The animals were subjected to circadian light–dark cycles, fed standard rabbit chow, and provided water ad libitum. The procedures used in this study were approved by the Animal Care and Research Committee of the University of Buenos Aires, and this investigation was in accordance with the American Physiological Society's *Guiding Principles in the Care and Use of Animals*, published by the U.S. National Institutes of Health.

Surgical procedures and coronary perfusion pressure recording

Rabbits were anesthetized with ketamine (75 mg/kg) and xylazine (0.75 mg/kg) and then euthanized with thiopental sodium (35 mg/kg). Hearts were excised and placed in a perfusion system according to the Langendorff technique. Perfusion medium consisted in Krebs–Henseleit buffer containing 118.5 mM NaCl, 4.7 mM KCl, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM glucose, pH 7.2–7.4, equilibrated with 95% O_2/5% CO_2 at 37 °C. Two electrodes were sutured and connected to a pacemaker to maintain a constant heart rate of 200 beats/min.

A latex balloon connected to a pressure transducer (Deltram II; Utah Medical System) via a polyethylene cannula was inserted into the left ventricle (LV) for measurement of LV pressure. The latex balloon was filled with water to achieve a left-ventricle end-diastolic pressure (LVEDP) of 8–10 mm Hg. The coronary perfusion pressure (CPP) was recorded through a pressure transducer connected to the perfusion line. All hearts were perfused with constant flow. Coronary flow was adjusted to obtain a CPP of 70.5 ± 4.2 mm Hg during the initial stabilization period and was then kept constant throughout the experiment. In a heart perfused at a constant coronary flow, the CPP indicates coronary vascular resistance. Left-ventricular pressure and CPP were recorded in real time using a computer with data acquisition hardware. The left-ventricular developed pressure (LVDP) was calculated as the difference between peak systolic pressure and LVEDP. Left-ventricular function was assessed at baseline and during ischemia/reperfusion.

Isolated rabbit heart and ischemia/reperfusion

After a 15-min stabilization period with a constant perfusion flow, a 15-min global ischemia followed by 30-min reperfusion was performed. The groups were divided according to I/R time. Samples were obtained before ischemia (0/0), after 15 min of ischemia (15/0), after 15 min of ischemia and 5 min of reperfusion (15/5), and after 15 min of ischemia and 30 min of reperfusion (15/30). After the above-mentioned I/R times, the left ventricle was separated from the heart and used for all biochemical tests.

This experimental protocol was carried out in the absence or in the presence of adenosine (0.03 μg/kg . min) in the perfusion medium (Krebs–Henseleit buffer). Adenosine was administered 10 min before ischemia and during reperfusion time. An additional group was used to evaluate the behavior of the ventricular function during a 65-min perfusion.

Infarct size measurement

After 2 h of reperfusion, performed to allow the washout of dehydrogenases and cofactors released from the necrotic tissue, hearts were frozen and cut into 2 mm transverse slices from apex to base. The slices were then incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution in isotonic pH 7.4 phosphate buffer at 37 °C for 20 min [18]. TTC reacts with electron donors (e.g. NADH) in the presence of dehydrogenases, causing the viable cells to stain a deep red color. Red-stained viable tissue was easily distinguished from the gray or white unstained necrotic tissue. The slices were subsequently fixed in 10% formalin solution.

Tissue O_2 uptake

Left ventricles were sectioned into 1 mm^3 cubes and the O_2 uptake of two to four cubes was determined polarographically with a Clark-type electrode (Oroboros Oxygraph, Graz, Austria) in a 1.5-ml chamber at 30 °C, in an air-saturated Krebs medium consisting of 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 5.5 mM glucose, pH 7.4. Respiration traces showed a linear relationship with the number of cubes (tissue mass) and during the initial 3–10 min of the measurement. The data were expressed in μmol O_2/min. g tissue.

Isolation of mitochondria

Left ventricles were excised and weighed. Organs were chopped and homogenized in an ice-cold homogenization medium (1/10) containing 230 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4 (MSTE) for 15 s with a blade homogenizer (Kendro-Sorvall-Du Pont Inst., Asheville, NC, USA) and by five strokes in a glass–Teflon homogenizer. All these operations were carried out at 2–4 °C. The homogenates were centrifuged at 600*g* for 10 min to discard nuclei and cell debris and the supernatant was centrifuged at 8000*g* for 10 min to precipitate mitochondria, which were washed with MSTE [19].

Preparation of mitochondrial membranes

Mitochondrial membranes were obtained by three cycles of freezing and thawing of the mitochondrial preparation and were homogenized by passage through a 25-gauge hypodermic needle [20]. Protein concentration was determined with the Folin reagent using bovine serum albumin as standard.