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Mitochondrial dysfunction associated with cardiac ischemia/reperfusion can be attenuated by oxygen tension control. Role of oxygen-free radicals and cardiolipin

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Abstract

Reactive oxygen species (ROS) are considered an important factor in ischemia/reperfusion injury to cardiac myocites. Mitochondrial respiration is an important source of ROS generation and hence a potential contributor to cardiac reperfusion injury. Appropriate treatment strategy could be particularly useful to limit this ROS generation and associated mitochondrial dysfunction. In the present study, we examined the effect of lowering the oxygen tension, at the onset of the reperfusion, on various parameters of mitochondrial bioenergetics in rat heart tissue. After isolation of mitochondria from control, ischemic, normoxic and hypoxic reperfused rat heart, various bioenergetic parameters were evaluated such as rates of mitochondrial oxygen consumption, complex I and complex III activity, H_2O_2 production and in addition, the degree of lipid peroxidation, cardiolipin content and cardiolipin oxidation. We found that normoxic reperfusion significantly altered all these mitochondrial parameters, while hypoxic reperfusion had a protective effect attenuating these alterations. This effect appears to be due, at least in part, to a reduction of mitochondrial ROS generation with subsequent preservation of cardiolipin integrity, protection of mitochondrial function and improvement of post-ischemic hemodynamic function of the heart.

Keywords: Mitochondrial respiration; Complexes I and III activity; ROS generation; Cardiolipin oxidation; Rat heart hypoxic reperfusion

1. Introduction

A large body of experimental evidences indicate that reactive oxygen species (ROS) play an important role in producing lethal cell injury associated with cardiac ischemia/reperfusion [1–6]. The mechanism for the enhanced ROS generation and cellular and subcellular target of ROS attack are not well established. Mitochondria are considered an important locus of ROS production mainly at the level of complexes I and III [7–10] of the respiratory chain and hence a potential contributor to heart reperfusion injury. As a major source of ROS production, mitochondria could be major targets of ROS attack. Given that ROS are highly reactive and short lived species, their effect should be greatest in immediate area surrounding their locus of production. It is conceivable that mitochondrial membrane constituents, including the complexes of the respiratory chain and phospholipid constituents, could be the major target of ROS attack.

Cardiolipin, a phospholipid of unusual structure localized almost exclusively within the mitochondrial inner membrane, is particularly rich in unsaturated fatty acids. Thus, mitochondrial cardiolipin molecules are a possible and early target of ROS attack, either because of their high content of unsaturated fatty acids or because of their location in the inner mitochondrial membrane near to the site of ROS production, mainly at the level of complexes I and III of the respiratory chain [9,10]. This phospholipid plays an important role in mitochondrial bioenergetics, optimizing the activity of the respiratory chain complexes [11–13].

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Previous studies from this and other laboratories have shown that the activity of the complexes I, III and IV of the respiratory chain is reduced in mitochondria from ischemic/reperfused rat heart [14–18]. This decrease has been ascribed to ROS-induced cardiolipin oxidation. The impairment of mitochondrial complexes I, III, IV activity due to ROS-induced cardiolipin damage may increase the electron leak from the electron transport chain generating more superoxide anion radical and perpetuating a cycle of oxygen–radical-induced damage, which ultimately leads to a decrease in oxidative phosphorylation and to heart failure on reperfusion.

It has been reported that the production of free radicals occurs during the first minutes after cardiac reperfusion [4,5,19]. Using electron magnetic resonance Zweier et al. identified a spectral peak in heart compatible with an oxygen-centred free radical which peaked during the first 10 s of the reperfusion [4]. Thus, the first few minutes of reperfusion constitute a critical phase as here lethal tissue injury, in addition to that already developed during ischemia, may be initiated; therefore modifications of the early phase of reperfusion conditions may represent an opportunity of cardioprotection. Appropriate treatment strategies could be particularly useful to limit ROS production and ROS-induced alterations to mitochondrial structure and function and hence to protect ischemic reperfused myocardium. Free radical scavengers have been shown to attenuate reperfusion injury following ischemia reperfusion [19]. As an alternative treatment to antioxidants, it s conceivable that post-ischemic reperfusion injury could be attenuated by oxygen tension control [20,21].

The present study examined the hypothesis that the alterations to mitochondrial oxidative metabolism associated to ischemia/reperfusion may be limited by lowering the oxygen tension during reperfusion. We studied the effect of lowering the pO_2 during the first minutes of heart reperfusion on various mitochondrial bioenergetic parameters such as oxygen consumption, respiratory complexes activity, mitochondrial ROS production rates, as well as on mitochondrial lipid peroxidation, cardiolipin content and cardiolipin oxidation. In addition, since ROS and mitochondrial damage play a role in contractility dysfunction associated with reperfusion of ischemic heart, we also tested the efficacy of lowering pO_2 to limit the impairment of ventricular mechanics in a reperfused heart model.

2. Materials and methods

2.1. Animal experiments

Animals received care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985), as well as with Italian laws on animal experimentation. A model of isolated and perfused heart was used according to Langendorff technique. Male Wistar rats weighing 250–300 g. were heparinized and anesthetized with Thiopental 35 mg i.p. given. Heart were excised and perfused at 37 °C under 10 ml·min^{-1} constant flow. Krebs–Henseleit solution was used as perfusion buffer, saturated with O_2/CO_2 (95/5) gas mixture. pO_2 was monitored by an oxygen electrode (Instech Dual Oxygen, Plymouth Meeting, PA) and maintained at a pO_2 level of 600 mm Hg, which is considered normal in Langendorff preparations, due to the lack of haemoglobin [22]. In a second reservoir Krebs–Henseleit solution had not been bubbled at all with the O_2/CO_2 mixture in order to maintain the solution at the atmospheric pO_2 level of 150 mm Hg, which is considered hypoxic for this model [22]. pH of the solutions was maintained between 7.35 and 7.45 by adding 1N HCl if required.

Left ventricular (LV) isovolumic pressure was recorded by a strain-gauge pressure transducer (Hewlett-Packard Medical Electronic Division, Model 1280C, Waltham, MA). The end-diastolic pressure (LVEDp) was adjusted to 5–10 mm Hg. Aortic root pressure (RAp) was also monitored by a strain-gauge pressure transducer (Hewlett-Packard Medical Electronic Division, Model 1280C, Waltham, MA), via a side-arm of the cannula. Pacing electrodes were attached to atrial appendages and the heart was paced at 300 beats/min.

Control hearts were perfused for 60 min. Ischemic hearts were perfused for 15 min and then subjected to global ischemia for 30 min by turning off the perfusion system. Normoxic reperfused hearts were reperfused for 15 min with a Krebs–Henseleit solution oxygen buffered at the same pO_2 level as before ischemia (normoxic group: pO_2 600 mm Hg). In the hypoxic reperfused group of hearts the second reservoir containing Krebs–Henseleit solution, in which the O_2/CO_2 mixture had not been bubbled at all, was connected to the perfusion system during the first 3 min of reperfusion (hypoxic group: pO_2 150 mm Hg). In this hypoxic group, at the end of the 3rd min after starting reperfusion, the perfusion system was connected again with the solution buffered at a pO_2 of 600 mm Hg until the end of reperfusion (Fig. 1). Throughout each protocol, including global ischemia, the temperature of the hearts was maintained at 37 °C by a water-jacketed chamber. Left ventricular pressure curves were recorded just before ischemia, and at the 1st, 2nd, 3rd, 4th, 5th, 10th, 15th min of reperfusion. The following parameters were measured: left ventricular end-diastolic pressure (LVEDp, mm Hg); systolic pressure (LVSp, mm Hg); developed pressure (LVDp = LVSp − LVEDp, mm Hg); dP/dt (expressed as percent change of the pre-ischemia value); root aortic pressure (RAp, mm Hg).

2.2. Isolation of mitochondria

Rat heart mitochondria were isolated in a medium of 250 mM sucrose, 10 mM Tris–HCl, 1 mM EGTA, pH 7.4, by differential centrifugation of heart homogenates essentially as described previously [17]. Mitochondria were resuspended in 250 mM sucrose, 10 mM Tris–HCl (pH 7.4) and stored in ice. The yield of mitochondrial proteins (mg/g heart wet wt.) within each group of animals was consistent, suggesting minimal variation in the preparations of the mitochondrial fraction.

Mitochondrial protein concentration was measured by the biuret method using serum albumin as standard.

2.3. Determination of mitochondrial H_2O_2 production

The rate of mitochondrial hydrogen peroxide production was estimated by measuring the linear fluorescence increase induced by H_2O_2 oxidation of dichlorofluorescin to the fluorescent dichlorofluorescein in the presence of horseradish peroxidase [23]. Rat heart mitochondria (0.3 mg protein) were suspended in 2.5 ml of a medium of 100 mM sucrose, 100 mM KCl, 5 mM Tris, pH 7.4, supplemented with 7.5 μg horseradish peroxidase and 1 μM dichlorofluorescein. The production of hydrogen peroxide was induced by addition of 5 mM malate + 2 mM pyruvate or 5 mM succinate as substrates (state 4). The amount of H_2O_2 produced was calculated by measuring the fluorescence changes upon addition of known amounts of H_2O_2.

2.4. Mitochondrial oxygen consumption

Mitochondrial ADP-dependent state 3 respiration was measured polarographically with an oxygen electrode at 25 °C. Respiration was initiated by the addition of 2 mM pyruvate + 5 mM malate or 5 mM succinate. After 2 min state 3 respiration was induced by the addition of 0.5 mM ADP.

2.5. Complex I activity

Complex I (NADH-CoQ reductase) activity was measured in mitochondrial particles prepared by sonicating, under nitrogen atmosphere, 1 mg of rat heart

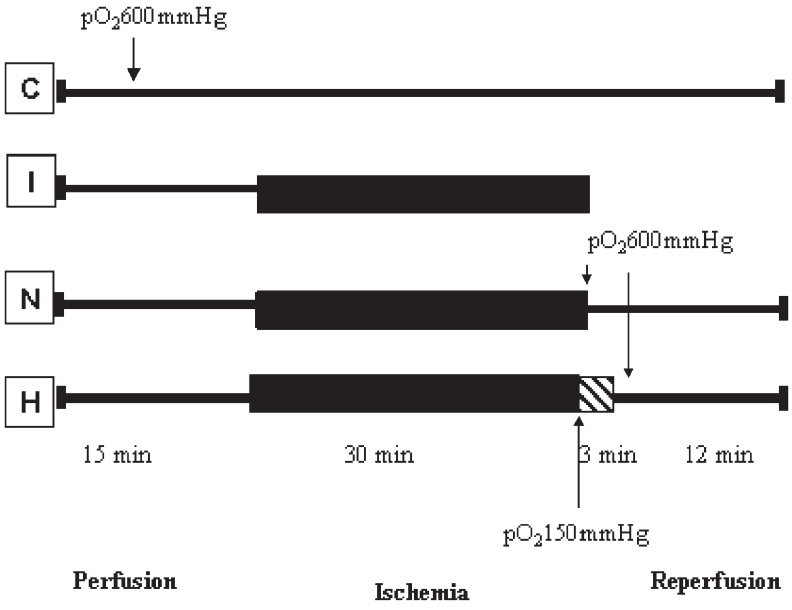

Fig. 1. Experimental protocol of ischemia and reperfusion in four groups of Langendorff rat heart preparations. Group C (control): hearts perfused for 60 min. Group I (ischemic): hearts perfused for 15 min followed by 30 min global ischemia. Group N (normoxic): after 30 min of ischemia hearts were reperfused for 15 min at 600 mm Hg pO_2. Group H (hypoxic): after 30 min ischemia hearts were reperfused at 150 mm Hg pO_2 in the first 3 min and at 600 mm Hg pO_2 afterwards.

mitochondria dissolved in 1 ml of 50 mM phosphate buffer pH 7.2. The assay mixture contained 3 mM sodium azide, 1.2 μM antimycin A, 50 μM decylubiquinone and 50 mM phosphate buffer pH 7.2. The mitochondrial sample (50 μg) was added to 3 ml of the assay mixture and the reaction was started by the addition of 60 μM NADH. The reaction was measured by following the decrease in absorbance of NADH at 340 nm with a diode array spectrophotometer. The activity was calculated using an extinction-coefficient of 6.22 $mM^{-1} \times cm^{-1}$ for NADH. The specific activity of the enzyme is expressed as nmol of NADH oxidized/min/mg of mitochondrial protein. The rotenone insensitive rate of NADH oxidation was measured and subtracted.

2.6. Complex III activity

The complex III activity (decylubiquinol/ferricytochrome c oxidoreductase) was measured in mitochondrial particles prepared by sonicating 1 mg of rat heart mitochondria dissolved in 1 ml of 50 mM phosphate buffer pH 7.2. The assay mixture contained 3 mM sodium azide, 1.5 μM rotenone, 50 μM ferricytochrome c and 50 mM phosphate buffer pH 7.2. The sample (10 μg) was added to 3 ml of the assay mixture and the reaction was started by the addition of 30 μM of decylubiquinol. The reaction was measured with a diode array spectrophotometer by following the increase in reduced cytochrome *c* absorbance at 550–540 nm. The activity was calculated using an extinction-coefficient of 19.1 $mM^{-1} \times cm^{-1}$.

The specific activity of the enzyme is expressed as nmol of cytochrome *c* reduced/min/mg of mitochondrial particles.

Decylubiquinol was synthesized by reduction of decylubiqionone (10 μM) with $NaBH_4$ in 2 ml of 1:1 ethanol/H_2O mixture (v/v, pH 2). The ubiquinol formed was extracted twice with 1 mm of diethylether/isooctane 2:1 (v/v). The combined organic phases were washed with 2 ml of 2 M NaCl and evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in ethanol and the resulting light yellow solution was acidified with 10 μl of 0.1 M HCl and stored at −20 °C.

2.7. Analysis of cardiolipin in mitochondrial membranes

Cardiolipin was analyzed by high-pressure liquid chromatography (HPLC) using a Hewlett Packard series 1100 gradient liquid chromatograph. Lipids from heart mitochondria were extracted with chloroform/methanol by the procedure of Bligh and Dyer [24]. Lipid extraction was carried out on ice immediate after the preparation of mitochondria in the presence of BHT and under nitrogen atmosphere. Phospholipids were separated by the HPLC method previously described [25] with an Lichrosorb Si60 column (4.6×250 mm). The chromatographic system was programmed for gradient elution using two mobile phases: solvent A, hexane/2-propanol (6:8, v/v) and solvent B, hexane/2-propanol/water (6:8:1.4, v/v/v). The percentage of solvent B in solvent A was increased in 15 min from 0% to 100%. Flow rate was 1 ml/min and detection at 206 nm. The peak of cardiolipin was identified by comparison with the retention time of standard cardiolipin and rechromatographed by TLC.

2.8. Lipid and cardiolipin peroxidation

Lipid peroxidation was estimated by the appearance of conjugated dienes as follows. Lipids were extracted from mitochondria by the Bligh e Dyer procedure [24]. Lipid extracts from 4 mg of mitochondrial membrane were dissolved in 2.5 ml of chloroform/methanol (1:1) and the absorption spectra were followed between 210 and 310 nm [26] with a Perkin-Elmer Lambda 3B spectrophotometer.

Peroxidized cardiolipin was identified by normal-phase HPLC, as described above, with UV detection at 235 nm, indicative of conjugated dienes [26,27]. The resulting peak was rechromatographed by TLC and used as standard.

2.9. Statistical analysis

Measures of ventricular function have been expressed as mean values±S.E. Statistical significance of the comparison between the time courses of values obtained in hypoxic and normoxic group was calculated by two-way ANOVA for repeated measures. All other results have been expressed as mean±S.E. and their statistical significance was determined by the Student's *t* test.

3. Results

Lipid peroxidation has been proposed to be a major mechanism of ROS attack. This process is accompanied by a rearrangement of unsaturated fatty acids double bonds, leading to the formation of conjugated dienes which absorb at 233 nm. Thus, the degree of mitochondrial membranes

lipid peroxidation can be readily assayed by recording the increase in absorbance of extracted membrane lipids at 233 nm. Fig. 2 shows absorbance values obtained from mitochondrial lipid extracts of control, ischemic, normoxic reperfused and hypoxic reperfused rat heart. Mitochondria from ischemic heart exhibited a 25% and those from normoxic reperfused heart a 44% increase in the level of lipid peroxidation, compared to the control heart. Hypoxic reperfusion had a protective effect reducing the level of lipid peroxidation.

Respiratory activities of mitochondria isolated from control, ischemic, normoxic and hypoxic reperfused rat heart, measured in the presence of pyruvate+malate or succinate as substrates and ADP to stimulate respiration (state 3) are reported in Table 1. The rate of state 3 respiration was markedly decreased in mitochondria isolated from normoxic reperfused rat heart, while hypoxic reperfusion attenuated this decrease. Almost similar results were obtained with succinate as substrate. State 4 rates of respiration were slightly decreased in all these preparations of mitochondria. The respiratory control ratio (RCR), an index of mitochondrial membrane integrity, was also decreased in mitochondria from reperfused rat heart, while low PO_2 reperfusion limited this decrease.

Complex I and complex III activities were measured in all these preparations of mitochondria. As shown in Fig. 3, mitochondria from normoxic reperfused rat heart exhibited a marked decrease in the activity of both these enzyme complexes, compared with the control heart. Hypoxic reperfusion had significant protective effect and actually attenuated the decline in the complex I and complex III activity.

It has been shown that addition of the respiratory substrates pyruvate+malate or succinate to aerobic mitochondria results in a generation of H_2O_2 which arises from superoxide anion formed mainly at the level of complexes I and III [7,28,16]. The capacity of mitochondria isolated from control, ischemic,

Table 1
Respiratory activities in mitochondria isolated from control, ischemic, normoxic and hypoxic reperfused rat heart

	Respiratory activities (natom O/min/mg prot)			
	Control	Ischemic	Normoxic reperfused	Hypoxic reperfused
Pyruvate+malate				
State 3	192±9.6	151±5.5*	103±4.4*	132±5.5**
State 4	46±3.2	42±2.5	35±1.3*	39±1.4
RCR	4.2±0.2	3.6±0. 25*	2.9±0.12*	3.4±0.11**
Succinate				
State 3	172±14.2	136±11.5*	85±3.7*	120±11.4**
State 4	50±4.2	48±3.4	37±2.8*	45±2.7**
RCR	3.4±0.26	2.8±0.21*	2.3±0.12*	2.7±0.11**

Mitochondrial respiratory activities were measured as described in Materials and methods. Each value represents the mean±S.E. of eight separate experiments.
* $P<0.01$ vs. control.
** $P<0.05$ vs. normoxic reperfused.

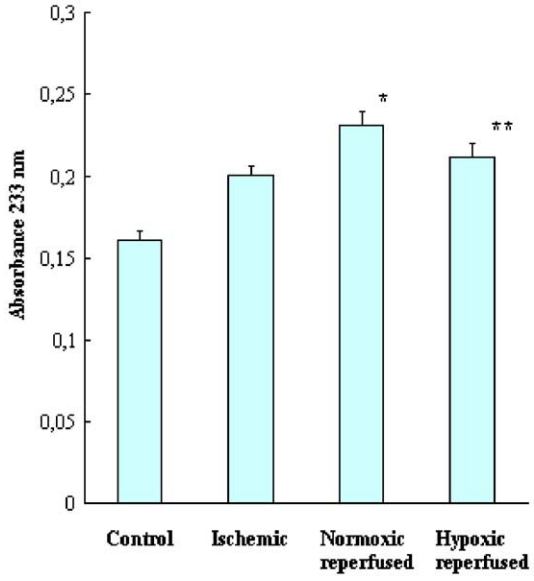

Fig. 2. Absorbance values of the conjugated dienes of mitochondrial lipids extracted from control, ischemic, normoxic and hypoxic reperfused rat heart. The conjugated dienes spectra were recorded as described in Materials and methods. Each value represents the mean±S.E. of eight separate experiments. *$P<0.05$ vs. control; **$P<0.05$ vs. normoxic reperfused.

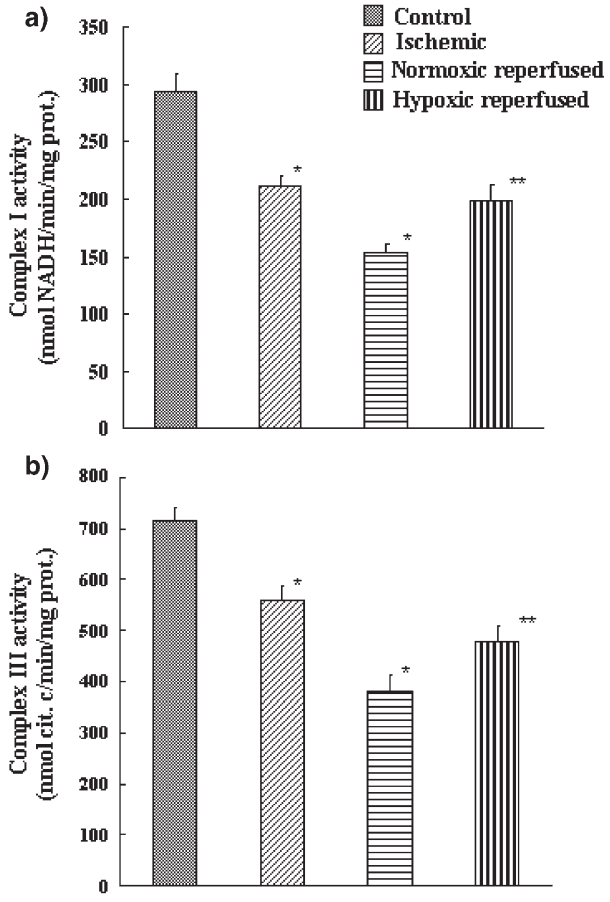

Fig. 3. Complex I and complex III activity in mitochondria isolated from control, ischemic, normoxic and hypoxic reperfused rat heart. Complex I (a) and complex III (b) activities were measured as described in Materials and methods. Each value represents the mean±S.E. of eight separate experiments. *$P<0.01$ vs. control; **$P<0.05$ vs. normoxic reperfused.

normoxic reperfused and hypoxic reperfused rat heart to generate oxygen radical in the presence of pyruvate+malate or succinate was evaluated. The basal rate of H_2O_2 production was significantly enhanced in mitochondria from normoxic reperfused rat heart, respiring with pyruvate+malate or succinate (Fig. 4). Under hypoxic reperfusion this H_2O_2 production was markedly decreased. We also measured the rate of mitochondrial ROS production during the early phase (2 min) of normoxic and hypoxic reperfusion. As shown in Fig. 4, at 2 min of normoxic reperfusion, the rate of mitochondrial

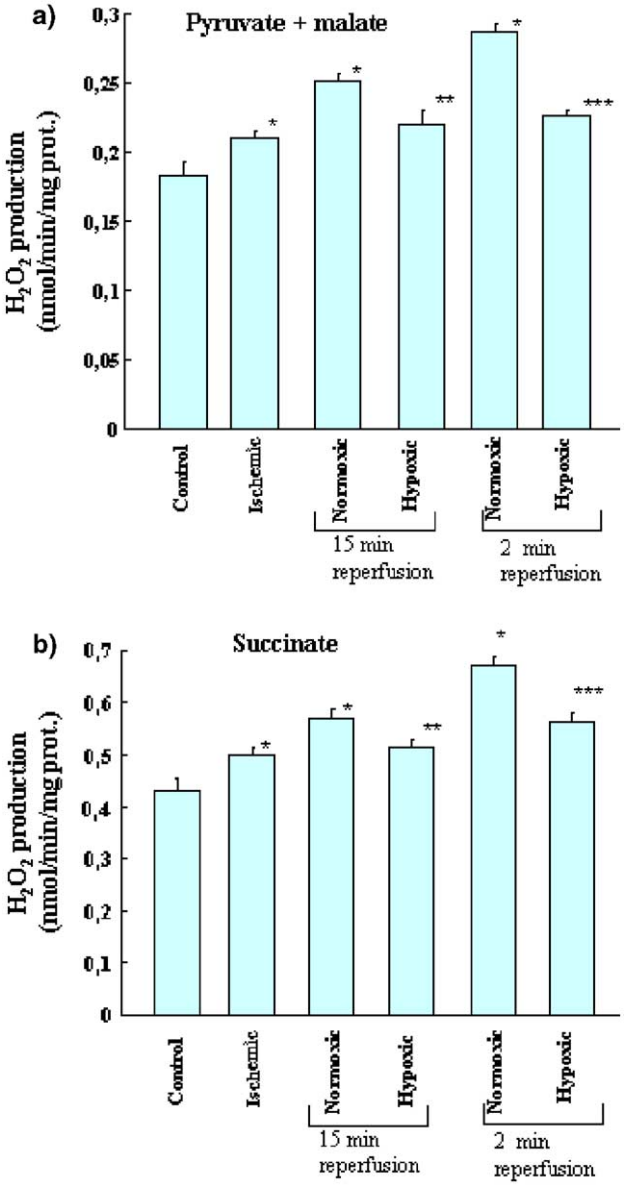

Fig. 4. H_2O_2 production in mitochondria isolated from control, ischemic, normoxic and hypoxic reperfused rat heart. Mitochondria were isolated from control, ischemic, and from normoxic and hypoxic reperfused hearts at 2 or 15 min of reperfusion. The H_2O_2 formation was induced by the addition of 2 mM pyruvate+5 mM malate (a) or 5 mM succinate (b) and measured as described in Materials and methods. Each value represents the mean±S.E. of eight experiments or four experiments for 2 min reperfusion. *$P<0.01$ vs. control; **$P<0.05$ vs. normoxic 15 min reperfused; ***$P<0.05$ vs. normoxic 2 min reperfused.

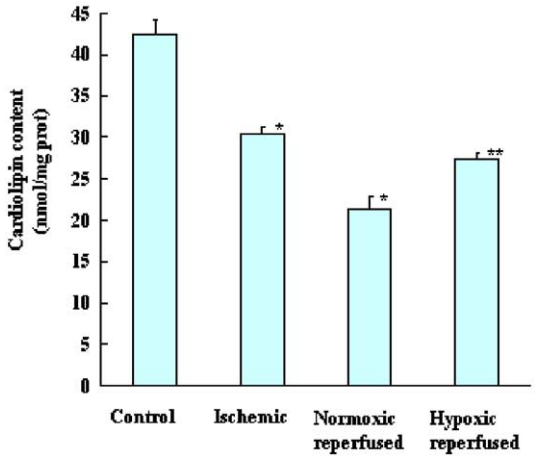

Fig. 5. Cardiolipin content in mitochondria isolated from control, ischemic, normoxic and hypoxic reperfused rat heart. Mitochondrial cardiolipin content was determined by the HPLC technique as described in Materials and methods. Each value represents the mean±S.E. obtained from eight different experiments. *$P<0.01$ vs. control; **$P<0.05$ vs. normoxic reperfused.

ROS production was greatly enhanced. This early ROS production was abolished under hypoxic condition, suggesting that the protective effect of hypoxia occurs during the early phase of reperfusion.

We have previously shown that mitochondrial cardiolipin loss is mainly responsible for the defect in complex I and complex III activity associated to heart reperfusion [16,17]. The effect of hypoxic reperfusion on mitochondrial cardiolipin content was tested. The content of mitochondrial cardiolipin was measured by a very sensitive HPLC technique set up in our laboratory with a detection limit of 0.5 nmol/

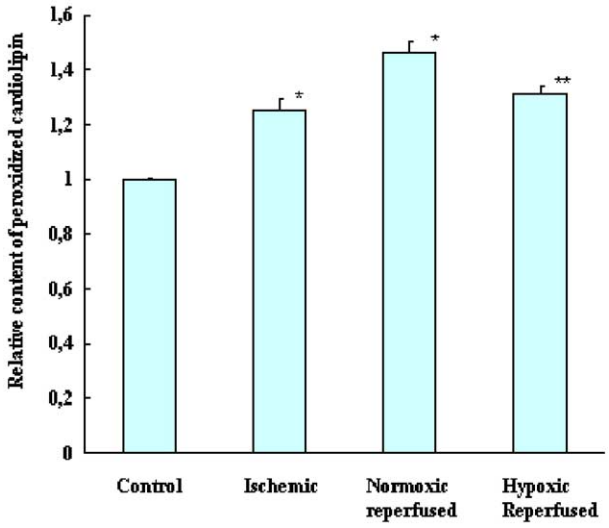

Fig. 6. Relative content of peroxidized cardiolipin in mitochondria from control, ischemic, normoxic and hypoxic reperfused rat heart. Mitochondrial content of peroxidized cardiolipin was determined by the HPLC technique described in Materials and methods. The content of peroxidized cardiolipin is expressed as peak area (at 235 nm) per mg of phospholipids and the peak area of the control is assumed as unit. Each value represents the mean±S.E. obtained from eight different experiments. *$P<0.05$ vs. control; **$P<<0.05$ vs. normoxic reperfused.

sample. As reported in Fig. 5, the content of cardiolipin was reduced by 28% in mitochondria from ischemic heart and by 50% and 36% in mitochondria from normoxic and hypoxic reperfused rat heart, respectively, compared to the control heart.

We also measured the content of peroxidized cardiolipin in these preparations of mitochondria. The content of peroxidized cardiolipin was measured by an HPLC method based on the absorbance at 233 nm, indicative of the formation of conjugated dienes. As shown in Fig. 6, the content of peroxidized cardiolipin was enhanced by 25%, 46% and 33% in mitochondria isolated from ischemic, normoxic reperfused and hypoxic reperfused rat hearts, respectively, compared to control heart.

The effect of hypoxic reperfusion on the left ventricular function was studied and the time course of LVEDp and LVDp in these two groups of hearts is shown in Fig. 7. An increase in LVEDp occurs during reperfusion which was lesser in hypoxic than normoxic group of hearts, at the 15th min the values being 62.3±5.8 vs. 86.9±4.8 mm Hg, respectively. After reperfusion LVDp was lower than before ischemia with a better recovery in hypoxic than in normoxic group (40.6±3.7 vs. 15.0±1.3 mm Hg). It is worth noting that differences between normoxic and hypoxic hearts are clearly evident since the first minutes of reperfusion (see Fig. 7), when the burst of ROS production occurs.

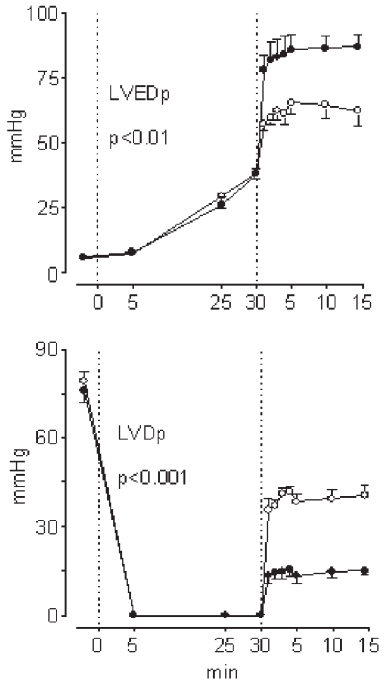

Fig. 7. Changes in left ventricle end-diastolic pressure (LVEDp) and left ventricle developed pressure (LVDp=systolic minus end-diastolic pressure), in two groups of isolated and perfused rat hearts during 30 min ischemia and 15 min reperfusion. Closed circles: hearts reperfused at 600 mm Hg pO_2 for 15 min. Open circles: hearts reperfused at 150 mm Hg pO_2 during the first 3 min followed by 12 min reperfusion at 600 mm Hg pO_2 (see Fig. 1). Data are expressed as mean values±S.E. Statistical significance calculated by two-way ANOVA for repeated measures.

4. Discussion

Reperfusion is a pre-requisite to salvaging viable myocardium following an acute episode of ischemia. Reperfusion of ischemic myocardium may paradoxically present some risk in that can result in myocyte death, a phenomenon termed reperfusion-injury. It is generally acknowledged that ischemic–reperfusion injury to cardiac myocytes is mediated, among other factors, by an overproduction of oxygen free radicals. These oxygen radicals can be generated by several mechanisms including the xanthine oxidase reaction [29–31], the activity of NADPH oxidase [32] and mitochondrial respiration [33,34].

Because of the abundance of mitochondria in cardiac myocytes, mitochondrial electron transport chain might be an important subcellular source of ROS and hence a potential contributor to heart reperfusion injury. Mitochondria consume >90% of the oxygen used by the cell, and the mitochondrial respiratory chain generates a continuous flux of oxygen radicals. It has been estimated that around 2% of the oxygen reacting with the respiratory chain leads to formation of superoxide radical. Subsequent dismutation of superoxide anion generates H_2O_2, which in turn can lead to production of OH. These oxygen radicals are normally inactivated by endogenous scavengers mechanism present within the cell such as antioxidant enzymes and lipid-soluble antioxidants including vitamin E and reduced coenzyme Q. This production of oxygen radicals can be greatly enhanced when mitochondrial respiration is stimulated under conditions of altered redox state and of decreased availability of ADP a condition that may occur in ischemic/reperfused heart.

Experimental evidence has shown that during the first minutes after cardiac post-ischemic reperfusion, a burst of ROS generation occurs [4,5,19]. Mitochondrial ROS generation leads to primary reaction and damage to mitochondrial constituents including lipids and proteins in the immediate area surrounding the locus of ROS production, given that these species are highly reactive and short lived. Previous studies demonstrated that reperfusion of ischemic heart is associated to alterations of several parameters of mitochondrial bioenergetics [14–18]. These alterations were ascribed to oxygen radicals attack to vital mitochondrial constituents including phospholipid species, specifically cardiolipin, and complexes of the respiratory chain.

It is conceivable that oxygen radical generation and subsequent impairment of mitochondrial function and hence of cardiac performance may be related to pO_2 during the reoxygenation. Thus, it is possible that lowering the oxygen tension at the beginning of the reperfusion, when a burst of oxygen radical occurs, may reduce the generation of these oxygen species and subsequently the mitochondrial oxidative damage. Our results demonstrate that lowering the pO_2 at the beginning of the reperfusion (first 3 min) from 600 to 150 mm Hg resulted in an attenuation of mitochondrial dysfunction induced by normoxic reperfusion of the heart. In fact, we found that hypoxic reperfusion of ischemic heart attenuated (1) the increase in mitochondrial lipid peroxidation; (2) the decrease in the state 3 respiration and the associated decrease in the

respiratory control ratio; (3) the decrease in complex I and complex III activities; (4) the increase in the H_2O_2 production; (5) the loss in cardiolipin and the increase in its level of peroxidation. It should be noted that the degree of protection afforded by hypoxic reperfusion on all these bioenergetics parameters was almost similar to the degree of protection observed on the heart contractility (Fig. 7).

Lipid peroxidation is considered a major mechanism of oxygen free radicals attack. This process initiates with oxygen radical attack to double bonds of polyunsaturated fatty acids leading to formation of conjugated dienes. Thus, the level of conjugated dienes formation may be indicative of the level of oxygen radical production. The fact that hypoxic reperfusion leads to a decrease of the degree of lipid peroxidation, measured by the level of conjugated dienes (Fig. 2), indicates that lowering the pO_2 in the beginning of the reperfusion results in a lower production of oxygen radical species.

The results reported in Table 1 show a significantly lower state 3 respiration rate in mitochondria isolated from normoxic reperfused heart compared to control, while state 4 rates of respiration were slightly decreased. This decline in state 3 respiration could be attenuated by lowering pO_2 during reperfusion. State 3 rates of respiration indicate an intact respiratory chain and ATP synthesis. Alterations of states 3 and 4 respiration determine changes in the respiratory control ratio (RCR). A decrease in the RCR is observed in mitochondria isolated from normoxic reperfused rat heart compared to the control. This decrease was attenuated when reperfusion was carried out at low pO_2. These results indicate that heart hypoxic reperfusion had a protective effect on the integrity and on the function of the mitochondria.

Complex I and complex III are considered the main producers of superoxide anion in mitochondria [7–10]. The formation of superoxide occurs via the transfer of free electrons to molecular oxygen. This reaction occurs at specific sites of the electron transport chain, which resides in the inner mitochondrial membrane. A defect in the activity of both these respiratory complexes can be considered a potential source of ROS production. We found that heart ischemia/reperfusion is associated to a defect in complex I and complex III activity which may account for the enhanced production of H_2O_2 in mitochondria [16,17]. Both these effects are attenuated by hypoxic reperfusion.

Cardiolipin is emerging as an important factor in the regulation of mitochondrial bioenergetics in that it interacts with several vital inner membrane proteins, including anion carriers and respiratory chain complexes [11–13,35,36]. An involvement of cardiolipin in the execution phase of the apoptosis process has been also suggested [37,38]. It has also been reported that this phospholipid is specifically required for the electron transfer in complex I and complex III of the mitochondrial respiratory chain [10,17,39–41]. In addition, an involvement of cardiolipin in higher-order organization of these components of the respiratory chain in a supercomplex in the mitochondrial inner membrane has been proposed [42]. Thus, changes in the mitochondrial content of cardiolipin due to alterations of one of the enzymatic steps involved in its biosynthetic process [43,44] or as consequence of oxidative damage by ROS attack may affect the activity of complex I and complex III. As reported in Fig. 5, a marked decrease in the cardiolipin content is observed in mitochondria isolated from normoxic reperfused rat heart which was partially prevented by hypoxic reperfusion. These changes in the cardiolipin content are due to ROS-induced cardiolipin peroxidation as shown by changes in the level of conjugated dienes (see Fig. 6). The preservation of mitochondrial cardiolipin content results in a reduced alteration in complex I and complex III activity and hence, in a lower production of mitochondrial oxygen radicals.

It has been reported that the production of oxygen radicals occurs during the early phase (first minutes) after cardiac reperfusion (4, 5, 19). Our results are in line with these data showing that a significant mitochondrial ROS production occurs during the first 2 min of reperfusion, followed by a persistently generation thereafter. This early ROS production is markedly reduced by hypoxic condition. These data suggest that the cardioprotective effect of hypoxia against the ischemia/reperfusion injury can be ascribed, at least in part, to the reduction of mitochondrial oxygen radical generation with subsequent preservation of cardiolipin integrity, prevention of respiratory chain complexes impairment and protection of mitochondrial function.

Mitochondrial permeability transition pore (mPTP) is considered an important factor in heart ischemia/reperfusion injury and subsequently a possible target of cardioprotection [45]. It has been reported that in isolated rat heart mPTP does not open during the ischemic period, but does open between 2 and 5 min after the onset of reperfusion [46]. Several factors may be involved in pore opening during reperfusion such as Ca^{++} overload, ATP depletion, increased Pi, low lactate (higher pH respect to ischemic condition) [46,47]. ROS generation is also considered a key factor in pore opening, although it is still unclear whether ROS generation is merely a consequence of pore opening or it is an integral part of the signalling machinery of mPTP opening. It has been reported that postconditioning inhibits mPTP and this effect might be related to an attenuation of oxygen-derived free radical production by the respiratory chain in the early minutes of reflow [48]. In addition, hypoxic postconditioning has been shown to reduce ROS production in cardiomyocytes due to limitation of substrate oxygen [49]. In line with these data, our results suggest a lower ROS generation under hypoxic reperfusion as shown by the decrease in conjugated dienes formation (an index of lipid peroxidation due to ROS attack) and by the attenuation of the basal production of H_2O_2 by complexes I and III, which are considered the main producers of peroxide in mitochondria. Due to the role played by ROS in mPTP opening, it is possible that a lower ROS generation under hypoxic reperfusion may delay or attenuate the probability of mPTP opening and this may also contribute to limit mitochondrial dysfunction and to prevent heart damage.

In conclusion, our results demonstrate that the restriction of oxygen delivery to the reperfused rat heart attenuates the alterations to mitochondrial oxidative metabolism and limits cardiac reperfusion injury. This effect appears to be due partly to

the reduction of mitochondrial oxygen radical generation during the early phase of reperfusion. The clinical application of oxygen radical scavengers to the treatment of the stunned myocardium has been disappointing due to the limited ability of most antioxidants to penetrate cell membranes. Controlling the oxygen tension during early reperfusion may represent a potential experimental strategy, in alternative to or in association with antioxidants, in preventing mitochondrial dysfunction and cardiac reperfusion injury.

5. Uncited reference

[30]

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