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Research Article

Effects of myocardial ischemia and reperfusion on mitochondrial function and susceptibility to oxidative stress

P. Venditti, P. Masullo and S. Di Meo*

Department of General and Environmental Physiology, University of Naples 'Federico II', Via Mezzocannone 8, 80134 Napoli (Italy), Fax: + 39 081 2535090, e-mail: dimeo@biol.dgbm.unina.it

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Abstract. We investigated the effects of ischemia duration on the functional response of mitochondria to reperfusion and its relationship with changes in mitochondrial susceptibility to oxidative stress. Mitochondria were isolated from hearts perfused by the Langendorff technique immediately after different periods of global ischemia or reperfusion following such ischemia periods. Rates of O_2 consumption and H_2O_2 release with complex I- and complex II-linked substrates, lipid peroxidation, overall antioxidant capacity, capacity to remove H_2O_2, and susceptibility to oxidative stress were determined. The effects of ischemia on some parameters were time dependent so that the changes were greater after 45 than after 20 min of ischemia, or were significantly different to the nonischemic control only after 45 min of ischemia. Thus, succinate-supported state 3 respiration exhibited a significant decrease after 20 min of ischemia and a greater decrease after 45 min, while pyruvate malate-supported respiration showed a significant decrease only after 45 min of ischemia, indicating an ischemia-induced early inhibition of complex II and a late inhibition of complex I. Furthermore, both succinate and pyruvate malate-supported H_2O_2 release showed significant increases only after 45 min of ischemia. Similarly, whole antioxidant capacity significantly increased and susceptibility to oxidants significantly decreased after 45 min of ischemia. Such changes were likely due to the accumulation of reducing equivalents, which are able to remove peroxides and maintain thiols in a reduced state. This condition, which protects mitochondria against oxidants, increases mitochondrial production of oxyradicals and oxidative damage during reperfusion. This could explain the smaller functional recovery of the tissue and the further decline of the mitochondrial function after reperfusion following the longer period of oxygen deprivation.

Key words. Ischemia-reperfusion; oxidative stress; hydrogen peroxide release; antioxidant capacity; mitochondrial function.

Although restoration of blood flow is necessary to salvage ischemic tissues, oxidative damage may occur during reoxygenation and contributes to ischemia-reperfusion injury. Intracellular calcium overload, contracture, and cellular necrosis, instead of the expected improvement in cardiac function, are found after reperfusion of ischemic hearts [1]. To date, mechanisms of cellular and subcellular derangement and its contribution to myocardial ischemia-reperfusion injury have not been well established. Mitochondria are a likely site of ischemia-reperfusion damage, because loss of mitochondrial function inevitably leads to cell death, whereas optimal energy metabolism is required to preserve cell viability. In fact, mitochondrial structure and function are altered by prolonged periods of ischemia. Functional changes include a decreased rate of state 3 (ADP-stimulated) respiration [2] associated with normal or slightly reduced ca-

* Corresponding author.

pacity for ATP production [3, 4]. Reperfusion has been reported to cause an extension of mitochondrial damage [5, 6], no change [7, 8], or an increase in the oxidative phosphorylation rate [9, 10]. The discrepancies in findings are attributable to differences in the age of the animals and in the protocols, in particular the duration of ischemia. Cardiac mitochondria exhibit an age-related increase in susceptibility to reperfusion-induced dysfunction [11]. Furthermore, ischemia reperfusion-induced increases in oxidative phosphorylation rate have been found especially after short periods of ischemia [9, 10].
The mechanisms underlying the functional response of mitochondria to ischemia-reperfusion are not yet known, but the involvement of reactive oxygen species (ROS), which are able to elicit specific damage to respiratory chain components [12], is likely. ROS production increases in mitochondria harvested from ischemic hearts [13] and signs of oxidative damage are present in mitochondria exhibiting reperfusion-induced functional decline [11]. Therefore, long-lasting ischemia may produce biochemical changes that predispose the mitochondria to free radical-mediated injury.
The aim of the present study was to assess the effects of ischemia duration on the functional response of mitochondria to reperfusion and to determine whether greater alteration of mitochondrial characteristics determining susceptibility to oxidative stress occurs as a result of long-lasting ischemia.

Materials and methods

Materials

All chemicals used were of the highest grade available, purchased from Sigma (Milan, Italy). Antioxidant capacity and response to oxidative stress were determined by reagents and instrumentation of the commercially available Amerlite System (Johnson & Johnson, Cinisello Balsamo, Italy).

Animals

Male Wistar rats (60 days old) were used in the experiments. The animals, purchased at weaning from Nossan (Correzzana, Italy) were housed in separate cages at 24 ± 1 °C, with an artificial lighting cycle (lights on 0800–2000 hours). All animals were provided with water ad libitum and a commercial chow diet (Nossan). The rats were maintained in compliance with the ‘Principles of Laboratory Animal Care,’ published by the National Institutes of Health (publication No. 85–23, revised 1985) and with current Italian laws.

Isolated heart preparation and perfusion protocol

The rats were anesthetized by intraperitoneal injection of chloral hydrate (40 mg/100 g body weight) combined with ether and subjected to electrocardiographic recording. After heparinization, a rapid thoracotomy was performed and the aorta cannulated retrogradely. The hearts were excised and to remove blood they were flushed for 1 min with Krebs-Henseilet (KH) buffer containing (mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, EDTA 0.5, glucose 11, pH 7.4, and gassed with 5% CO_2 in O_2. The hearts, paced at a fixed rate of 240 beats/min using a square-wave stimulator, were perfused with KH at 37 °C under 70 mm Hg pressure according to Langendorff [14] for a 20-min equilibration period before any experiment. Then the hearts were subjected to: no ischemia (I_0); 20 min ischemia (I_{20}); 45 min ischemia (I_{45}); no ischemia and 25 min normoxic perfusion (I_0-R); 20 min ischemia and 25 min reperfusion (I_{20}-R); 45 min ischemia and 25 min reperfusion (I_{45}-R). Ischemia was induced by global normothermic flow interruption. I_0 hearts, perfused only for the equilibration period, and I_0-R hearts, perfused for an additional 25 min period were used as controls for ischemic (I_{20} and I_{45}) and reperfused (I_{20}-R and I_{45}-R) hearts, respectively.
Functional performance was determined at the end of the equilibration and perfusion periods as previously described [15].

Preparation of mitochondrial fraction

At the end of the experiments, large heart vessels, valves, and atria were trimmed away and the ventricles were cut open, rinsed free of liquid, and weighed. The ventricles were placed into isolation buffer (IB) (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 20 mM Tris, pH 7.4) containing 0.1 mg/ml nagarse and 0.1% fatty acid-free albumin. Then the ventricles were finely minced, washed with IB, and homogenized in the same solution (10% w/v) using a glass Potter-Elvehjem homogenizer set at a standard velocity for 1 min. The homogenates were freed from debris and nuclei by centrifugation at 500 g for 10 min at 4 °C and the resulting supernatants were centrifuged at 3000 g for 10 min. The mitochondrial pellets were resuspended in IB and centrifuged at the same sedimentation velocity. Mitochondrial preparations were washed in this manner twice before final suspension in IB.
Basically, mitochondria were prepared by the procedure described by Tyler and Gonze [16], but the mitochondrial pellet was obtained at lower speed (3000 instead of 8000 g) to reduce both the contamination by cytoplasmic and microsomal material and the amount of damaged mitochondria.
Mitochondrial protein content was determined, upon solubilization in 0.5% deoxycholate, by the biuret method [17] with bovine serum albumin as standard.

Mitochondrial oxygen consumption

Mitochondrial respiration was monitored at 30 °C by a Gilson glass respirometer equipped with a Clark oxygen

electrode (Yellow Springs Instrument, Yellow Springs, Ohio) in 1.6 ml of incubation medium (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4) with 0.5 mg of mitochondrial protein per milliliter. Succinate (10 mM) (plus rotenone 5 µM) or pyruvate/malate (10/2.5 mM) were used as substrates, in the absence (state 4) and in the presence (state 3) of 500 µM ADP.

Mitochondrial H_2O_2 release

The rate of mitochondrial H_2O_2 release was measured at 30 °C following the linear increase in fluorescence (excitation at 320 nm, emission at 400 nm) due to oxidation of p-hydroxyphenylacetate by H_2O_2 in the presence of horseradish peroxidase [18] in a computer-controlled Jasko fluorometer. The reaction mixture consisted of 0.1 mg/ml mitochondrial proteins, 6 U/ml horseradish peroxidase, 200 µg/ml p-hydroxyphenyl-acetate, and 10 mM succinate (plus rotenone 5 µM) or 10 mM pyruvate/2.5 mM malate added at the end to start the reaction in the same incubation buffer used for oxygen consumption measurements. Measurements with the different substrates in the presence of 10 µM antimycin A were also performed. Known concentrations of H_2O_2 were used to establish the standard concentration curve.

ROS generation is generally accepted to rise with the concentration and the reduction degree of autoxidisable respiratory carriers, occurring at complex I and complex III [19]. In the presence of antimycin A, both autoxidisable electron carriers become completely reduced and their concentration is the only factor affecting ROS production rate.

Lipid peroxidation, response to oxidative stress, and whole antioxidant capacity

The extent of peroxidative reactions was determined by measuring hydroperoxides (HPs) according to Heath and Tappel [20].

Response to oxidative stress was determined as previously described [21]. Briefly, several dilutions of the mitochondrial suspensions in a protein concentration range from 20 to 0.005 mg/ml were prepared with 15 mM Tris (pH 8.5). The assays were performed in microtiter plates. Enhanced chemiluminescence reactions were initiated by adding 250 µl of the reaction mixture to 25 µl of the samples. The reaction mixture was obtained by dissolving a tablet containing substrate in excess (sodium perborate) and signal-generating reagents (sodium benzoate, indophenol, and luminol) (Amerlite Signal Reagent Tablets) in buffer at pH 8.6 (Amerlite Signal Reagent Buffer). The plates were incubated at 37 °C for 30 s under continuous shaking and then transferred to a luminescence analyzer (Amerlite Analyzer). The emission values were fitted to dose-response curves using the statistical facilities of the Fig.P graphic program (Biosoft, Cambridge, UK).

Determination of the overall antioxidant capacity (CA) was performed according to Di Meo et al. [22]. Briefly, 250 µl of the above reaction mixture was added to 10 µl of 110 ng/ml peroxidase plus 15 µl of either desferrioxamine, at concentrations ranging from 0.01 to 3 mM, in 15 mM Tris (pH 8.5), or buffer alone. Equal volumes of reaction mixture were also added to both 10 µl of 110 ng/ml peroxidase plus 15 µl of mitochondrial samples (5 mg of protein per milliliter) (samples) and 10 µl of 15 mM Tris (pH 8.5) plus 15 µl of the same mitochondrial samples (blanks). The emission values obtained from the mixture of peroxidase and desferrioxamine were reported against the desferrioxamine concentration on logarithmic coordinates supplying a standard curve. The differences between the emission values obtained from the samples and those obtained from the relative blanks were referred to those of the standard curve, allowing the mitochondrial antioxidant capacity to be expressed as equivalent desferrioxamine concentration.

Capacity to remove H_2O_2

The capacity to remove H_2O_2 (CR) was determined by comparing the ability of mitochondrial samples to reduce H_2O_2-linked fluorescent emission with that of desferrioxamine solutions [23]. H_2O_2 was generated by glucose oxidation catalyzed by glucose oxidase (GOX). The non-fluorescent substrate p-hydroxyphenylacetate (PHPA) was oxidized to the stable fluorescent product 2,2′-dihydroxy-biphenyl-5,5′-diacetate $(PHPA)_2$ [18] by the enzymatic reduction of H_2O_2 catalyzed by horseradish peroxidase (HRP).

The fluorescence was monitored on a Jasko fluorometer (excitation wavelength 320 nm, emission wavelength 400 nm), equipped with a thermostatically controlled cell-holder and interfaced with an IBM-compatible PC. Assays were performed in quartz fluorometer cuvettes containing a magnetic stirrer and maintained at 30 °C. The reaction was started by adding 10 µl of 80 µg/ml GOX to a mixture containing 0.2 mg/ml PHPA, 6 U/ml HRP, 5 mM glucose in 145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4. After 100 s, 10 µl of desferrioxamine solution (containing between 1 and 12 nmol), or mitochondrial samples (containing from 0.1 to 1 mg of mitochondrial proteins) was added to 2.0 ml final volume. The additions were made to the cuvettes via externally mounted syringes. The values of fluorescence change for unit of time obtained after addition of desferrioxamine or mitochondria were converted to relative percentage of the values obtained before the addition. The values for desferrioxamine were used to fit standard curves by the Fig.P program (Biosoft). The values for samples plotted on standard curves were used to evaluate their capacity to remove H_2O_2, expressed as equivalent desferrioxamine concentration.

Statistical analysis

The data obtained from eight different experiments are expressed as mean values ± SE. Data were analyzed with two-way analysis of variance (ANOVA). When a significant F ratio was found, the Student-Newman-Keuls multiple-range test was used to determine the statistical significance of differences between mean values. The level of significance was chosen as $p < 0.05$. In figure 3, the results of at least six experiments are presented as sample curves.

Results

Functional recovery from ischemia-reperfusion

Functional recovery of the Langendorff preparations after ischemia-reperfusion was evaluated in terms of coronary flow and inotropic parameters such as left ventricular developed pressure (LVDP) and maximal rate of developing left ventricular pressure (dP/dt_{max}) after 5 and 25 min reperfusion following the different periods of ischemia. The duration of ischemia significantly affected the functional recovery during reperfusion (fig. 1). Within 5 min of reperfusion, the values of all parameters were independent of ischemia duration, although they were lower than those seen in nonischemic hearts. However, after 25 min of reperfusion, in the hearts made ischemic for 20 min, the coronary flow and inotropic parameters were no different from preischemic and control values, while in the hearts made ischemic for 45 min, they remained significantly lower.

Oxygen consumption

Using succinate or pyruvate/malate as substrates, the rates of the state 4 oxygen consumption of mitochondria were not dependent on either treatment or time. In contrast, differences were shown in the rates of state 3 oxygen consumption (table 1). In the presence of succinate, the ischemia produced a reduction in this rate only after a period of 45 min. Reperfusion led to a further reduction in the respiration rates which were lower than that in mitochondria from nonreperfused hearts subjected to an equal period of ischemia. Furthermore, the lowest rate was found in mitochondria from hearts reperfused after 45 min of ischemia.

In the presence of pyruvate/malate, a similar trend was shown, but the respiration rate was not significantly different in mitochondria from I_{20}-R and I_{45}-R hearts. With both substrates, changes in the ratio between state 3 and state 4 respiration rates (respiratory control ratio, RCR) were found, reflecting changes in the state 3 respiration rates.

Mitochondrial H_2O_2 release

The rate of mitochondrial H_2O_2 release in the presence of succinate alone (state 4) was not affected by 20 min of ischemia, but was significantly increased by 45 min of ischemia. A similar pattern was found after reperfusion, because this did not significantly modify the rates obtained after the different periods of ischemia (table 2). When pyruvate/malate were used as substrates, the rate of H_2O_2 release was not affected by 20 min of ischemia and

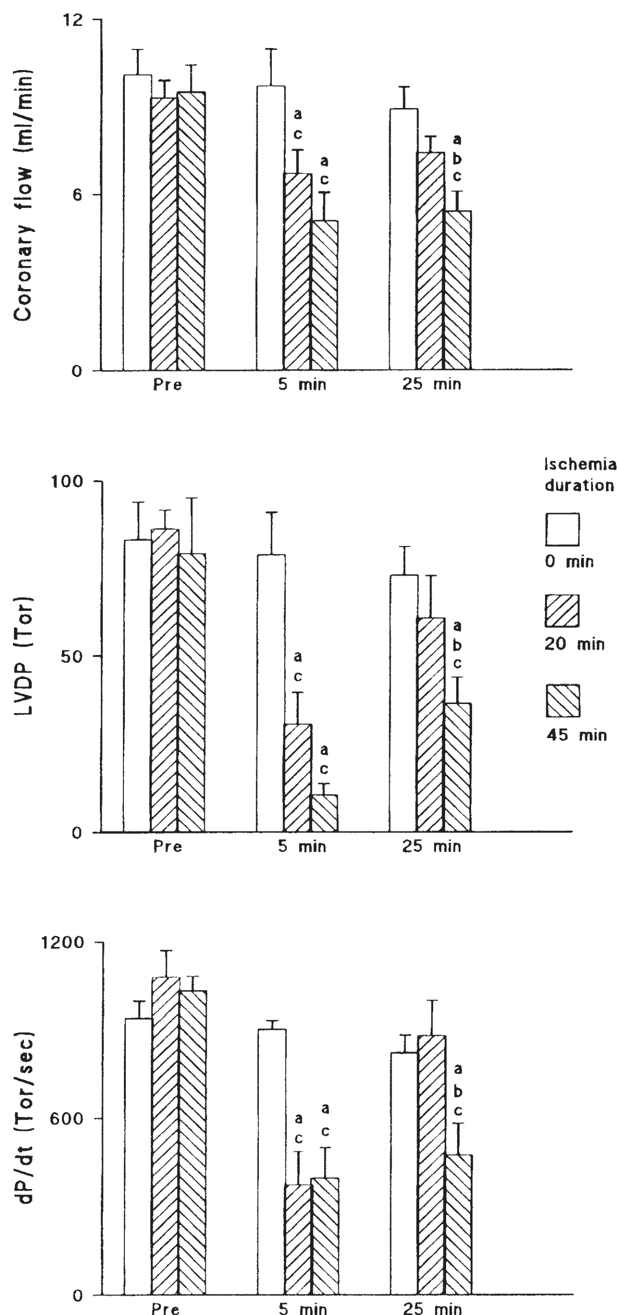

Figure 1. Coronary flow and left ventricular performance of Langendorff preparations. Coronary flow, left ventricular developed pressure (LVDP), and maximal rate of developing left ventricular pressure (dP/dt_{max}) were measured during the preischemic phase and after 5 or 25 min of reperfusion in hearts subjected to periods of ischemia lasting 0, 20 or 45 min. a, significant ($p < 0.05$) vs their respective nonischemic control; b, significant vs hearts made ischemic for less time; c, significant versus preischemic values.

Table 1. Effect of ischemia and reperfusion on oxygen consumption in mitochondria from rat heart.

Group	Oxygen consumption rate (ng atoms O_2/min/per milligram protein)					
	succinate			pyruvate/malate		
	State 4	State 3	RCR	State 4	State 3	RCR
I_0	110 ± 4	336 ±9	3.2 ± 0.1	40 ± 4	162 ± 5	4.1 ± 0.1
I_{20}	104 ± 3	290 ± 6[a]	2.9 ± 0.1	42 ± 4	149 ± 8	3.2 ± 0.2[a]
I_{45}	100 ± 6	222 ± 9[a,b]	2.3 ± 0.1[a,b]	45 ± 3	123 ± 8[a,b]	2.8 ± 0.2[a]
I_0-R	110 ± 3	324 ± 7	3.0 ± 0.1	42 ± 4	164 ± 7	4.0 ± 0.1
I_{20}-R	111 ± 5	240 ± 8[a,c]	2.2 ± 0.1[a,c]	48 ± 4	117 ± 7[a,c]	2.2 ± 0.1[a,c]
I_{45}-R	101 ± 3	186 ± 10[a,b,c]	1.8 ± 0.1[a,b,c]	48 ± 5	97 ± 7[a,c]	1.8 ± 0.1[a,c]

Data represent the mean ± SE of eight different experiments. Hearts subjected to: no ischemia (I_0); 20 min ischemia (I_{20}); 45 min ischemia (I_{45}); no ischemia and 25 min normoxic perfusion (I_0-R); 20 min ischemia and 25 min reperfusion (I_{20}-R); 45 min ischemia and 25 min reperfusion (I_{45}-R). RCR (respiratory control ratio), ratio between state 3 respiration rate and state 4 respiration rate.
[a] Significant vs their respective nonischemic control; [b] significant vs hearts made ischemic for less time; [c] significant vs hearts made ischemic for the same time. Level of significance, $p < 0.05$.

Table 2. Effect of ischemia and reperfusion on H_2O_2 release by mitochondria from rat heart.

Groups	H_2O_2 release (pmoles/min/per milligram protein)			
	succinate	succinate + AA	pyruvate/malate	pyruvate/malate + AA
I_0	106 ± 6	1328 ± 54	135 ± 12	1370 ± 72
I_{20}	110 ± 3	1359 ± 71	149 ± 6	1429 ± 85
I_{45}	141 ± 7[a,b]	1414 ± 47	174 ± 6[a]	1431 ± 65
I_0-R	113 ± 6	1343 ± 49	138 ±11	1314 ± 57
I_{20}-R	128 ± 7	1364 ± 70	168 ± 6[a]	1384 ± 48
I_{45}-R	151 ± 5[a,b]	1526 ± 53	205 ± 6[a,b,c]	1546 ± 30

Data represent the mean ± SE of eight different experiments. AA, antimycin A. Hearts subjected to: no ischemia (I_0); 20 min ischemia (I_{20}); 45 min ischemia (I_{45}); no ischemia and 25 min normoxic perfusion (I_0-R); 20 min ischemia and 25 min reperfusion (I_{20}-R); 45 min ischemia and 25 min reperfusion (I_{45}-R).
[a] Significant vs their respective nonischemic control; [b] significant vs hearts made ischemic for less time; [c] significant vs hearts made ischemic for the same time. Level of significance, $p < 0.05$.

was significantly increased by 45 min of ischemia. However, after reperfusion, mitochondria from hearts subjected to both 20 and 45 min of ischemia exhibited a higher rate of H_2O_2 release compared with their control (I_0-R). Neither ischemia nor reperfusion modified the antimycin-stimulated rates in the presence of either succinate or pyruvate/malate.
The release of H_2O_2 and O_2 consumption were measured in the same buffer, using the same concentrations of substrates and were assayed at the same temperature. This allowed us to calculate the fraction of the O_2 turned into H_2O_2 instead of being reduced to water. Although the statistical analysis showed that the effect of ischemia duration on such a fraction was significant during both succinate- and pyruvate/malate-supported respiration, significant differences between groups were found only when succinate was used as substrate. In particular, after both ischemia and reperfusion, the fraction of O_2 converted into H_2O_2 was higher in mitochondria from hearts made ischemic for 45 min than in those from both control and 20-min ischemic hearts (fig. 2).

Lipid peroxidation, whole antioxidant capacity and capacity to remove H_2O_2

As shown in table 3, the ischemia-reperfusion protocol significantly affected lipid peroxidation. HP levels remained unchanged with ischemia, but increased after reperfusion compared with controls and hearts subjected to the same period of ischemia. Moreover, lipid peroxidation after reperfusion was higher in hearts made ischemic for 45 min.
Whole antioxidant capacities of mitochondria from I_{20} and I_{20}-R hearts were not significantly different from their respective controls. Mitochondrial CA was higher in I_{45} hearts than in I_0 hearts, while it was lower in I_{45}-R hearts than in both I_0-R and I_{45} hearts.
The mitochondria from I_{45}-R hearts were less able to remove H_2O_2.

Response to oxidative stress

The relationship between light emission (E) and protein concentration (C) of mitochondria stressed with sodium perborate was described by the same equation [E =

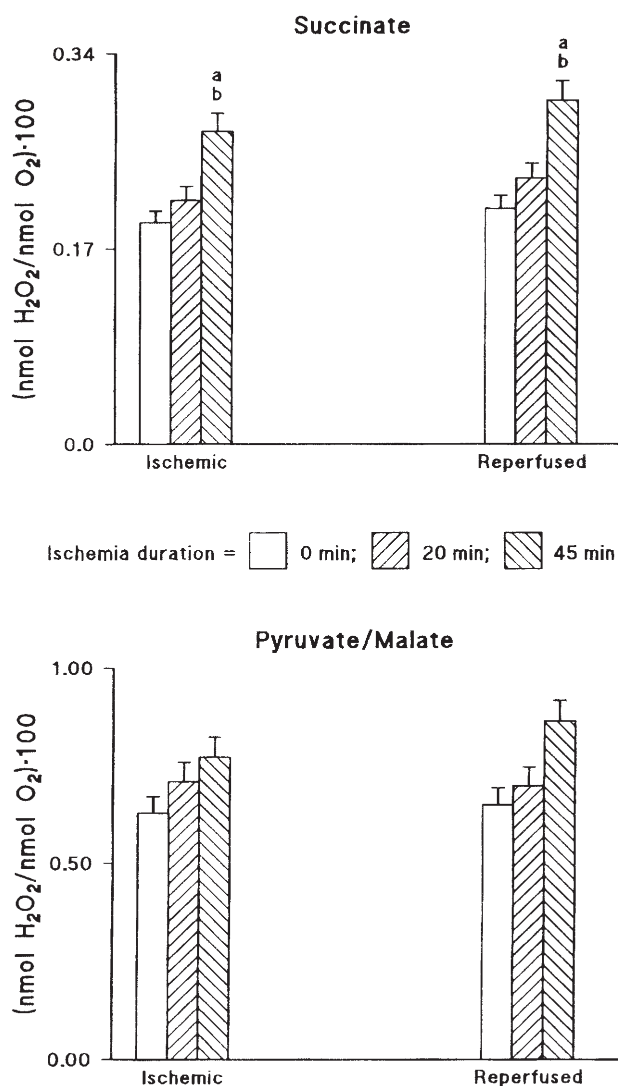

Figure 2. Effect of ischemia and reperfusion on the percentage of total oxygen released as H_2O_2 by heart mitochondria supplemented with complex I- or somplex II-linked substrates during state 4 (ADP-independent) respiration. Values are means ± SE of eight different experiments. Mitochondrial preparations from hearts subjected to: no ischemia, 20 min ischemia, 45 min ischemia (Ischemic) or to the same periods of ischemia and then to 25 min of normoxic perfusion (Reperfused). a, significant ($p < 0.05$) vs their respective not ischemic control; b, significant vs hearts made ischemic for less time; c, significant vs hearts made ischemic for the same time.

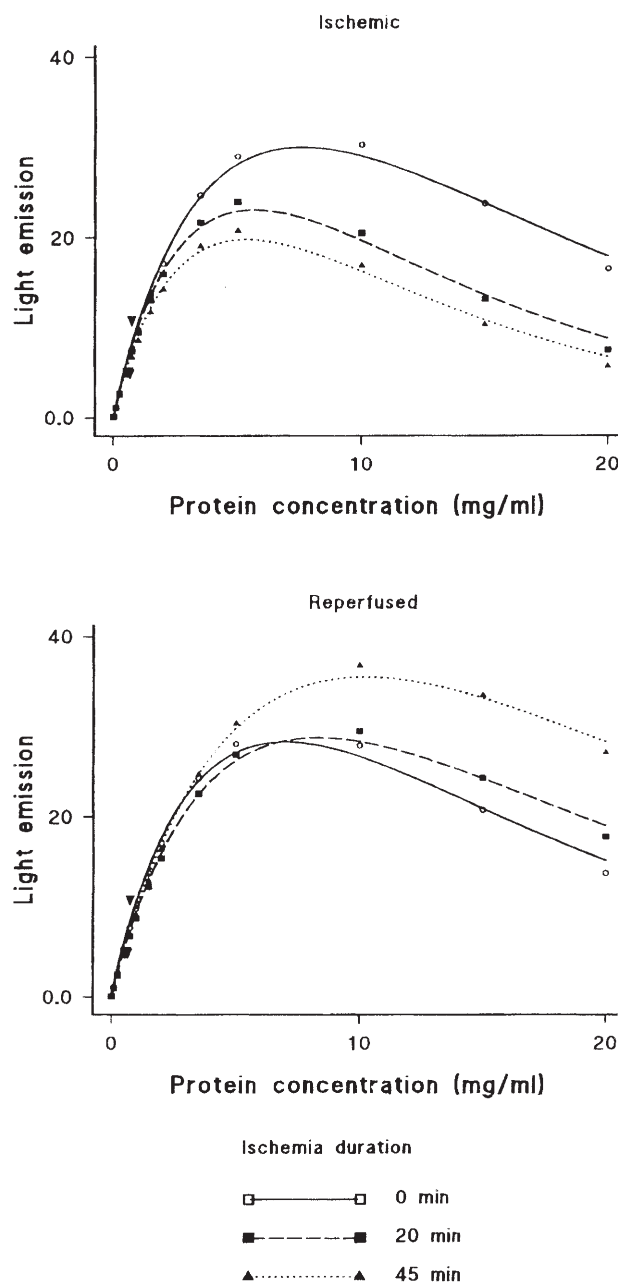

Figure 3. Effect of ischemia and reperfusion on response to oxidative stress in vitro of mitochondria from rat heart. The susceptibility to stress was evaluated by determining the variations with mitochondrial protein concentrations (C) of light emission (E) from a luminescent reaction. Emission values are given as a percentage of an arbitrary standard (22 ng/ml peroxidase). The curves are computed from experimental data using the equation: $E = a \times C/\exp(b \times C)$. Mitochondrial preparations from hearts subjected to: no ischemia, 20 min ischemia, 45 min ischemia (Ischemic) or to the same periods of ischemia and then to 25 min of normoxic perfusion (Reperfused).

$a \times C/\exp(b \times C)$] used for mitochondria isolated from hearts not subjected to the ischemia-reperfusion protocol [22]. In such an equation, the a value depends on the cytochrome content, the b value on the antioxidant level, while the emission maximum ($E_{max} = a/e \times b$) can indicate the susceptibility of the preparations to oxidative challenge. The curves in figure 3 show that the susceptibility to oxidative stress of the I_0 mitochondria is the highest and the ischemia-linked reduction in such a susceptibility depends on the duration of ischemia. Interestingly, the pattern is changed by reperfusion, so that the susceptibility of I_0-R mitochondria is the lowest and that of I_{45}-R mitochondria is the highest. This qualitative evaluation was confirmed by the E_{max} values reported in table 4. Examination of the other data in the table shows that differ-

Table 3. Effect of ischemia and reperfusion on lipid peroxidation, antioxidant capacity, and capacity to remove H_2O_2 of mitochondria from rat heart.

Groups	Parameters		
	HPs	CA	CR
I_0	19.2 ± 1.6	31.3 ± 2.6	2.56 ± 0.10
I_{20}	18,7 ± 0.8	37.9 ± 2.7	2.76 ± 0.26
I_{45}	17.2 ± 1.1	44.7 ± 3.0[a]	2.75 ± 0.14
I_0-R	18.7 ± 1.5	30.9 ± 2.2	2.68 ± 0.12
I_{20}-R	30.4 ± 1.4[a,c]	29.9 ± 2.0	2.43 ± 0.14
I_{45}-R	41.3 ± 1.7[a,b,c]	23.1 ± 1.8[a,c]	2.13 ± 0.11[a,c]

Data represent the mean ± SE of eight different experiments. Hearts subjected to: no ischemia (I_0); 20 min ischemia (I_{20}); 45 min ischemia (I_{45}); no ischemia and 25 min normoxic perfusion (I_0-R); 20 min ischemia and 25 min reperfusion (I_{20}-R); 45 min ischemia and 25 min reperfusion (I_{45}-R). Hydroperoxides (HPs) were measured in nmol NADP/min/per milligram mitochondrial proteins. Whole antioxidant capacity (CA) was expressed as equivalent concentration of desferrioxamine (µmol/l). Capacity to remove H_2O_2 (CR) was expressed as equivalent level of desferrioxamine (nmol/mg mitochondrial proteins).
[a] Significant vs their respective nonischemic control; [b] significant vs hearts made ischemic for less time; [c] significant vs hearts made ischemic for the same time. Level of significance, $p < 0.05$.

Table 4. Effect of ischemia and reperfusion on parameters characterizing susceptibility to oxidants of mitochondria from rat heart.

Groups	Parameters		
	a	b	E_{max}
I_0	11.1 ± 1.1	0.13 ± 0.01	32.0 ± 2.6
I_{20}	11.2 ± 1.0	0.17 ± 0.01[a]	24.2 ± 2.0
I_{45}	10.2 ± 0.9	0.18 ± 0.01[a]	20.8 ± 1.8[a]
I_0-R	11.3 ± 1.0	0.14 ± 0.01	29.1 ± 2.3
I_{20}-R	9.8 ± 0.4	0.12 ± 0.02[b]	28.5 ± 2.2
I_{45}-R	10.0 ± 1.0	0.10 ± 0.01[b]	36.8 ± 2.8[b]

Data represent the mean ± SE of eight different experiments. Hearts subjected to: no ischemia (I_0); 20 min ischemia (I_{20}); 45 min ischemia (I_{45}); no ischemia and 25 min normoxic perfusion (I_0-R); 20 min ischemia and 25 min reperfusion (I_{20}-R); 45 min ischemia and 25 min reperfusion (I_{45}-R).The relationship between light emission (E) and protein concentration (C) of mitochondria is described by the equation: $E = a \times C/\exp(b \times C)$. $E_{max} = a/e \times b$).
[a] Significant vs their respective nonischemic control; [c] significant vs hearts made ischemic for the same time. Level of significance, $p < 0.05$.

ences in the emission curves and in the emission peak are due to different b values, which, at least in part, reflect changes induced by ischemia and reperfusion in antioxidant content in mitochondrial preparations.

Discussion

Our results show that 20 min of myocardial ischemia significantly reduces state 3 mitochondrial oxygen consumption when succinate is used as the substrate. In contrast, 45 min of ischemia reduces this consumption, regardless of whether pyruvate/malate or succinate are used as substrates.

There are controversial results concerning ischemia effects on mitochondrial function of isolated hearts. Jennings et al. [2] showed that mitochondria from ischemic dog hearts are unable to utilize pyruvate and α-ketoglutarate, whereas succinate oxidation is almost unaffected. Subsequent studies on pig [24] and dog [25] hearts supported the observation that complex I is especially sensitive to ischemic injury. Moreover, the finding that mitochondrial ATPase activity decreases during sustained ischemia of pig heart [24] suggested that complex V is an important site of cellular injury in ischemic pig heart. However, an inhibition of complex II-dependent respiration was found after 20 [7] or 25 min [11] of rat heart ischemia and 1 h of rabbit heart ischemia [26]. Furthermore, inhibition of mitochondrial ATPase did not appear to contribute significantly to the decline in mitochondrial respiration rate induced by rat heart ischemia [7, 11]. The differences in the ischemia effects on the different sites of the respiratory chain and the influence of the duration of oxygen deprivation may be attributed to species-linked differences. Therefore, our finding that rat heart ischemia produces an impairment of mitochondrial respiration due to early inhibition of complex II and late inhibition of complex I is not in contrast with previous reports. The causes for the different time course of the inhibition of the respiratory complexes in ischemic heart are not yet known. However, during global ischemia of rat heart, the rate of glutamate/malate oxidation exhibits an early increase followed by a progressive decline owing to the destruction of a component subsequent to the NADH dehydrogenase [27].

Although ischemia-induced alterations in mitochondrial function have long been known [2], the causes for impairment of the respiratory complexes are still unclear. Ca^{2+} overload occurs during myocardium ischemia [28] and mitochondrial Ca^{2+} rises in parallel with cytosolic Ca^{2+} following hypoxia-induced contracture in isolated cardiomyocytes [29]. Because Ca^{2+} mitochondrial accumulation depresses mitochondrial function [30], the above results suggest that failure of mitochondrial function could result from a disturbance of cellular calcium homeostasis.

Signs of increased radical formation were found in ischemic hearts [31, 32]. Because ROS are able to damage respiratory chain components [12], this has suggested another possible mechanism for impairment of the respiratory chain [33]. However, although ROS production can increase in hypoxic heart [34], this seems unlikely to happen during global ischemia. Moreover, evidence of mitochondrial oxidative damage in ischemic heart is poor. An electron spin resonance study indicated increased ROS production in submitochondrial particles prepared from

subsarcolemmal mitochondria from an ischemic region of the myocardium [35]. However, during ischemia there is likely not enough oxygen to enhance the ROS generation by intermyofibrillar mitochondria. This view is supported by the finding that 4-hydroxy-2-nonenal, a product of lipid peroxidation, does not increase in a whole mitochondrial population upon cardiac ischemia [11]. Further support is supplied by our results which show that signs of oxidative stress are lacking in mitochondria from ischemic hearts. The mitochondrial levels of HP after 20 or 45 min of ischemia were no different from controls. Furthermore, gradual increases in whole antioxidant capacities of mitochondria and decreases in their susceptibility to oxidants are associated with an increase in ischemia duration. It is not clear whether changes in antioxidant enzyme activities contribute to the above changes, because opposite changes in glutathione peroxidase and superoxide dismutase mitochondrial activities have been reported after ischemia [36]. In contrast, the increased mitochondrial capacity to oppose oxidative stress is likely due to accumulation of reduced pyridine nucleotides occurring during prolonged ischemia [34]. These may provide a source of reducing equivalents needed to remove peroxides and to maintain thiols, such as glutathione, in a reduced state [34]. The high state of reduction of mitochondrial components may be protective against an oxidative challenge but, at the same time, can lead to detrimental effects on mitochondrial function during reoxygenation. In effect, we are inclined to think that the further functional decline found in mitochondria from reperfused hearts is due to ROS production strictly related to the accumulation of reducing equivalents induced by ischemia in mitochondria. In turn, altered mitochondrial function may be responsible for the irreversible changes that take place in reperfused myocardium after prolonged periods of ischemia.

Growing evidence indicates that mitochondrial production of ROS contributes to the deleterious effects of reperfusion of ischemic myocardium. Oxygen radicals increase in concentration upon reperfusion of ischemic cardiac tissue [37–39]. Although ROS may be produced through the operation of xanthine oxidase which is activated during hypoxia [40, 41] the respiratory chain represents a major source of oxygen radicals during reperfusion of ischemic myocardium [13, 42]. Because mitochondrial ROS generation depends on reduction of mitochondrial components [19], the increased reduction of the respiratory chain associated with ischemia is currently assumed to promote ROS generation upon the resumption of respiration. It has also been proposed that ROS generation is induced by interaction of O_2 with ubisemiquinone, which accumulates in ischemic mitochondria as a result of respiratory chain inhibition [43]. ROS generation ends rapidly as the electron carriers of the respiratory chain are reoxidized, but it is long-lasting in mitochondria that have accumulated large amounts of reducing equivalents. Our finding that, in the absence of changes in CR, H_2O_2 mitochondrial release increases significantly after 45 min of ischemia, whereas no difference is found among antimycin-treated mitochondria, supports this view. Further support is supplied by reperfusion-induced changes in parameters which can be considered indices of oxidative damage. ROS can interact with and damage various cellular components [44], but oxyradicals, such as $^{\bullet}OH$, which are highly reactive and short-lived species, would be expected to cause damage at or near the site of their formation. Thus, mitochondria are a likely site of reperfusion-induced oxidative damage, the severity of which might increase with ischemia duration. Our results show that in mitochondria from reperfused hearts, the hydroperoxide levels increase gradually with the ischemia duration. Moreover, whole antioxidant capacity, capacity to remove H_2O_2, and resistance to oxidants exhibit significant decreases, likely due to glutathione and reducing equivalents depletion, only after reperfusion following 45 min of ischemia. In addition, the above changes are well related to a gradual decline in mitochondrial respiration, which reflects damage to electron transport chain components.

Because of the multifactorial nature of ischemia-reperfusion injury, the mechanisms by which ROS mediate the decline in mitochondrial function are not entirely clear. Recent reports support the hypothesis that such a decline is due, at least in part, to modification of specific mitochondrial proteins by 4-hydroxy-2-nonenal, a product of the lipid peroxidation [11]. Nitric oxide (NO) is another putative species responsible for altered mitochondrial function in myocardial ischemia-reperfusion. NO induces reversible inhibition of respiration in isolated mitochondria [45, 46], and recent reports suggest that NO generated on reperfusion causes mitochondrial dysfunction, damaging complexes I and II of the respiratory chain [7]. High concentrations of or long-term exposure to NO [47] induce irreversible inhibition of respiration, even though it is unclear whether this inhibition is due to NO or peroxynitrite [48], a potent oxidant in which NO may be converted by mitochondria-generated superoxide [49]. Inhibition of mitochondrial function could also be due to a combination of oxidative stress with an increase in Ca^{2+} concentration that happens in myocardial cells during ischemia-reperfusion [50]. In the presence of Ca^{2+}, oxidative alterations of mitochondrial inner membrane protein thiols promote an inner membrane permeabilization referred to as mitochondrial permeability transition [51, 52] that leads to cytochrome c release and then to inhibition of the respiratory chain [53]. Such effects could explain the CR decrease and H_2O_2 generation increase, respectively, found after reperfusion following 45 min of ischemia. It is worth noting that release of cytochrome c from mitochondria to the cytoplasm appears to be an

early event in the apoptotic pathway of cell death [54]. On the other hand, if the insult caused by oxygen deprivation is not very great, because of the short ischemic phase, mitochondrial pores which open in the early phase of reperfusion can rapidly reseal [50], allowing mitochondrial function and heart performance to recover. However, whatever the mechanisms by which ROS impair mitochondrial function, our results suggest that the dependence of the mitochondrial alterations on the ischemia duration can be due to the degree of reductive stress associated with the different periods of oxygen deprivation.

1 Braunwald E. and Kloner R. A. (1985) Myocardial reperfusion: a double-edged sword? J. Clin. Invest. **76:** 1713–1719

2 Jennings R. B., Herdson P. B. and Sommers H. M. (1969) Structural and functional abnormalities in mitochondria isolated from ischemic dog myocardium. Lab. Invest. **20:** 548–557

3 Borutaite V., Mildaziene V., Brown G. C. and Brand M. D. (1995) Control and kinetic analysis of ischemia-damaged heart mitochondria: which parts of the oxidative phosphorylation system are affected by ischemia? Biochim. Biophys. Acta **1272:** 154–158

4 Kay L., Saks V. A. and Rossi A. (1997) Early alteration of the control of mitochondrial function in myocardial ischemia. J. Mol. Cell. Cardiol. **29:** 3399–34121

5 Ferrari R., Lisa F. di, Raddino R. and Visioli O. (1982) The effects of ruthenium red on mitochondrial function during postischaemic reperfusion. J. Mol. Cell. Cardiol. **14**: 737–740

6 Kane J. J., Murphy M. L., Bisset J. K., De Soyza N., Doherthy J. E. and Straub K. D. (1975) Mitochondrial function, oxygen extraction, epicardial ST-segment changes and tritiated digoxin distribution after reperfusion of ischemic myocardium. Am. J. Cardiol. **36:** 218–224

7 Abe K., Hayashi N. and Terada H. (1999) Effect of endogenous nitric oxide on energy metabolism of rat heart mitochondria during ischemia and reperfusion. Free Radic. Biol. Med. **26:** 379–387

8 Weishaar R., Tschurtschenthaler G. V., Ashikawa K. and Bing R. (1979) The relationship of regional coronary blood flow to mitochondrial function during reperfusion of the ischemic myocardium. Cardiology **64:** 350–364

9 Kotaka A., Miyzaki Y., Ogawa K., Satake T., Sugiyama S. and Ozawa T. (1982) Reversal of ischaemia-induced mitochondrial dysfunction after coronary reperfusion. J. Mol. Cell. Cardiol. **14:** 223–231

10 Piper H. M., Schwartz A., Spahr R., Hütter J. F. and Spiekermann P. G. (1984) Absence of reoxygenation damage in isolated heart cells after anoxic injury. Pflügers Arch. **401:** 71–76

11 Lucas D.T. and Szweda L.I. (1998) Cardiac reperfusion injury: aging, lipid peroxidation, and mitochondrial dysfunction. Proc. Natl. Acad. Sci. USA **95:** 510–514

12 Zhang Y., Marcillat O., Giulivi C., Ernster L. and Davies K. J. (1990) The oxidative inactivation of mitochondrial electron transport chain components and ATPase. J. Biol. Chem. **265:** 16330–16336

13 Das D. K., George A., Liu X. K. and Rao P. S. (1989) Detection of hydroxyl radical in the mitochondria of ischemic-reperfused myocardium by trapping with salicylate. Biochem. Biophys. Res. Commun. **165:** 1004–1009

14 Langendorff O. (1895) Untersuchungen am uberlebenden Saugethierherzen. Arch. Ges. Physiol. (Pflüger) **61:** 291–332

15 Venditti P., Masullo P., Agnisola C. and Di Meo S. (2000) Effect of vitamin E on the response to ischemia-reperfusion of Langerdorff heart preparations from hyperthyroid rats. Life Sci. **66:** 697–708

16 Tyler D. D. and Gonze J. (1967) The preparation of heart mitochondria from laboratory animals Methods Enzymol. **10:** 75–77

17 Gornall A. G., Bardawill C. J. and David M. M. (1949) Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177:** 751–766

18 Hyslop P. A. and Sklar L. A. (1984) A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular activation processes. Anal. Biochem. **141:** 280–286

19 Boveris A. and Chance B. (1973) The mitochondrial generation of hydrogen peroxide. Biochem. J. **134:** 707–716

20 Heath R. L. and Tappel A. L. (1976) A new sensitive assay for the measurement of hydroperoxides. Anal. Biochem. **76:** 184–191

21 Venditti P., De Leo T. and Di Meo S. (1999) Determination of tissue susceptibility to oxidative stress by enhanced luminescence technique. Methods Enzymol. **300:** 245–252

22 Di Meo S., Venditti P. and De Leo T. (1996) Tissue protection against oxidative stress. Experientia **52:** 786–794

23 Venditti P., Masullo P. and Di Meo S. (2001) Hemoproteins affects H_2O_2 removal from rat tissues. Int. J. Biochem. Cell. Biol. **33:** 293–301

24 Rouslin W. and Millard R. W. (1981) Mitochondrial inner membrane enzyme defects in porcine myocardial ischemia. Am. J. Physiol. **240:** H308–H313

25 Rouslin W. and Ranganathan S. (1983) Impaired function of mitochondrial electron transfer complex in canine myocardial ischemia: loss of flavin mononucleotide. J. Mol. Cell. Cardiol. **15:** 537–542

26 Shlafer M., Myers C. L. and Adkins S. (1987) Mitochondrial hydrogen peroxide generation and activities of glutathione peroxidase and superoxide dismutase following global ischemia. J. Mol. Cell. Cardiol. **19:** 1195–1206

27 Veitch K., Hombroeckx A., Caucheteux D., Pouleur H. and Hue L. (1992) Global ischaemia induces a biphasic response of the mitochondrial respiratory chain. Anoxic pre-perfusion protects against ischaemic damage. Biochem J. **281:** 709–715

28 Marban E., Koretsune Y., Corretti M., Chacko V. P. and Kusuoka H. (1989) Calcium and its role in myocardial cell injury during ischemia and reperfusion. Circulation **80:** 17–22

29 Griffiths E. J., Ocampo C. J., Savage J. S., Rutter G. A., Hansford R. G., Stern M. D. et al. (1998) Mitochondrial calcium transporting pathways during hypoxia and reoxygenation in single rat cardiomyocytes. Cardiovasc. Res. **39:** 423–433

30 McCormack J. G., Halestrap A. P. and Denton R. M. (1990) Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol. Rev. **70:** 391–426

31 Arroyo C. M., Kramer J. H., Lieboff R. H., Mergner G. W., Dickens B. F. and Weglicki W. B. (1987) Spin trapping of oxygen and carbon centered free radicals in the ischemic canine myocardium. Free Radic. Biol. Med. **3:** 313–316

32 Eaton P., Li J.-M., Hearse D. J. and Shattock M. J. (1999) Formation of 4-hydroxy-2-nonenal-modified proteins in ischemic rat heart. Am. J. Physiol. **276:** H935–H943

33 Di Lisa F., Menabò R., Canton M. and Petronilli V. (1998) The role of mitochondria in the salvage and the injury of the ischemic myocardium. Biochim. Biophys. Acta **1366:** 69–78

34 Kehrer J. P. and Lund, L. G. (1993) Cellular reducing equivalents and oxidative stress. Free Radic. Biol. Med. **17:** 65–75

35 Ueta H., Ogura R., Sugiyama M., Kagiyama A. and Shin G. (1990) O_2^- spin trapping on cardiac submitochondrial particles isolated from ischemic and non-ischemic myocardium. J. Mol. Cell. Cardiol. **22:** 893–899

36 Arduini A., Mezetti A., Porreca E., Lapenna D., DeJulia J., Marzio L. et al. (1988) Effect of ischemia and reperfusion on antioxidant enzymes and mitochondrial inner membrane pro-

teins in perfused rat heart. Biochim. Biophys. Acta **970:** 113–121
37 Ambrosio G., Zweier J. L. and Flaherty J. T. (1991) The relationship between oxygen radical generation and impairment of myocardial energy metabolism following post-ischemic reperfusion. J. Mol. Cell. Cardiol. **23:** 1359–1374
38 Henry T. D., Archer S. L., Nelson D., Weir E. K. and From A. H. L. (1993) Postischemic oxygen radical production varies with duration of ischemia. Am. J. Physiol. **264:** H1478–H1484
39 Xia Y., Khatchikian G. and Zweier J. L. (1996) Adenosine deaminase inhibition prevents free radical-mediated injury in the postischemic heart. J. Biol. Chem. **271:** 10096–10102
40 McCord J. M. (1988) Free radicals and myocardial ischaemia: overview and outlook. Free Radic. Biol. Med **4:** 9–14
41 Nishino T. (1994) The conversion of xanthine dehydrogenase to xanthine oxidase and the role of the enzyme in reperfusion injury. J. Biochem. (Tokyo) **116:** 1–6
42 Ambrosio G., Zweier J. L., Duilio C., Kuppusamy P., Santoro G., Elia P. P. et al. (1993) Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. J. Biol. Chem. **268:** 18532–18541
43 Halestrap A. P., Griffiths E. J. and Connern C. P. (1993) Mitochondrial calcium handling and oxidative stress. Biochem. Soc. Trans. **21:** 353–358
44 Esterbauer H., Schaur R. J. and Zollner H. (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. **11:** 81–128
45 Borutaite V. and Brown G. C. (1996) Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. Biochem. J. **315:** 295–299
46 Cassina A. and Radi R. (1996) Differential inhibitory actions of nitric oxide and peroxynitrite on mitochondrial electron transport. Arch. Biochem. Biophys. **328:** 309–316
47 Nathan C. (1992) Nitric oxide as a secretory product of mammalian cells. FASEB J. **6:** 3051–3064
48 Lizasoain L., Moro M. A., Knowles R. G., Darley-Usmar V. and Moncada S. (1996) Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. Biochem. J. **314:** 877–880
49 Poderoso J. J., Carreras M. C., Lisdero C., Riobo N., Scopfer F. and Boveris A. (1996) Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. Arch. Biochem. Biophys. **328:** 85–92
50 Halestrap A. P. (1999) The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. Biochem. Soc. Symp. **66:** 181–203
51 Zoratti M. and Szabo I. (1995) The mitochondrial permeability transition. Biochim. Biophys. Acta 1241: 139–176
52 Vercesi A. E., Kowaltowski A. J., Grijalba M. T., Meinicke A. R. and Castilho R. F. (1997) The role of reactive oxygen species in mitochondrial permeability transition. Biosci. Rep. **17:** 43–52
53 Borutaite V., Morkuniene R. and Brown G. C. (1999) Release of cytochrome c from heart mitochondria is induced by high Ca^{2+} and peroxynitrite and is responsible for Ca^{2+}-induced inhibition of substrate oxidation. Biochim. Biophys. Acta **1453:** 41–48
54 Yang J., Liu X., Bhalla K., Kim C. N., Ibrado A. M., Cai J. Y. et al. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science **275:** 1129–1132

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