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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.

Mitochondrial O_2 consumption

Mitochondrial O_2 uptake was measured with a Clark-type electrode (Oroboros Oxygraph) in a 1.5-ml chamber at 30 °C in an air-saturated reaction medium ($[O_2] = 220$ μM) consisting of 230 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl, 1.0 mM EDTA, 5.0 mM KH_2PO_4/K_2HPO_4, 2.0 mM $MgCl_2$, pH 7.4, and 0.2–0.4 mg mitochondrial protein/ml. State 4 respiration rates were determined using 6 mM malate and 6 mM glutamate or 8 mM succinate as substrates of complex I or complex II, respectively; state 3 respiration rate was established by addition of 0.5 mM ADP [21]. Respiration was expressed in ng-at O/min . mg protein, and respiratory control was calculated as the ratio of state 3/state 4 respiration rates.

To determine mtNOS functional activity in the inhibition of O_2 consumption, 1 mM L-arginine or 2 mM N^{ω}-monomethyl-L-arginine (L-NMMA) was added to state 3 respiring mitochondria [22].

Mitochondrial electron transfer activities

The enzyme activities of complexes I, II, and IV were determined spectrophotometrically (Beckman DU 7400 spectrophotometer; 550 nm, $\varepsilon = 19\ mM^{-1}\ cm^{-1}$) at 30 °C with mitochondrial membranes suspended in 100 mM KH_2PO_4/K_2HPO_4, pH 7.4 [23,24]. Complex I was determined as NADH-cytochrome *c* reductase and complex II was determined as succinate-cytochrome *c* reductase. Mitochondrial membranes were added with 0.2 mM NADH or 5.0 mM succinate as substrate, 25 μM cytochrome c^{3+}, and 0.5 mM KCN. Enzymatic activities were expressed as nmol reduced cytochrome *c*/min . mg protein. Complex IV (cytochrome oxidase) was determined in the same buffer supplemented with 60 μM cytochrome c^{2+}. Reduced cytochrome *c* was prepared by reduction of cytochrome c^{3+} with $Na_2S_2O_4$, followed by Sephadex G-25 chromatography. The rate of cytochrome c^{2+} oxidation was calculated as the pseudo-first-order reaction constant k'/mg protein.

Manganese-superoxide dismutase activity and concentration

Manganese-superoxide dismutase (Mn-SOD) activity was assayed at 550–540 nm by monitoring the reduction rate of partially acetylated ferricytochrome *c* [25,26]. The reaction medium consisted of 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 10 μM partially acetylated ferricytochrome *c*, 50 μM xanthine, and sufficient xanthine oxidase (XO; ~5 nM) to reach an absorbance increase of 0.025/min at 25 °C, in the absence of SOD. The reaction was started by the addition of XO and the absorbance was followed continuously for 2 min, in the absence or in the presence of four different amounts of mitochondrial membranes (0.3–1.0 mg/ml). One unit of SOD was defined as the amount of enzyme that inhibits by 50% the reduction of ferricytochrome *c* and corresponds to 4.0 pmol of Mn-SOD. Control experiments in the presence of 0.5 mM KCN were carried out to discount the inhibition of cytochrome *c* reduction associated with the Cu,Zn-SOD activity. Under our experimental conditions, the addition of KCN to the reaction medium did not modify the rate of cytochrome *c* reduction. Results were expressed as Mn-SOD activity (U/mg protein) and as Mn-SOD concentration (μM) in the mitochondrial matrix.

Hydrogen peroxide production

Mitochondrial H_2O_2 production was determined fluorimetrically at 365–450 nm (Hitachi F-3010 fluorescence spectrophotometer) in state 4 mitochondrial suspensions (0.1–0.3 mg protein/ml) using the scopoletin–horseradish peroxidase (HRP) assay [27]. The reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl, pH 7.4, 8 mM succinate or 6 mM malate and 6 mM glutamate, 0.5 μM Cu,Zn-SOD, 1 μM HRP, and 1 μM scopoletin. A calibration curve was made using H_2O_2 (0.05–0.35 μM) as standard to express the fluorescence changes as nmol H_2O_2/min . mg protein. To distinguish the H_2O_2 production associated with $O_2^{\bullet-}$ generation from complex I, H_2O_2 production was determined in the absence or in the presence of 1 μM rotenone. Similarly, and concerning complex III, the determination was performed in the absence or in the presence of 1 μM antimycin.

Nitric oxide production

Nitric oxide production was measured in mitochondrial membranes by following the oxidation of oxyhemoglobin to methemoglobin at 37 °C and at 577–591 nm ($\varepsilon = 11.2\ mM^{-1}\ cm^{-1}$) using a Beckman DU 7400 diode array spectrophotometer [20,28]. The reaction medium consisted of 50 mM KH_2PO_4/K_2HPO_4, pH 7.4, 1 mM L-arginine, 1 mM $CaCl_2$, 100 μM NADPH, 10 μM dithiothreitol, 2 μM Cu,Zn-SOD, 0.1 μM catalase, 20 μM oxyhemoglobin heme, and 0.2–0.4 mg of protein/ml. Control experiments adding 1 mM L-NMMA as an inhibitor of mtNOS were performed to consider only the L-NMMA-sensitive hemoglobin oxidation as due to NO formation. Addition of L-NMMA inhibited by about 90–92% the rate of hemoglobin oxidation. The absorbance changes that were inhibited by L-NMMA were expressed as nmol NO/min . mg protein.

Thiobarbituric acid-reactive substances (TBARS)

Phospholipid oxidation was determined by the fluorescence assay of TBARS [29]. An aliquot (100 μl) of left-ventricle mitochondrial suspension (35 mg protein/ml) was added to 2 ml of 0.1 N HCl, 0.3 ml of 10% w/v phosphotungstic acid, and 1 ml of 0.7% w/v 2-thiobarbituric acid. The mixture was heated in boiling water for 1 h. TBARS were extracted with 3 ml of *n*-butanol. After a brief centrifugation, the fluorescence of the butanolic phase was measured in a Hitachi F-3010 spectrofluorimeter at 515–553 nm. A calibration curve was performed using malondialdehyde (0.06–0.6 μM) as standard to express the fluorescence changes as pmol TBARS/mg protein.

Protein carbonyl content

The mitochondrial content of carbonyl groups from oxidatively modified proteins was assessed by determining the amount of 2,4-dinitrophenyl hydrazone formed upon reaction of the mitochondrial membranes with 2,4-dinitrophenyl hydrazine (DNPH) [30]. Mitochondrial membranes were supplemented with 4 ml of 10 mM DNPH and left 1 h for incubation at room temperature and in the dark. Samples were vortexed every 15 min. Proteins were precipitated adding 5 ml of 20% trichloroacetic acid (TCA). The tubes were left at 4 °C for 10 min and centrifuged for 5 min to collect the protein precipitates. The supernatants were discarded and another wash was performed using 4 ml 10% TCA. Finally, the pellets were washed three times with 4 ml of ethanol: ethyl acetate (1:1) to remove free DNPH, dissolved in 2 ml of 6 mM guanidine hydrochloride (pH 2.5), and left for 10 min at 37 °C. Carbonyl content was calculated considering the absorbance maximum of 2,4-dinitrophenyl hydrazone at 360 nm ($\varepsilon = 22\ mM^{-1}\ cm^{-1}$). Protein carbonyls were expressed as nmol/mg protein.

Western blot assays: mtNOS expression and nitrotyrosine detection

Mitochondrial membrane fractions were prepared using a protease inhibitor mixture during mitochondrial isolation. The proteins were subjected to 7% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Then, they were blotted into nitrocellulose films and probed with either 1/500 diluted rabbit polyclonal anti-neuronal NOS (H-299; Santa Cruz Biotechnology) and a secondary goat anti-rabbit IgG antibody (1:5000) or 1/4000 diluted mouse monoclonal anti-nitrotyrosine (Millipore) and a secondary anti-mouse IgG antibody (1:7500). The secondary antibodies were conjugated with horseradish peroxidase and revealed using an ECL assay. Densitometric analysis of the bands

was performed by Image 1.62 software (Wayne Rasband, NIH, Bethesda, MD, USA).

Statistics

Results are expressed as means ± SEM of four to six independent experiments. One-way analysis of variance followed by Bonferroni multiple comparisons test was used to analyze the significance of differences between paired groups. Statistical analysis was done using GraphPad Instat 4 (GraphPad Software, La Jolla, CA, USA). The probability level of 0.05 or lower was used as a criterion for biological significance.

Results

Ventricular functional parameters and effect of adenosine

Fig. 1 shows LVDP (Fig. 1A), LVEDP (Fig. 1B), LV maximal rate of pressure increase ($+dP/dt_{max}$; Fig. 1C), and CPP (Fig. 1D) during a sustained 60-min perfusion (normoxic) or during 15 min stabilization followed by I/R (15/30) in the absence or in the presence of adenosine. The contractile state was evaluated through the LVDP and LV + dP/dt_{max}. LVDP of hearts exposed to I/R recovered only 63% of the initial preischemic values (107 ± 7 mm Hg, Fig. 1A). The treatment with adenosine before ischemia and during reperfusion significantly improved the postischemic recovery, reaching a value of 97 ± 6 mm Hg ($p<0.05$ vs I/R group). A similar behavior was observed in the LV + dP/dt_{max} (Fig. 1C). Fig. 1B shows that I/R produced a fourfold increase in LVEDP, and the administration of adenosine abolished the enhancement of myocardial stiffness, reaching 12 ± 1 mm Hg at 30 min of reperfusion instead of 35 ± 3 mm Hg as was detected in perfused heart in the absence of adenosine. Finally, Fig. 1D illustrates the behavior of CPP, which increased 44% during 30 min reperfusion. The administration of adenosine before ischemia produced the well-known vasodilator effect evidenced by a drop of about 20% in CPP during the first 7 min of stabilization. Adenosine attenuated the CPP enhancement and maintained values similar to those of the preischemic state.

Fig. 2 shows representative photographs of cross-sectional slices stained with TTC, from a rabbit heart subjected to 15 min of global ischemia followed by 120 min of reperfusion. The nonappearance of TTC-negative areas confirms the absence of myocardial infarction in this experimental model.

Tissue and mitochondrial O_2 consumption

Left-ventricle respiration rates were assessed in 1-mm^3 tissue cubes, a thickness that allows O_2 diffusion to the center of the cube, avoiding anaerobic areas. Control samples (0/0) had a respiratory rate of 1.57 ± 0.10 μmol O_2/min g tissue (Fig. 3). This rate represents mainly the respiratory rate of resting mitochondria (state 4) within the tissue accounting for the respiratory activity of partially quiescent heart, i.e., without workload. Tissue O_2 uptake decreased successively in the steps of ischemia (15/0), early reperfusion (15/5), and reperfusion (15/30), reaching an overall decrease of 46% in the last step of the protocol (0.85 ± 0.08 μmol O_2/min . g tissue).

The same phenomenon of respiratory impairment in rabbit left ventricle was observed in isolated mitochondria, with successive decreases in O_2 uptake along the steps of I/R. Mitochondrial state 3

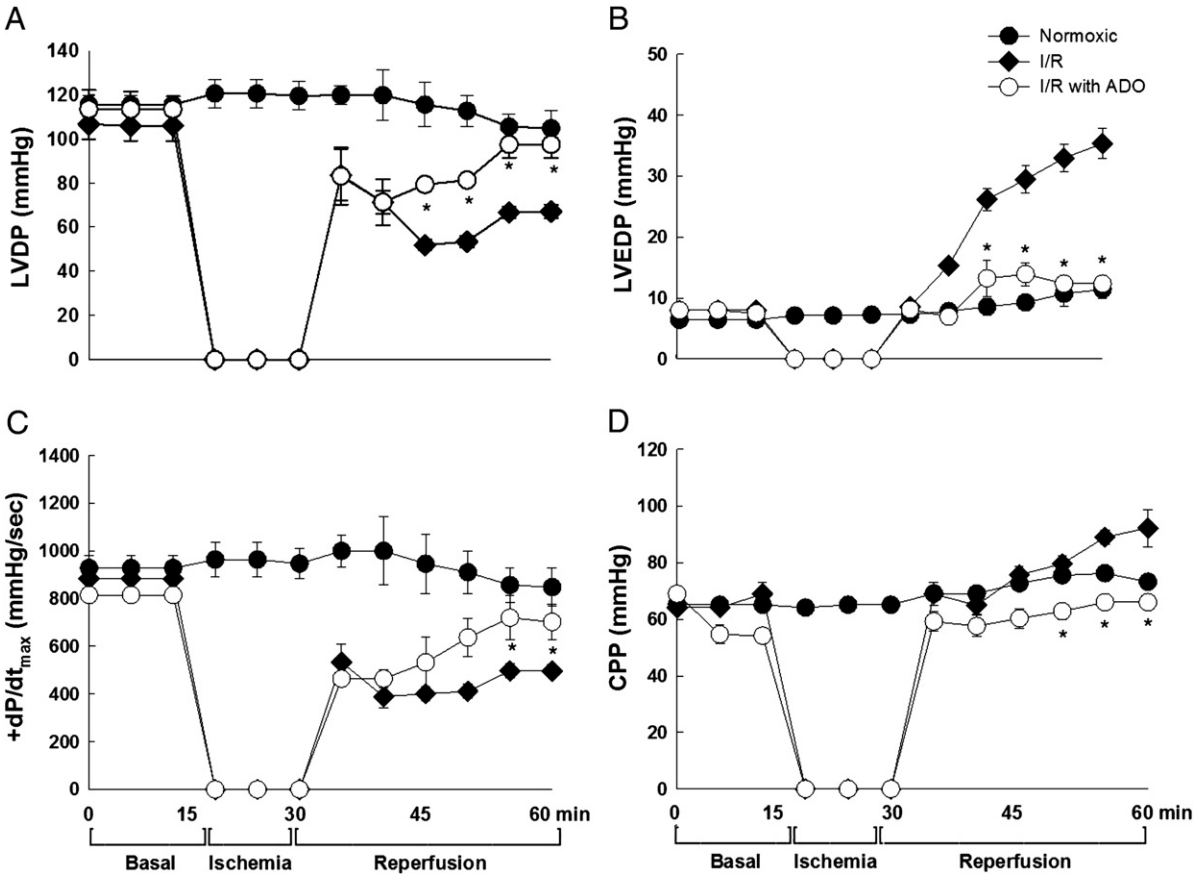

Fig. 1. Hemodynamic variables (A) left-ventricle diastolic pressure, (B) left-ventricle end-diastolic pressure (C) LV maximal rate of pressure increase ($+dP/dt_{max}$), and (D) coronary perfusion pressure determined in isolated rabbit heart during sustained perfusion (normoxia), ischemia/reperfusion, and ischemia/reperfusion + adenosine (ADO). *$p<0.05$, statistically different I/R vs I/R + adenosine.

Fig. 2. Representative slices stained with triphenyltetrazolium showing the absence of myocardial infarction. Viable areas are stained brick red, whereas infarcted areas are gray or white (not detected).

respiration, the active respiration yielding ATP, was decreased by 32% when malate–glutamate was used as complex I substrate, with no changes in state 4 respiration and with the corresponding decrease in respiratory control (Table 1). These effects were not observed when succinate was used as complex II substrate (Table 1), indicating that mild I/R selectively affects complex I activity.

Mitochondrial complexes and mtNOS activity, mtNOS functional activity, and mtNOS expression

The respiratory impairment of the left ventricle was further investigated by assaying the activity of mitochondrial respiratory complexes. Successive decreases with a maximal 28% inhibition after 30 min reperfusion were observed in complex I activity, whereas complex II and IV activities were not modified by ischemia/reperfusion (Table 2). Additionally, the rate of mitochondrial NO production, i.e., mtNOS activity, decreased 28% in the whole I/R sequence (Table 2).

The expression of mtNOS was analyzed by Western blotting using anti-nNOS antibodies. The detected protein level was not modified along the reperfusion process (Fig. 4), indicating the occurrence of protein damage and inactivation but not of protein degradation during the short time (45 min) of the I/R process.

Mitochondrial NOS functional activity was determined through the inhibition of O_2 consumption produced by NO [22,31]. State 3 respiratory rates were determined under two limit conditions of intramitochondrial NO concentration: at maximal and minimal levels. The first condition was achieved by supplementation with L-arginine and the second by addition of L-NMMA. The difference between the O_2 consumption rates under these two conditions is expressed directly as a

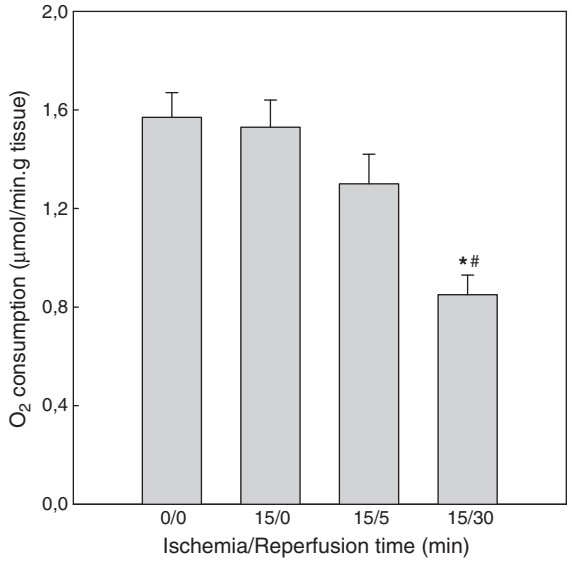

Fig. 3. Tissue O_2 uptake of rabbit left ventricle upon isolated heart ischemia/reperfusion. $^{*}p<0.01$, statistically different compared to 0/0; $^{\#}p<0.01$, statistically different compared to 15/0.

Table 1
Mitochondrial O_2 uptake of rabbit left ventricle upon isolated heart ischemia/reperfusion.

Ischemia/reperfusion (min)	Oxygen consumption (ng-at O/min . mg protein)			
	0/0	15/0	15/5	15/30
Substrate malate–glutamate				
State 4	23 ± 2	22 ± 2	23 ± 2	22 ± 2
State 3	142 ± 5	125 ± 6	115 ± 5*	96 ± 5***,†
Respiratory control	6.2	5.7	5.0	4.4
Substrate succinate				
State 4	67 ± 4	69 ± 5	65 ± 5	68 ± 5
State 3	219 ± 9	215 ± 9	218 ± 9	221 ± 7
Respiratory control	3.3	3.1	3.4	3.3

$^{*}p<0.05$ or $^{***}p<0.001$, statistically different compared to 0/0.
$^{\dagger}p<0.05$, statistically different compared to 15/0.

difference or referred to as a fraction of state 3 O_2 consumption rate. Table 3 shows that the supplementation of state 3 mitochondria with L-arginine decreased the state 3 respiration, whereas the addition of L-NMMA to the reaction medium increased O_2 uptake. Mitochondrial NOS activity gradually decreased along I/R steps and reached a maximal level of inhibition after 30 min reperfusion. Mitochondrial NOS functional activity using malate–glutamate as substrate (51%) was reduced with ischemia (34%) and with 5 (30%) and 30 min (26%) reperfusion. A similar effect was observed using succinate as substrate but the lowest values of the functional activity reached were only 27% lower than the initial values, showing a milder decrease than that observed with malate and glutamate (50%). The determination of mtNOS functional activity confirmed the decrease in mtNOS activity observed in the determination of enzymatic activity.

As shown in Fig. 5, linear correlations were obtained between mitochondrial complex I activity and mtNOS activity ($r^2 = 0.99$) and between mitochondrial complex I activity and mtNOS functional activity ($r^2 = 0.66$), indicating that the pattern observed for the decline in complex I activity was also observed for the reduction in mtNOS biochemical and functional activity. These results are in accordance with the reported physical and functional interaction between complex I proteins and mtNOS [32–34].

Hydrogen peroxide production

Mitochondrial H_2O_2 production was measured using malate–glutamate and succinate as substrates and adding the specific inhibitors rotenone and antimycin to reach maximal H_2O_2 production by complex I and complex III, respectively. The specificity of the impairment at complex I activity was evidenced by the successive increases in H_2O_2 production rates observed using malate–glutamate but not succinate as substrate (Table 4). The rate of H_2O_2 release in state 4 was 78% higher in the last step of the process (15/30) than in control mitochondria (0/0) (Table 4). In addition, ischemia and 30 min reperfusion produced a 46% enhancement in the ratio between the state 4 H_2O_2 production measured in the absence and in the presence of rotenone, suggesting again structural modification of complex I proteins associated with oxidative stress.

Mn-SOD activity and concentration

The activity of Mn-SOD was measured and the Mn-SOD concentration in the mitochondrial matrix was calculated. Table 5 shows that in rabbit heart Mn-SOD activity is 53 ± 5 U/mg protein. This activity corresponds to a Mn-SOD concentration of 7.4 ± 0.7 µM (expressed as homotetrameric enzyme) in the mitochondrial matrix. Mn-SOD concentration was not modified by either ischemia/reperfusion (Table 5) or adenosine supplementation (data not shown), suggesting that the observed enhancement in H_2O_2 production is a consequence of a primary increase in mitochondrial $O_2^{\bullet -}$ generation.

Table 2
Mitochondrial complexes and mtNOS activity of rabbit left ventricle upon isolated heart ischemia/reperfusion.

Ischemia/reperfusion (min)	Complex I (nmol/min . mg protein)	Complex II (nmol/min . mg protein)	Complex IV (min^{-1}/mg protein)	mtNOS (nmol/min . mg protein)
0/0	577 ± 25	128 ± 10	49.9 ± 2.6	0.90 ± 0.05
15/0	541 ± 24	119 ± 10	48.8 ± 2.0	0.86 ± 0.06
15/5	515 ± 22	117 ± 9	52.0 ± 3.1	0.80 ± 0.05
15/30	413 ± 22***,†	120 ± 10	50.2 ± 2.5	0.65 ± 0.05**

**$p<0.01$ or ***$p<0.001$, statistically different compared to 0/0.
†$p<0.05$, statistically different compared to 15/0.

Phospholipid and protein oxidation and tyrosine nitration

Mitochondrial proteins and phospholipids were affected by ischemia/reperfusion as evidenced by the moderate increase in the content of protein carbonyls (Fig. 6), phospholipid oxidation products (Fig. 6) and nitrated proteins (Figs. 7A and C), in a gradual process wherein 30 min reperfusion was associated with increases of 17% (protein carbonyls), 42% (phospholipid oxidation products, TBARS) (Fig. 6), and 70% (protein nitration) (Figs. 7A and C).

Effect of adenosine on left-ventricle tissue O_2 consumption, complex I and mtNOS activities, phospholipid oxidation, and protein nitration

Adenosine supplementation protected rabbit left ventricle from the described respiratory dysfunction. The effect of adenosine on mitochondrial dysfunction was analyzed considering tissue O_2 uptake, complex I and mtNOS activities, phospholipid oxidation, and protein nitration. The protection conferred by adenosine was expressed as a percentage of control values (0/0) reached after 30 min reperfusion and resulted in 97, 94, 85, and 100% for the parameters listed in Table 6. In addition, the supplementation of the perfusion medium with adenosine reduced by 80% the protein nitration detected as nitrotyrosines that was observed after 15 min ischemia and 30 min reperfusion (Figs. 7B and C).

Discussion

The results presented here provide evidence that a state of mitochondrial dysfunction is produced in rabbit heart in a mild condition of I/R that simulates the condition of human stunned myocardium. The mitochondrial impairment develops gradually along the steps of ischemia, early reperfusion, and late reperfusion, with changes that can be described as almost no change, slight change, and moderate change in each phase [8]. It has been reported by others [38], and also shown in this work, that in myocardial stunning the infarct area is not significant (Fig. 2) and the functional injury is completely reversible (Fig. 1). The mitochondrial dysfunction is properly described as "complex I syndrome" with decreased tissue O_2 uptake, decreased malate–glutamate-supported mitochondrial respiration, reduced complex I (NADH-dehydrogenase) activity, increased phospholipid and protein oxidation and protein nitration products, and increased $O_2^{\bullet-}$ and H_2O_2 production rates [39]. Interestingly, high doses of vitamin E were able to restore to normal the age-dependent complex I syndrome in hippocampus and brain cortex [40]. In terms of cardiomyocyte contraction and energetics, complex I syndrome implies a significant decrease in the cellular capacity to generate enough biochemical energy for keeping ionic composition for myocardial contraction. This reversible condition establishes a period in which energy production is a limiting step for cardiomyocyte homeostasis and function. Previous reports identified complex I as a major site of mitochondrial damage in I/R [41,42]. In this work, consecutive decreases with a maximal 28% inhibition after 30 min reperfusion were observed in complex I activity, whereas complex II and complex IV activities were not modified. The reduction observed in complex I activity was associated with a reduction in mtNOS activity (Table 2) and mtNOS functional activity (Table 3) in accordance with the reported physical and functional interaction between complex I proteins and mtNOS [34]. Several reports show that complexes I, III, and IV interact to form supercomplexes with a defined stoichiometry in mammalian mitochondria [43–46]. Schäfer et al. [47] reported the stoichiometry of I_1III_2 and $I_1III_2IV_1$ supercomplexes based on biochemical data from bovine heart mitochondria and suggested that the supercomplex $I_1III_2IV_1$ is a major physiological component of the respiratory chain in mammalian mitochondria. In addition, Persichini et al. [48] reported a physical interaction of mtNOS with the C-terminal peptide of the Va subunit of cytochrome oxidase, using electron microscopic immunolocalization and coimmunoprecipitation studies. The proximity between mtNOS and cytochrome oxidase allows a tight functional coupling between the mitochondrial NO production and the oxygen consumption by complex IV, leading to a fine-tuning of cytochrome oxidase activity by NO. Poderoso's group [34] showed that not only complex IV but also complex I proteins immunoprecipitate with intramitochondrial and translocated nNOS, which indicates a direct molecular interaction between mtNOS and

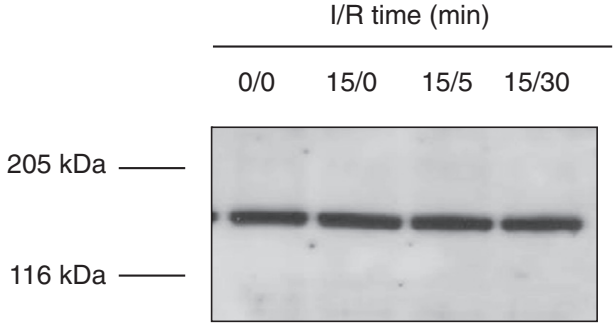

Fig. 4. Western blot analysis of left-ventricle mitochondrial membranes isolated from rabbit heart subjected to ischemia/reperfusion, using anti-nNOS antibodies (Santa Cruz Biotechnology; H299).

Table 3
Mitochondrial nitric oxide synthase functional activity, as inhibition of oxygen uptake, of rabbit left ventricle upon isolated heart ischemia/reperfusion.

Ischemia/reperfusion (min)	Oxygen consumption (ng-at O/min . mg protein)			
	0/0	15/0	15/5	15/30
Substrate malate–glutamate				
State 3	142 ± 5	125 ± 6	115 ± 5	96 ± 5
+ L-Arginine (a)	76 ± 6	81 ± 5	87 ± 6	92 ± 4
+ L-NMMA (b)	149 ± 10	123 ± 8	121 ± 8	117 ± 8
$b-a$ (ng-at O/min . mg protein)	73 ± 12	42 ± 9	34 ± 10	25 ± 9*
mtNOS functional activity (%)	51	34	30	26
Substrate succinate				
State 3	219 ± 9	215 ± 9	218 ± 9	221 ± 7
+ L-Arginine (a)	184 ± 10	189 ± 10	193 ± 10	198 ± 9
+ L-NMMA (b)	273 ± 11	268 ± 10	258 ± 10	264 ± 10
$b-a$ (ng-at O/min . mg protein)	89 ± 15	79 ± 14	65 ± 14	66 ± 13
mtNOS functional activity (%)	41	37	30	30

mtNOS functional activity was calculated as $[(b-a)/\text{state 3}]\times 100$.
*$p<0.05$, statistically different compared to 0/0.

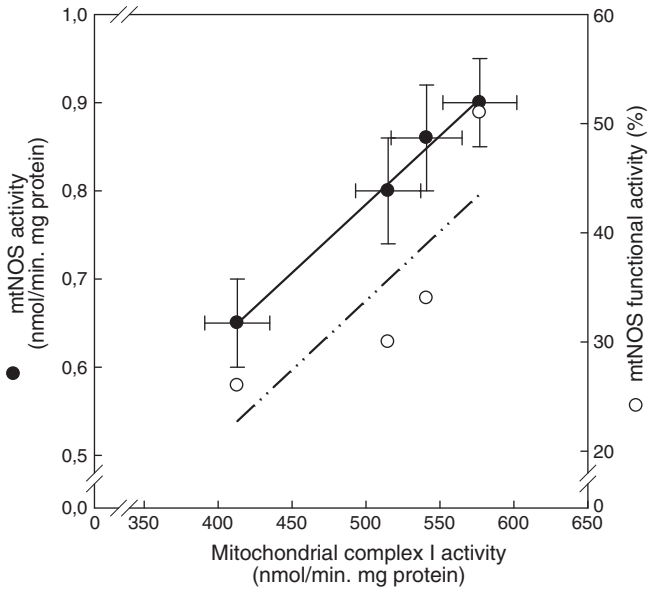

Fig. 5. Linear correlations between mitochondrial complex I activity and mtNOS activity ($r^2 = 0.99$) and between mitochondrial complex I activity and mtNOS functional activity ($r^2 = 0.66$). Data were taken from Tables 2 and 3.

complexes I and IV [44,46,49]. In addition, the obtained results are in agreement with the recent observations by Parihar et al. [32,33] and by Navarro et al. [50], who proposed that mtNOS is structurally adjacent to complex I.

The mtNOS and complex I functional association in brain has been linked to the development of neurodegenerative diseases [50]. The so-called complex I syndrome was classically observed in brain mitochondria in Parkinson disease and in other neuropathological situations [51–53]. Interestingly, the mitochondrial dysfunction observed in the present model of rabbit myocardial stunning is in agreement with the complex I syndrome described by Boveris, Carreras, and Poderoso [39]. The lack of the last electron acceptor, molecular oxygen, during ischemia leads to a high reduction state of the components of the mitochondrial respiratory chain. Upon reoxygenation, a burst of $O_2^{\bullet-}$ and H_2O_2 production occurs owing to the increased autoxidation rate of the most important intramitochondrial sources of $O_2^{\bullet-}$ ($UQH^{\bullet}$ and $FMNH^{\bullet}$), as was observed in early reperfusion in rat liver by in situ organ chemiluminescence [8].

Ischemia/reperfusion is associated with mitochondrial protein oxidation and nitration and with phospholipid oxidation. As has been earlier proposed [54], these changes in complex I proteins are certainly an explanation for the observed increase in H_2O_2 production and they are consistent with the observation that the lack of O_2 deactivates mitochondrial complex I [55].

Table 4
Mitochondrial production of hydrogen peroxide in rabbit left ventricle upon isolated heart ischemia/reperfusion.

Ischemia/ reperfusion (min)	H_2O_2 production (nmol/min . mg protein)			
	0/0	15/0	15/5	15/30
Malate–glutamate				
State 4	0.18 ± 0.02	0.20 ± 0.02	0.26 ± 0.04	0.32 ± 0.04*
+ Rotenone	0.39 ± 0.03	0.44 ± 0.03	0.46 ± 0.04	0.48 ± 0.05
State 4/rotenone	0.46	0.45	0.56	0.67
Succinate				
State 4	0.50 ± 0.04	0.52 ± 0.04	0.51 ± 0.05	0.53 ± 0.05
+ Antimycin	0.60 ± 0.05	0.62 ± 0.05	0.64 ± 0.06	0.65 ± 0.06
State 4/antimycin	0.83	0.84	0.80	0.82

*$p < 0.05$, statistically different compared to 0/0.

Table 5
Mn-superoxide dismutase activity and concentration in rabbit left-ventricle mitochondria upon isolated heart ischemia/reperfusion.

Ischemia/ reperfusion (min)	Mn-SOD		
	Activity (U_{SOD}/mg protein)	Concentration (μM)	
		Active centers	Enzyme
0/0	53 ± 5	30 ± 3	7.4 ± 0.7
15/0	51 ± 6	31 ± 4	7.9 ± 0.9
15/5	55 ± 7	33 ± 6	8.1 ± 1.2
15/30	56 ± 3	32 ± 3	7.9 ± 0.4

The concentration of Mn-SOD active centers was calculated taking into account the value of Mn-SOD activity, the amount of commercial SOD that inhibits 50% ferricytochrome *c* reduction by each SOD unit (1 U SOD corresponds to 4 pmol SOD), the sample protein concentration (0.8–1.0 mg mitochondrial protein/ml), and a volume of 7 μl mitochondrial matrix/mg protein [35]. The concentration of Mn-SOD (μM enzyme) in the mitochondrial matrix was calculated as (μM active center)/4, because mammalian Mn-SOD is a homotetramer with a manganese ion per subunit [36,37].

The molecular mechanism responsible for complex I syndrome is probably accounted for by a sum of processes and reactions that lead synergistically to complex I inactivation. The involved processes and reactions are, in the first place, the lipid peroxidation process and the reactions of the derived reactive free radical intermediates (mainly $ROO^{\bullet}$) and of the aldehydes produced in such process (4-HO-nonenal and malonaldehyde) with allylic carbons and with amino groups, respectively, of the polypeptide chain of the complex I proteins. In the second place, formation of $ONOO^-$ by the intramitochondrial reaction of NO and $O_2^{\bullet-}$ at the vicinity of NADH-dehydrogenase active center [56] provides another pathway leading to complex I nitration and inactivation. Interestingly, complex I inactivation is accompanied by increased autoxidation and $O_2^{\bullet-}$ production rate and subsequently an increased generation of H_2O_2 [40,54]. It is understood that the reactions that inactivate complex I, mediated by free radicals ($ROO^{\bullet}$), aldehydes, and $ONOO^-$, change the weak native noncovalent intermolecular bonding forces and synergistically promote covalent cross linking with protein inactivation [57,58]. It has been known for a long time that complex I is highly sensitive to steroids and detergents [59], a fact that is now interpreted as the impairment of the noncovalent bonding that holds complex I proteins together.

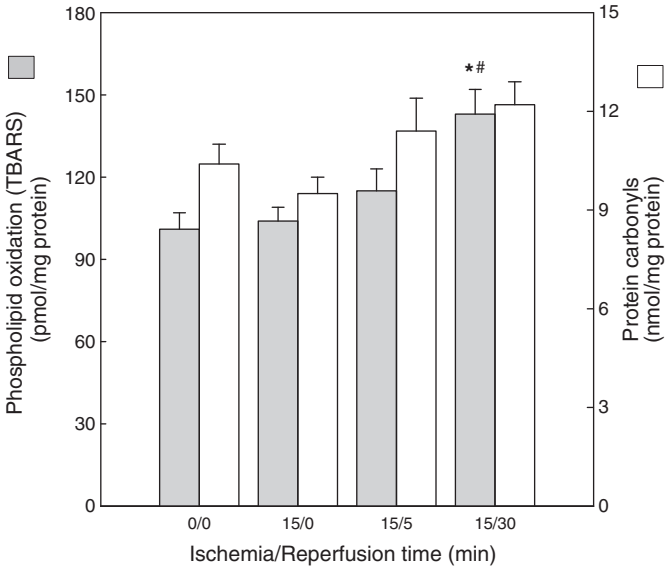

Fig. 6. Protein carbonyls and phospholipid oxidation products in rabbit left-ventricle mitochondria upon isolated heart ischemia/reperfusion. *$p < 0.05$, statistically different compared to 0/0; #$p < 0.05$, statistically different compared to 15/0.

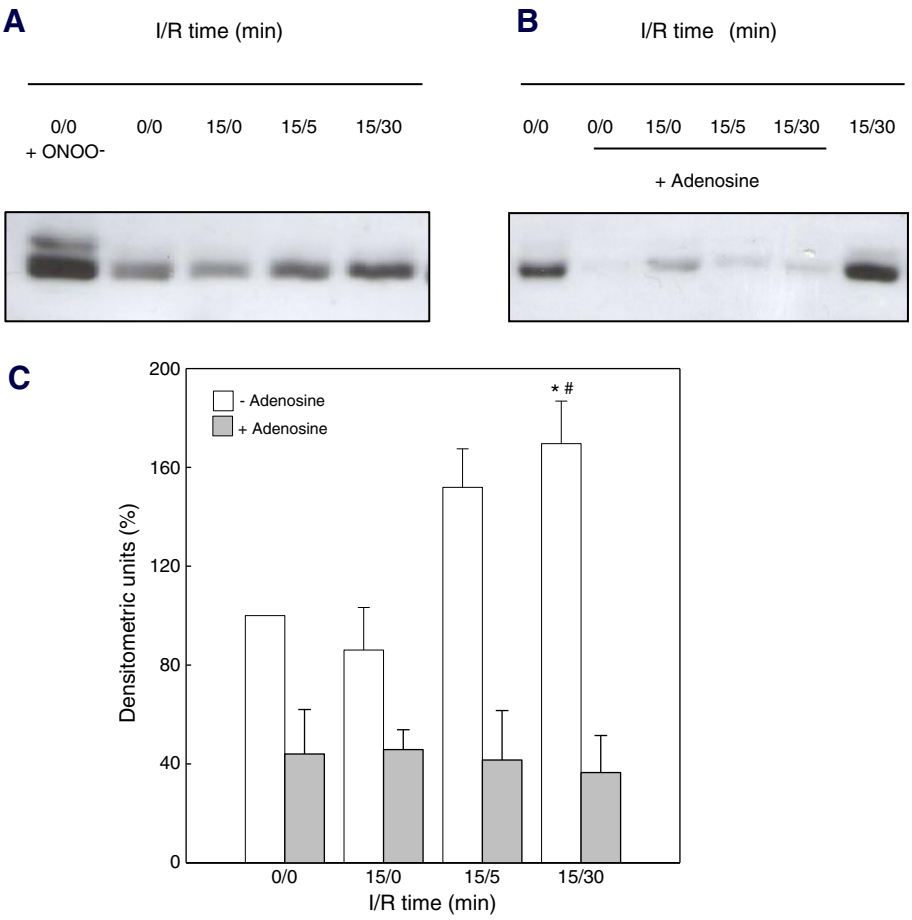

Fig. 7. Western blot analysis of left-ventricle mitochondrial membranes isolated from rabbit heart subjected to ischemia/reperfusion, using anti-nitrotyrosine antibodies (Millipore; clone 1A6). (A) Representative gel showing nitrotyrosine levels in mitochondrial membranes of hearts perfused in the absence of adenosine. Positive control (left lane) was performed by adding 100 μM $ONOO^-$ to mitochondrial membranes from 0/0 hearts. (B) Effect of adenosine on protein nitration of mitochondrial membranes isolated from rabbit left ventricle upon isolated heart ischemia/reperfusion. (C) Densitometric units ($n=4$) were expressed as a percentage of 0/0 and plotted all together in the same graph. $^*p<0.05$, statistically different compared to 0/0; $^\#p<0.05$, statistically different compared to 15/0.

The Mn-SOD activity determined in rabbit heart mitochondria (53 ± 5 U/mg protein) agrees with the value reported by Ferrari and co-workers (40.6 ± 3.2 U/mg protein) [60]. Because Mn-SOD activity is not modified by I/R, the observed increase in H_2O_2 generation (78%) by complex I implies a markedly enhanced production of $O_2^{\bullet-}$ that explains an increased level of $ONOO^-$ even with a decreased NO generation by mtNOS. Moreover, alternative NOS-independent mechanisms of NO synthesis may operate in situations in which conventional NO production is impaired [61,62].

Concerning mtNOS regulation, it is currently known that this enzyme, like other metabolically relevant enzymes, is subjected to multiple regulations. The activity of this enzyme is regulated by substrate availability (NADPH, L-arginine, and oxygen) [63], by Ca^{2+} levels [64], by phosphorylation [65], and by inner mitochondrial membrane potential [66,67]. Considering that mtNOS activity determination is performed in mitochondrial membranes (and not in coupled mitochondria) in the presence of an excess of substrates and Ca^{2+}, the observed decrease in mtNOS activity after reperfusion cannot be associated with changes in membrane potential or in calcium concentration. The decrease in mtNOS biochemical activity is attributed to an inactivation by nitration.

It has been suggested that a transient reversible inhibition of the mitochondrial electron transfer minimizes I/R injury and that blockade of electron transport at complex I preserves respiration during reperfusion [68]. Studies have shown that complex I inhibition by nitrosation protects mitochondria during hypoxia and reoxygenation

Table 6
Effect of adenosine on tissue O_2 consumption, complex I and mtNOS activities, and phospholipid oxidation products (TBARS) in rabbit left ventricle upon isolated heart ischemia/reperfusion.

Ischemia/reperfusion	Tissue O_2 uptake (μmol O_2/min . g heart)	Complex I (nmol/min . g heart)	mtNOS (nmol/min . g heart)	Phospholipid oxidation (pmol/mg protein)
Control (0/0)	1.57 ± 0.10	577 ± 25	0.90 ± 0.05	101 ± 6
Adenosine				
0/0	1.56 ± 0.08	559 ± 22	0.85 ± 0.10	104 ± 6
15/30	1.52 ± 0.09	541 ± 28	0.77 ± 0.09	108 ± 8
Adenosine protection (%)[a]	97%	94%	85%	100%

[a] Adenosine protection was calculated as the ratio of the values observed for adenosine-treated rabbit hearts (15/30) to the values observed in control animals (0/0) and it is expressed as a percentage.

and protects cardiomyocytes during ischemia/reperfusion [69,70]. There is evidence that S-nitrosation of complex I proteins is associated with cytoprotection from the damage caused by I/R [71,72].

Adenosine has been recognized as protective in I/R. Supplementation of the perfusion solution with adenosine reduces the size of infarction [73] and improves ventricular function after reperfusion, attenuating myocardial stunning [74]. The previous evidence adds to the results reported here, in that adenosine protected from the decline in tissue O_2 consumption, the impairment of complex I and mtNOS activities, and the enhancement of mitochondrial phospholipid oxidation and tyrosine nitration. The ameliorating effect of adenosine on myocardial contractility upon reperfusion occurs immediately (10 min; Fig. 1), whereas the onset of complex I syndrome proceeds gradually along the reperfusion phase (30 min; Fig. 3 and Tables 1–4). Thus, the adenosine protection on heart contractility is not to be attributed to the preservation of mitochondrial function and ATP levels [74,75]. On the other hand, it is well known that Ca^{2+} overload is responsible, at least in part, for myocardial stunning [76]. Thus, adenosine may attenuate myocardial stunning by inhibiting Ca^{2+} influx through stimulation of A_1 receptors [74,77]. According to Venardos and co-workers [78], the improvement in the contractile response by adenosine treatment is associated with a decrease in nitrotyrosine formation simultaneous with increases in Akt and troponin I phosphorylation.

In summary, the ventricular dysfunction observed in myocardial stunning after I/R is associated with a mitochondrial dysfunction that shows a complex I syndrome with a partial inactivation of mtNOS and complex I activities and oxidative and/or nitrosative damage and with an increased H_2O_2 production rate and $ONOO^-$ formation. Moreover, adenosine proved to be effective in attenuating ventricular dysfunction with reduction of myocardium stunning and protection from the complex I syndrome distinguished under reperfusion.

Acknowledgments

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