Enzyme Release and Mitochondrial Activity in Reoxygenated Cardiac Muscle: Relationship with Oxygen-Induced Lipid Peroxidation

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Abstract. The aim of this work was to precisely determine the sites of the peroxidative action on unsatured lipids by oxygen-derived free radicals and the lytic cell damage on reoxygenated perfused hearts. The cellular load of lipid peroxidation products (malondialdehyde) during the reoxygenation was dependent on PO_2. This unfavorable biochemical response was linked to creatine kinase leakage, alteration of coronary flow and mitochondrial injury. When an enzymatic (superoxide dismutase, 290 IU/minute) or tripeptide scavenger of oxygen radicals (reduced glutathione, 0.5 mmol/l) was administered at the end of hypoxia and during reoxygenation, the abnormal intolerance of hypoxic heart to molecular oxygen was significantly weakened; the load of lipid peroxides load, enzyme release, and vascular alteration were all reduced. Moreover, mitochondrial activity was enhanced and the oxygen-induced uncoupling of mitochondrial remained limited: both the respiratory control ratio (RCR) and the ADP/O ratio were higher than in control reoxygenated hearts. The inhibition by rotenone (100 μmol/l) of reoxidation of electron chain transfer during oxygen readmission also reduced the unfavorable cardiac accumulation of lipid peroxidation products and the release of creatine kinase.

These data demonstrate that in the oxygen paradox, the peroxidative attack on lipids plays an important role in inducing alterations of sarcolemmal permeability and mitochondrial activity. An uncontrolled reactivation of oxidative function of mitochondria during reoxygenation enhances the synthesis of oxygen-derived free radicals and triggers the peroxidation of cardiac lipids resulting in irreversible injury to cellular and intracellular membranes.

Key words: Oxygen paradox — Lipid peroxidation — Mitochondrial function

Introduction

Myocardial reperfusion of reoxygenation injury may be ascribed to an abnormal oxygen intolerance (Hearse et al. 1973; Gauduel et al. 1979; Shlafer et al. 1982; Tilton et al. 1983; Jolly et al. 1985; Mc Cord 1985). In particular toxic effects of oxygen are observed upon its renewed supply to hypoxic myocardium (Hearse 1978). It is now well established that reoxygenation of hypoxic myocardium induces structural abnormalities (Ganote et al. 1980; Humphrey et al. 1984) and release of cellular enzymes (Ganote and Kaltenbach 1979). The sudden cellular injuries, triggered by oxygen readmission (the oxygen paradox phenomenon), are dependent on oxygen tension and on reoxidation of the mitochondrial electron transfer chain (Hearse 1978; Ganote et al. 1980). Recent studies have shown that the "oxygen paradox" is also characterized by an abnormal intracellular accumulation of lipid hydroperoxides and by a deleterious generation of oxygen free radicals: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$) (Rao et al. 1983; Gauduel and Duvelleroy 1984; Hess and Mason 1984; Hammond and Hess; Myers et al. 1985). The above cytotoxic oxygen metabolites (lipid peroxides, oxygen-free radicals) have been suspected to be the cause of oxygen-induced cellular injury (Meerson and Ustinova 1982; Stewart et al. 1983; Scott et al. 1985). However, the exact mechanism of biochemical responses leading to free radical-initiated lipid peroxidation and to irreversible cellular injury remains to be clarified.

The aim of this study was to establish the effects of oxygen tension on lipid peroxidative action in reoxygenated hearts and to investigate the temporal relationship between oxygen-induced lipid peroxidation and cellular or subcellular damage.

Oxygen-derived free radical scavengers (reduced glutathione and superoxide dismutase) were used to assess the effects of cytotoxic oxygen metabolites on sarcolemmal permeability and mitochondrial function in reoxygenated heart. In addition, experiments were performed with rotenone to precise the role of mitochondrial reoxidation on the peroxidative attack on unsatured lipids during the oxygen paradox.

Materials and Methods

Experimental system and procedure

Male rats of the Wistar strain were anesthetized with diethylether inhalation. Heparin (100 IU) was injected into the femoral veins and the hearts were quickly removed, washed with cold isotonic saline solution and cannulated on a non-recirculating Langendorff system (1895). Immediately after mouting, the heart was perfused with oxygenated Krebs-Henseleit buffer (pH 7.4) delivered to the

aortic inflow canula at a mean pressure of 10 kPa. The solution used during this initial aerobic perfusion contained (in mmol/l): NaCl 115, KCl 5.6, $MgSO_4$ 0.5, NaH_2PO_4 1.17, $NaHCO_3$ 28, $CaCl_2$ 2.5 and glucose 11. This buffered solution (pH 7.4) was equilibrated with 95 % O_2 and 5 % CO_2 and maintained at 37 °C (PO_2: 650 mm Hg).

Perfusion procedure

After a 20 minutes stabilization period, the heart was subjected to hypoxia for various intervals (15,30,60 minutes) by perfusing it modified Krebs Henseleit bicarbonate buffer without substrate. The solution used during the hypoxic perfusion contained (in mmol/l): NaCl 105, KCl 16, $MgSO_4$ 0.5, $NaHPO_4$ 1.17, $NaHCO_3$ 28, $CaCl_2$ 2.5. The elevation of potassium from 5.6 to 16 mmol/l with a corresponding decreases in Na^+ concentration prevented the occurrence of hypoxia-induced cardiac arrhythmias and providen more reproducible experimental conditions. The buffer solution was gassed with a 95 % N_2, 5 % CO_2 mixture, yielding an arterial pO_2 lower than 5 mm Hg. The heart was protected from atmospheric gas contamination by using glass connections and enclosing the heart in a water jacketed chamber continuously gassed with the same gas mixture as that used for the perfusion fluid. pO_2 of the perfusion medium was monitored in the aortic cannula using standard electrodes (Radiometer pHM-72).

After various intervals of hypoxia, the heart was reoxygenated for 20 minutes with Krebs bicarbonate buffer at 37 °C, containing 11 mmol/l glucose and gassed to reach various level of arterial pO_2 (10, 150 and 650 mmHg).

Administration of antioxidants

Two antioxidants were used in these studies: the reduced from of glutathione (GSH) and superoxide dismutase (SOD, EC 1.11.1.1) purified from bovine erythrocytes. The reduced from of glutathione (0.5 mmol/l) was administered in the perfusion medium during the last ten minutes of hypoxia and during reoxygenation. Superoxide dismutase with perfusate as diluent (specific activity: 2800 IU/mg protein) was pulse administered (280 IU in 0.1 ml) through the aortic cannula every minute during the last ten minutes of hypoxia and every minute during reoxygenation (total injection of 84.000 IU).

Analytical procedures

Lipid peroxidation

Malondialdehyde (MDA) produced in the perfused hearts as a result of free-radical mediated generation of lipid peroxidation products was determined in heart homogenates and venous effluent by the thiobarbituric acid method (Bernheim et al. 1948). Samples of ventricular tissue (500 mg) were homogenized and precipitated with 20 °% trichloracetic acid (2 ml per 500 mg tissue). The homogenate was centrifuged in an RC5 refrigerated centrifuge (Sorvall) for 15 minutes at 1100*xg* and the resulting supernatant was added to 1 ml of aqueous thiobarbituric acid (0.75 %). The mixture was then incubated at 100 °C for 20 minutes. After cooling down to 21 $\pm$ 1 °C, the amount of MDA in the samples was determined by measuring optical density at 535 nm using a dual beam spectrophotometer PERKIN ELMER 551 and ε_{551} equal to 156 l/mol/cm (Sinnhuber and Yu 1958). The reference control sample contained glucose to avoid artifactural readings of assays of reoxygenated hearts. The amount of effluent malodialdehyde was expressed in nmol/min/g wet wt and the cellular MDA content in nmol/g cardiac protein.

Mitochondrial preparation

After the required interval of perfusion, the hearts were plunged into an ice-cold mitochondrial isolation medium (KCl 0.18 mol/l, EDTA 10 mmol/l, albumin 0.5 %, pH adjusted to 7.4 at 4 °C) and homogenized with ultra-turrax (15 seconds at maximun speed). Mitochondria were subsequently isolated by the method of Sordahl et al. (1971). The mitochondrial pellet was suspended in the isolation medium at a final concentration of 15 mg protein/ml. Protein was determined by the biuret method, using bovine serum albumin as a standard. Mitochondrial function was determined polarographically at 30 °C using an oxymeter (OXY Y53) equipped with a Clark O_2 electrode. The incubation medium contained sucrose (250 mmol/l), K_2HPO_4 (10 mmol/l), tris HCl (pH 7.0) and glutamate 5 mmol/l. The final reaction volume was 2.5 ml and it contained 1.5 mg mitochondrial protein/ml. The following indices of mitochondrial function were determined: oxygen uptake (state 3 or 4) (n.atoms oxygen uptake/mg mitochondrial protein/minute); respiratory control ratio (RCR): ratio of oxygen consumed in the presence of ADP (350 nmoles) to that taken up after ADP phosphorylation (nmol of ATP produced/n.atoms oxygen consumed).

Enzyme assays

The release of enzyme in the venous effluent was studied to define the sarcolemmal permeability of reoxygenated cardiac cells. Creatine kinase (CK) activity was determined with the optimized method of Siegel and Cohen (1974) using an automatic dual beam spectrophotometer PERKIN-ELMER 551, equipped with thermostable cuvettes (25 °C) and enzymatic PERKIN-ELMER 5—100 calculator. Enzymic activity was expressed in international units. The amount of CK released during reoxygenation was quantified by integrating the area under the curves.

All the solutions were prepared with deionized-distilled water. The enzymes and chemical reagents used were of the finest grade and were purchased from Sigma Chemical Company, St. Louis and Boehringer Mannheim.

Statistical analysis

All values are reported as mean ± SEM; two-way analysis of variance and Scheffe test for multiple comparisons among pairs of means were used for statistical analysis and the 5 % level was used as the probability limit.

Results

Lipid peroxidation and sarcolemmal permeability

Peroxidation of polyunsatured cardiac lipids was studied in relation of MDA accumulation in tissue and MDA release in the coronary effluent. During hypoxic perfusion, the peroxidative action of lipids remained very low (Table 1). By contrast, readmission of oxygen interacted upon both the cellular MDA content and MDA leakage. The results summarized in Table 1 show that the extent of lipid peroxidation was dependent on the duration of the hypoxic interval and on arterial pO_2 during the reoxygenation phase. In particular, a

Table 1. Effect of oxygen tension on cellular MDA content and MDA release during reoxygenation of hypoxic hearts. Each value (mean ± SEM) represents the average of five separate experiments.

Conditions	MDA Content (nmol/g cardiac protein)	MDA Release (nmol/20 min reox/g wet weight)
	80 minutes oxygention	
(pO_2 = 650 mm Hg)	48 ± 2	—
	80 minutes hypoxia	
(pO_2 < 5 mm Hg)	50 ± 1	—
	30 minutes hypoxia + 20 minutes reoxygenation	
pO_2 Reox.		
10 mm Hg (1)	52 ± 2	12 ± 0.8
150 mm Hg (2)	61 ± 2*	18 ± 1*
650 mm Hg (3)	97 ± 3$	102 ± 5$
	60 minutes hypoxia + 20 minutes reoxygenation	
pO_2 Reox.		
10 mm Hg (4)	54 ± 3	14 ± 1
150 mm Hg (5)	92 ± 4*	26 ± 3*
650 mm Hg (6)	170 ± 5$	157 ± 9$

(1) vs (2) and (4) vs (5) *: $p < 0.05$
(2) vs (3) and (5) vs (6) $: $p < 0.05$

significant lipid peroxidation was observed in hearts submitted to 60 minutes of oxygen deprivation with subsequent 20 minutes of reoxygenation at a high pO_2 (650 mm Hg). The inhibition of the generation of abnormal oxygen radicals in reoxygenated hearts by exogenous antioxidants (SOD or GSH) reduced MDA release upon oxygen readmission at high pO_2 (Fig. 1).

In our experimental conditions, the deleterious effect of oxygen readmission on the cardiac lipid peroxidation was directly related with the sudden extension of cell membrane damage demonstrated by the creatine kinase leakage (Fig. 2). This figure illustrates the relationship between cellular injury of reoxygenated cardiac muscle and the amount of MDA produced following the start of reoxygenation in the presence or absence of antioxidants. Regardless of the duration of hypoxia (15, 30, 60 minutes) both the CK release and MDA concentration in heartes reoxygeneted in the presence of SOD or GSH were significantly lower than in hearts reoxygenated with oxygen radiacal scavenger-free solution. Figure 3 shows that the free radical scavengers prevented the development of the "no reflow" phenomenon in reoxygenated hearts.

Lipid peroxidation and mitochondrial function

Representative samples of mitochondria isolated from each experimental group

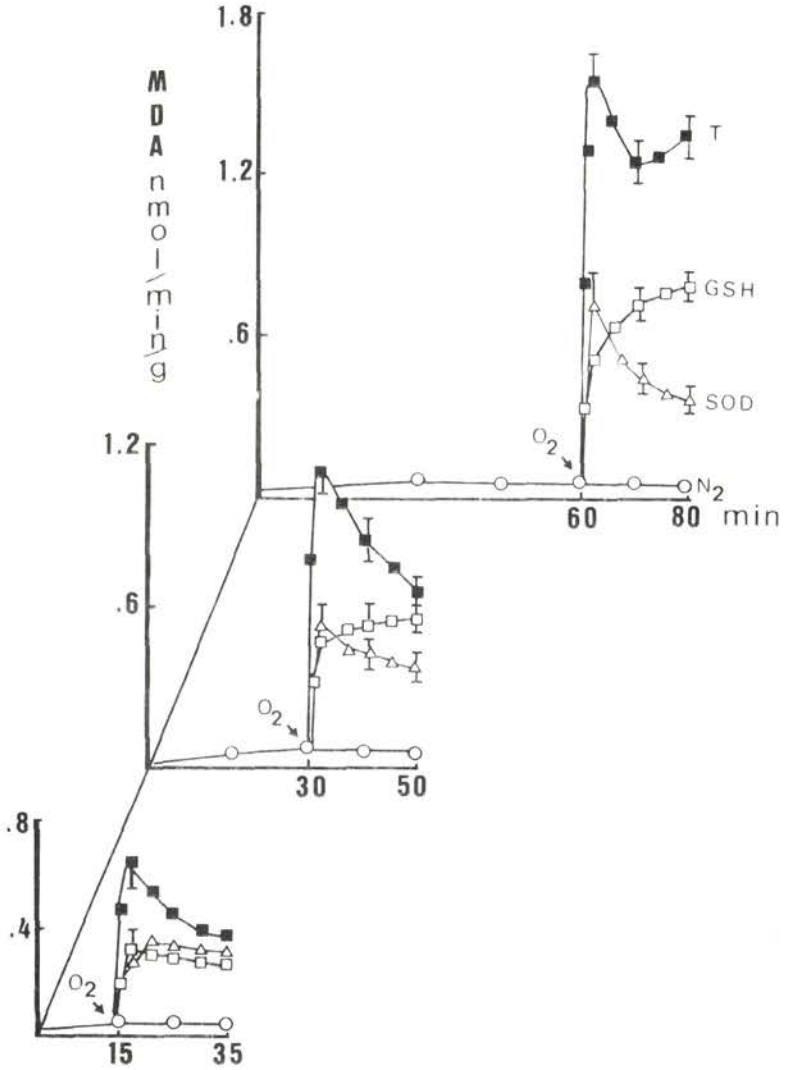

Fig. 1. Malondialdehyde release curves during oxygen deprivation (15,30 or 60 minutes) and following reoxygenation (20 minutes) with high arterial pO_2 (650 mm Hg). ■ Untreated hearts ($n = 6$), □ hearts reoxygenated in the presence of GSH ($n = 5$), △ hearts reoxygenated in the presence of SOD ($n = 5$), ○ hypoxic hearts alternatively perfused without (15, 30 or 60 minutes) and with glucose for 20 minutes. The results are expressed in nmol/min/g wet weight.

were studied polarographically. The results shown in Table 2 demonstrate that hypoxic injury results in progressive alteration of mitochondrial function. Reoxygenation of hypoxic hearts deprived of oxygen for 15,30 or 60 minutes

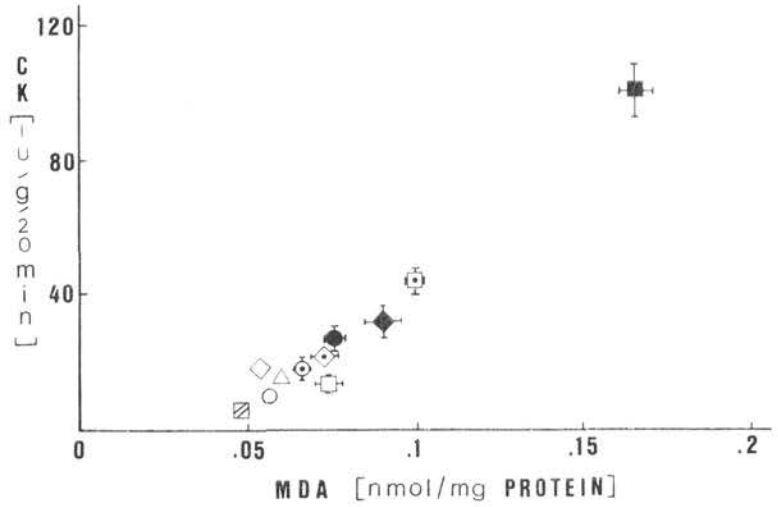

Fig. 2. Relationship between creatine kinase release during reoxygenation (20 minutes, pO_2 = 650 mm Hg) and tissue MDA concentration at the end of reoxygenation. The points for earch group represent mean ± SEM of five experiments. ▨: hypoxic hearts (60 minutes). Triangle: hypoxic hearts (60 minutes) reoxygenated at pO_2 = 150 mm Hg. Squares: hearts reoxygenated at pO_2 = 650 mm Hg after □ 15, ⊡ 30, ■ 60 minutes of hypoxia. Diamonds: heart reoxygenated in the presence of GSH (pO_2 = 650 mmHg) after ◇ 15, ◈ 30, ◆ 60 minutes of hypoxia. Circles: hearts reoxygenated with SOD (pO_2 = 650 mm Hg) after ○ 15, ◉ 30, ● 60 minutes of hypoxia.

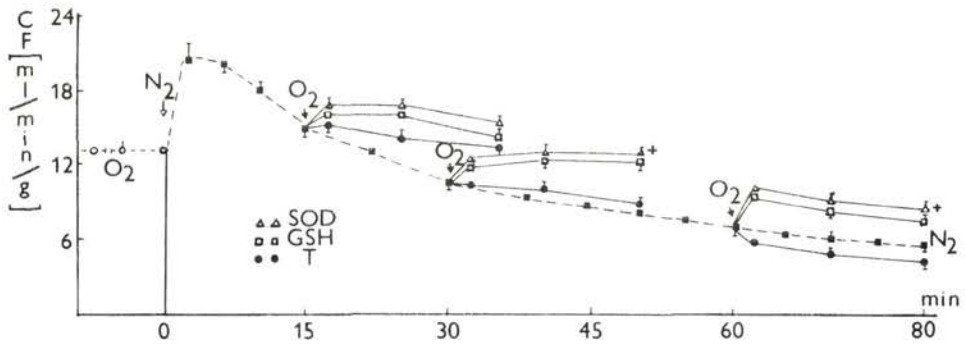

Fig. 3. Coronary flow in isolated perfused hearts following the start of hypoxia (15, 30 or 60 minutes) and reoxygenation (20 minutes) at high arterial pO_2 (650 mm Hg). The arrows indicate the start of reoxygenation. ■ : untreated hypoxic hearts (n = 5); ● untreated reoxygenated hearts (n = 5); □ : hearts reoxygenated in the presence of GSH (n = 5); △ : hearts reoxygenated in the presence of SOD (n = 5).

resulted in additional mitochondrial injury characterized by a fall in RCR due to partial uncoupling and a decrease of the respiratory capacity (state 3 rate). When hearts were reoxygenated in the presence of reduced glutathione or

Table 2. Average values of oxygen consumption of mitochondria isolated from hearts subjected to various intervals of hypoxia followed by reoxygenation at high pO_2 (650 mm Hg). Values are means ± SEM.

Conditions of perfusion	RCR	ADP/O	STATE 3 rate (mA 0/min/g cardiac protein)	STATE 4 rate
		Aerobic heart (non perfused) ($N = 4$)		
	15.1 ± 1	3.24 ± 0.09	132 ± 7	8.7 ± 0.5
		Hypoxic heart ($N = 5$)		
35 minutes	8.4 ± 0.2	2.65 ± 0.08	94 ± 3.8	11.1 ± 0.3
50 minutes	7.1 ± 0.6	2.37 ± 0.1	66 ± 3.4	9.2 ± 0.6
80 minutes	3.7 ± 0.1	1.75 ± 0.06	24 ± 0.5	6.5 ± 0.2
		Reoxygenated heart ($N = 5$)		
		15 minutes N2 + 20 minutes 02		
Control	7.5 ± 0.5§	2.8 ± 0.1	79 ± 7§	10.6 ± 0.9
GSH	9.5 ± 0.2§	2.9 ± 0.1	107 ± 7*	11.2 ± 0.9
SOD	10.2 ± 0.6*	2.9 ± 0.05	105 ± 6*	10.9 ± 0.6
		30 minutes N2 + minutes 02		
Control	4.4 ± 0.2§	1.9 ± 0.1§	60 ± 4	15 ± 0.5§
GSH	8.2 ± 0.4*	2.6 ± 0.1*	88 ± 6*	10 ± 0.6*
SOD	7.4 ± 0.3*	2.7 ± 0.1*	84 ± 7*	11 ± 0.8*
		60 minutes N2 + 20 minutes 02		
Control	1.6 ± 0.1§	1.5 ± 0.05§	11 ± 0.3§	7.2 ± 0.2
GSH	5.2 ± 0.2*	2.5 ± 0.1*	35 ± 1*	6.9 ± 0.1
SOD	5.6 ± 0.1*	2.6 ± 0.1*	38 ± 1*	6.9 ± 0.1

For all conditions: GSH vs Control and SOD vs Control.*: $p < 0.05$.
Hypoxic heart vs Control reoxygenated hearts. §: $p < 0.05$.

superoxide dismutase, mitochondrial function was significantly improved in comparison with untreated reoxygenated hearts as evidenced by the higher values of the respiratory control ratio, the state 3 and the ADP/O ratio.

For the different experimental groups (untreated hearts, hearts reoxygenated in the presence of GSH or SOD, the mitochondrial respiratory control ratio and the cellular content of MDA (Fig. 4). The high correlation coefficient ($r = 0.95$) suggests that a direct linear relationship exists between the uncoupling of mitochondrial function and the extent of oxygen-induced MDA production in cardiac cells.

In a separate set of experiments, we studied the effect of reoxydation of mitochodria on the peroxidative action on reoxygenated cardiac muscle. Hearts were deprived of oxygen for 15, 30 or 60 minutes (pO_2 less than 5 mm Hg) and then reoxygenated for 20 minutes under different arterial pO_2 (10, 150 or

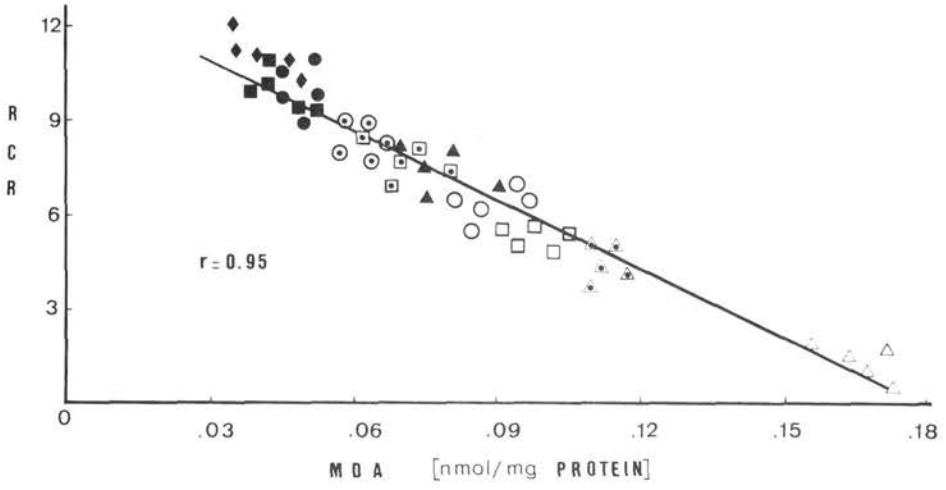

Fig. 4. The linear relationship between the mitochondrial RCR and the MDA tissue levels. The hearts were alternatively submitted to hypoxia (△ 15, △ 30 or ▲ 60 minutes) and reoxygenation (20 minutes, pO_2: 650 mm Hg). Diamonds: aerobic perfused hearts; triangles: untreated heartes; circles: heartes reoxygenated in the presence of SOD; squares; hearts reoxygenated in the presence of GSH.

Table 3. Effect of rotenone (100 μmol/l) on MDA concentration in the reoxygenated cardiac muscle. The concentration of MDA is expressed in nmol/g cardiac protein. Each value is the mean + SEM of five experiments. Rotenone (R) vs control (C) *: $p < 0.05$.

Conditions	Arterial pO_2 during reoxygenation					
	10 mm Hg		150 mm Hg		650 mm Hg	
	C	R	C	R	C	R
15 min. N2 + 20 min. 02	51 ± 1	52 ± 3	55 ± 3	52 ± 5	63 ± 5	53 ± 4 *
30 min. N2 + 20 min. 02	52 ± 2	50 ± 2	61 ± 2	55 ± 1 *	97 ± 3	58 ± 3 *
60 min. N2 + 20 min. 02	54 ± 3	55 ± 6	92 ± 4	62 ± 7 *	170 ± 8	99 ± 5 *

650 mm Hg). During oxygen readmission, mitochondrial respiratory activity was blocked with rotenone (100 µmol/l). The results summarized in Table 3 demonstrate that oxygen-induced cardiac lipid peroxidation was partially de-

pendent on events mediated by the resumption of the mitochondrial respiratory activity. Even under a high arterial pO_2, reoxygenation in the presence of rotenone of hypoxic hearts did not enhance the peroxidative attack on lipids and the CK leakage remained reduced (55 ± 5 IU) in comparison with untreated reoxygenated hearts (105 ± 8 IU).

Discussion

The experimental data presented in this study provide evidence that the peroxidative action on lipids has deleterious consequences on reoxygenated cardiac muscle. Under different conditions of reoxygenation (normal or high pO_2) the extent of lipid peroxidation, measured as MDA production and the rate of creatine kinase release, are dependent on arterial oxygen tension (pO_2). This relationship suggests that the peroxidative action on cardiac lipids induces alterations of sarcolemmal permeability and release of cellular enzymes.

SOD and GSH-treated hearts showed greater post-ischemic coronary flow than reoxygenated control hearts. Lipid hydroperoxides that result from the action of free-radicals on membranes have been reported to impair postaglandin synthesis in a way that promotes the vasoconstrictive thromboxane A2 (Shlafer et al. 1982; Stewart et al. 1983). Thus, a more effective inactivation of the oxygen radical load due to GSH and SOD supplementation might account for the better preservation of posthypoxic coronary flow patterns in these hearts.

Whatever the duration of hypoxia, exogenous SOD reduced the deleterious effects of oxygen readmission on the peroxidative action on lipids, sarcolemmal permeability, vascular bed and mitochondrial function. In accordance with the report of Zimmermann et al. (1973), SOD reduced MDA accumulation during the oxygen paradox because this enzyme is able to inhibit lipid peroxidation mediated by the superoxide anion. The ability of this enzyme to reduce the development of oxygen paradox can be also explained by the inhibitory effect of the enzyme on the production of the hydroxyl radical ($OH^{\bullet}$) through a Haber-Weiss reaction, iron-catalyzed superoxide dependent OH formation or autoxidation of enzymatic systems such as xanthine-oxidase system localized in the vascular endothelium (Kellogg and Fridovich 1975; Korthuis et al. 1985). Thus, the protective effects of SOD against oxygen-mediated sarcolemmal damages during reoxygenation seems to be twofold: i) inactivation of superoxide anion (Fridovich 1975); ii) avoidance of peroxidative action on unsatured lipids or denaturation of proteins in sarcolemma or endothelium.

The beneficial effect of exogenous GSH during reoxygenation may be due to the improvement of the cellular glutathione status (GSH/GSSG ratio) which is normally reduced by hypoxia (Guarnieri et al. 1980). Consequently, the

exogenous GSH adminstration at the end of hypoxia and during reoxygenation helps preventing both the decrease of the GSH/GSSG ratio (Haugaard 1968; Halliwell 1978) and induction of the decomposition of lipid peroxides (ROO•) (Chance et al. 1979).

In agreement with the available date in the literature (Nohl and Hegner 1978); Narabayashi et al. 1982; Nakanishi et al. 1984), the relation observed in own experiments between MDA levels and RCR of mitochondrial suspensions ($r = 0.95$) suggests that abnormal peroxidation of cardiac lipids may be induced by the functional alterations of reoxidized mitochondria during the oxygen paradox.

Recent studies have demonstrated that mitochondria can be one of the most important sources of superoxide anion (Loschen et al. 1973; Cadenas et al. 1981), especially from the ubiquinone-reduced form and NADH dehydrogenase (Takeshige 1979). The direct effect of pO_2 on the functional damages of mitochondria during the oxygen paradox, the extent of lipid peroxidation and membrane permeability changes suggests that reoxidation of the respiratory mitochondrial chain is the principal triggering mechanism of the generation of oxygen-derived free radical at the subcellular level. These toxic oxygen metabolites favour peroxidative action on unsatured fatty acids in myocytes. Owing to the fact that rotenone inhibits the formation of O_2^- and H_2O_2 in complex NADH-ubiquinone reductase (Forman and Boveris 1982) it is likely that during reoxygenation these two free radicals interfere with the intracellular lipid peroxidation, MDA accumulation and sarcolemmal permeability alterations. In accordance with previous works (Augustin et al. 1979; Nakanishi et al. 1984), it can be suggested that the protective effect of GSH on MDA accumulation and mitochondrial function is linked to an increase of glutathione peroxidase activity and a better control of permeability of mitochondria for calcium during oxygen readmission.

It should be stressed that GSH and SOD, which have different molecular weights and diffusion coefficients, have beneficial effects on the integrity of membranes and subcellular organels. The presented findings suggest that i) the scavengers tested could at least gain access to the interstitial space and probably penetrate into the cells; ii) the chain reaction of lipid peroxidation mediates membrane damage.

In conclusion, our results provide additional evidence that the toxic effects of oxygen readmission on hypoxic myocardium is due to the generation of oxygen derived free radicals in the cellular compartment. The abnormal oxidative reactivation of mitochondria will be one of the factors involved in their production. The toxic radicals have a deleterious peroxidative action on unsatured lipid components of cellular and mitochodrial membranes. The

propagation of the peroxidative injury to membranes during oxygen readmission might trigger the development of ionic abnormalities and arrhythmias.

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