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Brief Communication

OXIDATIVE INJURY IN REOXYGENATED AND REPERFUSED HEARTS

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Abstract—In this study, we separated the effects of low oxygen supply and low coronary flow in isolated perfused rat hearts to focus on the genesis of free radicals-induced reperfusion injury. Hearts were exposed to either hypoxemia/reoxygenation or ischemia/reperfusion in various sequences, with hypoxemia and ischemia matched for duration (20 min), temperature (37°C), and oxygen supply (10% of baseline). Hypoxemia/reoxygenation ($n = 7$) resulted in lower (developed pressure) × (heart rate) ($p < 0.001$) and higher end-diastolic pressure ($p < 0.001$) than ischemia/reperfusion ($n = 9$). The presence of 40 IU/ml superoxide dismutase and 104 IU/ml catalase nearly blunted the rise of the end-diastolic pressure ($p = 0.02$ vs. baseline), but could only partially prevent the depression of myocardial contractility ($p < 0.001$ vs. baseline, $n = 7$). Similar patterns were observed when hearts were made ischemic after hypoxemia, eliminating the intermediate reoxygenation step. We conclude that the major determinant of the reperfusion injury is associated with low oxygen supply rather than low coronary flow. Part of the injury is mediated by oxygen-derived free radicals, but a substantial portion of it is associated with energetic processes.

Keywords—Ischemia, Hypoxemia, Reperfusion, Reoxygenation, Ultrastructure, Oxygen, Coronary flow, Free radicals

INTRODUCTION

Myocardial ischemia is a multifactorial situation characterized by unmatched demand of energy and supply of blood. Ischemia (low coronary flow) normally implies a situation of low supply of O_2 (hypoxemia). Although overlapped, the two conditions are distinct, and in distinguishing between them it may be important to focus on the role of O_2 and its metabolites in the reperfusion-induced injury,[1] and hence to design suitable therapeutic approaches to protect the ischemic and/or hypoxemic myocardium. Comparing the characteristics of ischemic and hypoxemic hearts may provide a tool for this task, but is difficult due to the necessity of matching the two stresses. However, the isolated heart perfused with buffer at fixed coronary flow may be a suitable experimental model because one can control several of the factors that determine the size of the reperfusion injury, i.e., the duration of the stress, the temperature and the O_2 gradient.[2] The last factor was here expressed as the total O_2 supply to the heart: (coronary flow) × (O_2 content).

The aim of this study was to test whether the injury caused by hypoxemia is comparable to that caused by ischemia when the two stresses are similar for the O_2 supply. With such an approach, one can study the separate roles of coronary flow and oxygen tension within an ischemic episode. We show that the two situations were different in our model because the injury associated with hypoxemia/reoxygenation was much more severe than that observed during ischemia/reperfusion. Further, we assess the role of the O_2-derived free radicals and show that their effect was more pronounced when investigating the coronary function rather than the ventricular function.

MATERIALS AND METHODS

Reagents and apparatus

The buffer was 115.6 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM ethylenediaminetetraacetic

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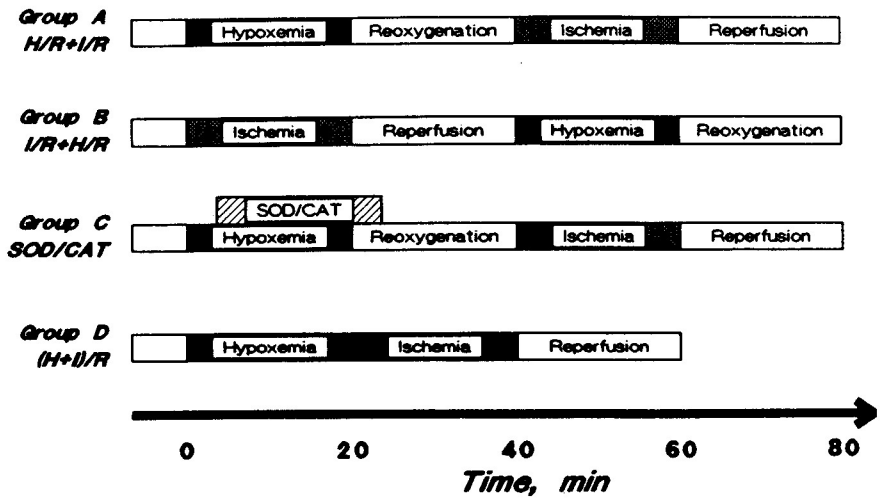


Fig. 1. Scheme of the experimental protocols devised for this study. During baseline, reperfusion, and reoxygenation, hearts were perfused at coronary flow = 15 ml/min and PO_2 = 670 mmHg. During hypoxemia, the PO_2 was reduced to 67 mmHg. During ischemia, the coronary flow was reduced to 1.5 ml/min. Each phase was 20 min long, and all the experiments were performed at 37°C.

acid (EDTA), 1.2 mM Na_2SO_4, 28.5 mM $NaHCO_3$, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 16.6 mM glucose, pH 7.4 at 37°C. Superoxide dismutase (EC 1.15.1.1, bovine erythrocytes) and catalase (EC 1.11.1.6, beef liver) were purchased from Boehringer Biochemia Robin (Milano, Italy). The buffer was equilibrated in two bubble oxygenators (Shiley model S-100A, Irvine, CA) with either 94% O_2 + 6% CO_2 or 9.4% O_2 + 6% CO_2 (balance N_2, nominal accuracy ± 0.01%) to yield PO_2 = 670 or 67 mmHg, respectively, at constant PCO_2 (43 mmHg) and pH (7.4). A roller pump (Watson Marlow model 503-S, Falmouth, England) forced the buffer through 8 μm pore size, 47 mm diameter polycarbonate filters (Nuclepore Corp., Pleasanton, CA) to the aortic cannula at flow rates of either 15 or 1.5 ml/min. The oxygenators, the aortic cannula, the O_2 electrode, and the heart chamber were kept at 37°C by an external water bath with the heart submerged in a buffer-containing water jacket kept at the same temperature. The maximum temperature oscillation, measured by a thermocouple in the right atrium, never exceeded ± 0.2°C regardless of the coronary flow.

Hearts from male Sprague Dawley rats (weight 250–280 g), anesthetized by intraperitoneal heparinized sodium thiopental (10 mg/100 g body weight), were mounted onto the aortic cannula, and the retrograde perfusion was started immediately (ischemia time < 30–45 s). The coronary sinus return was collected through an outflow cannula in the pulmonary artery, and an inflatable, saline-filled Latex balloon was introduced into the left ventricle. The volume of the balloon was such that the rise of pressure was negligible when inflated by 150 μl.

Measurements

The coronary pressure (CP) was monitored by a pressure transducer (Harvard Apparatus model 52-9966, Natick, MA) connected to the aortic cannula. An additional transducer connected to the balloon provided the end-diastolic pressure (EDP), left-ventricle developed pressure (LVDP) and heart rate (HR). The product LVDP · HR is an integrated index of the myocardial contractility[3] because the hearts were not paced. The coronary sinus (cs) return was analyzed for the PO_2 (YSI model 5300 Oxygen Monitor, Yellow Springs Instrument Co. Inc., Yellow Springs, OH) and PCO_2/pH (IL 1304, Paderno Dugnano, Italy).

Ultrastructure analysis was performed on all hearts. Three or four specimens from each left ventricle were fixed for 2–3 h in 2.5% cold glutaraldehyde, rinsed in 0.1 M cacodylate buffer (pH 7.4), postfixed with 1.5% OsO_4, dehydrated in ethyl alcohol and propylene oxide, and embedded in an epoxy resin (Epon-Araldite). Ten semithin sections from each specimen were stained with toluidine blue and safranine to choose areas free of artifacts, and thin sections, stained with uranyl acetate and lead citrate, were finally observed in a CEM 902 (Zeiss Ikon, Germany) electron microscope. To evaluate the ultra-structural damage, we considered nuclear margination and clumping of chromatin, mitochondrial enlargement, changes in myocardial fibers, interstitial edema, and

Table 1. Myocardial Performance During Baseline, Hypoxemia (Group A), and Ischemia (Group B) at Volume of the Intraventricular Balloon Set to Yield EDP ≈ 10 mmHg During Baseline

Parameter	Baseline	Hypoxemia	p	Ischemia
n	33	7		9
Coronary flow, ml/min	15	15	na	1.5
Buffer PO_2, mmHg	670	67	na	670
O_2 supply, μmoles/min	14.1	1.41	na	1.41
EDP, mmHg	10.6 ± 0.5	53.8 ± 3.9	< 0.0001	6.0 ± 0.4
CP, mmHg	70.9 ± 3.0	69.7 ± 3.1	< 0.0001	8.1 ± 0.5
HR, min^{-1}	262 ± 5	213 ± 20	0.0006	109 ± 14
LVDP, mmHg	134.7 ± 4.7	26.1 ± 3.3	NS	27.9 ± 4.4
LVDP · HR, mmHg × 10^3/min	34.9 ± 1.2	5.1 ± 0.3	0.0004	2.7 ± 0.4
$P_{cs}O_2$, mmHg	222 ± 10	10 ± 2	0.027	33 ± 8
$P_{cs}CO_2$, mmHg	51 ± 1	46 ± 1	0.004	62 ± 4
pH_{cs}	7.29 ± 0.01	7.33 ± 0.01	0.001	7.19 ± 0.03
O_2 uptake, μmoles/min	9.41 ± 0.21	1.19 ± 0.05	0.01	1.33 ± 0.02

NS = not significant. na = not applicable, cs = coronary sinus.

Ischemia and hypoxemia were equivalent for duration (20 min), temperature (37°C), and O_2 supply (10% of baseline). Data taken at the end of 20 min periods under stable conditions. The significance refers to the ischemia versus hypoxemia comparison (Student's t-test for unpaired observations).

hemorrhage, assigning scores from 1 (no or mild changes) to 4 (marked or severe changes).[4]

Experimental design

The hearts were stabilized for 30 min at coronary flow rate = 15 ml/min and perfusate PO_2 = 670 mmHg. The O_2 supply to the heart (14.1 μmoles/min) was calculated from the assumed O_2 solubility coefficient in water at 37°C (1.4×10^{-6} M O_2/mmHg [ref. 5]). The balloon was inflated to yield EDP = 10 ± 1 mmHg and all data were subsequently determined at that volume of the balloon. Therefore, the increase of EDP represents the diastolic contracture, and the loading characteristics of the hearts were the same throughout.

Ischemia or hypoxemia were applied for 20 min at 37°C, either reducing the flow to 1.5 ml/min or

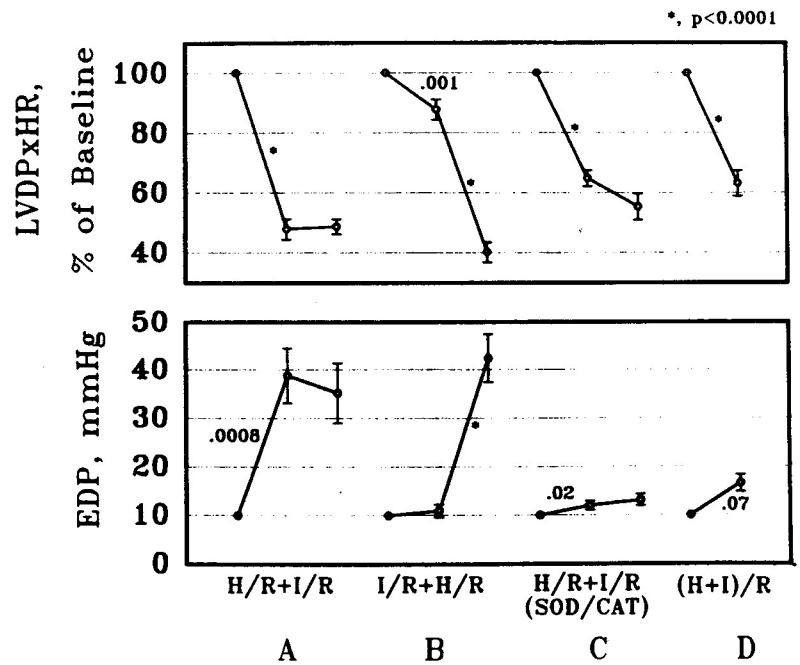


Fig. 2. EDP and LVDP · HR at the recovery from either hypoxemia or ischemia in various sequences. All data obtained in hearts perfused at 15 ml/min with buffer at PO_2 = 670 mmHg. One-way ANOVA analysis of these data yielded $p < 0.007$, and the Bonferroni's test was used to define the significance of the changes. Abbreviations: H—hypoxemia; I—ischemia; R—recovery.

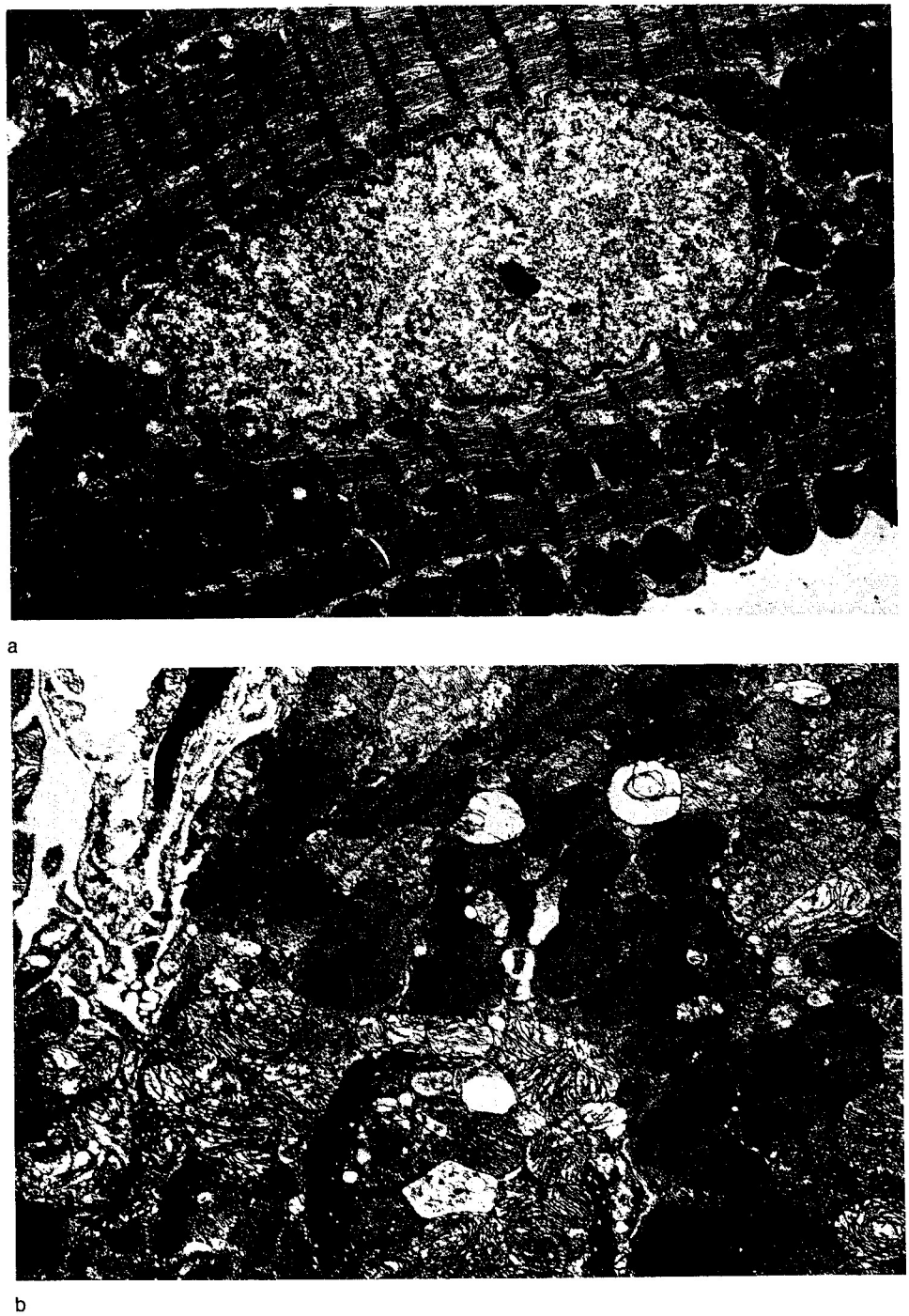
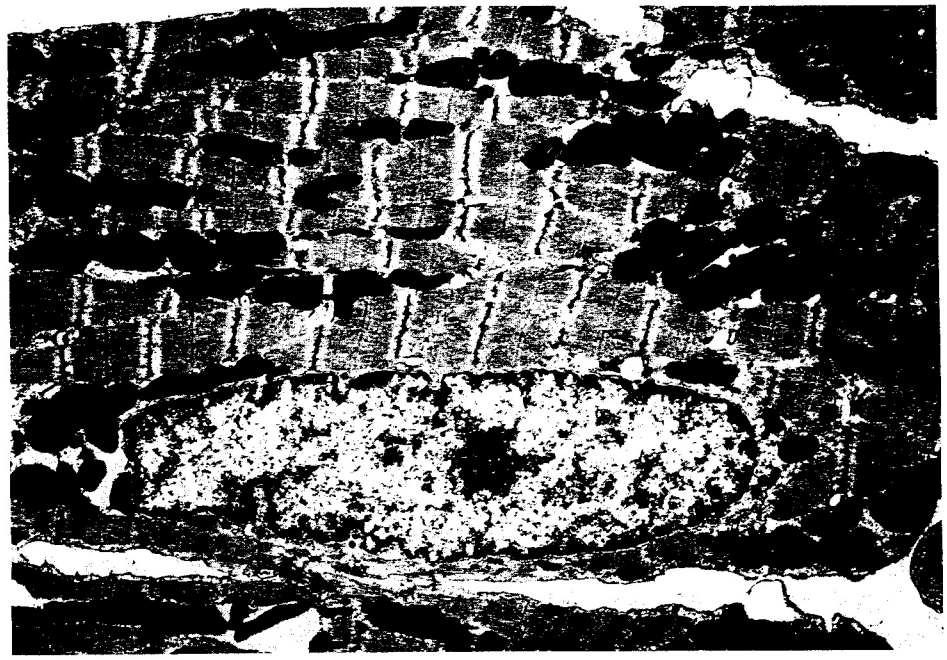


Fig. 3. Microphotographies of left ventricle (original magnification 4400×) after 2 h perfusion at coronary flow 15 ml/min and PO_2 = 670 mmHg (a, score 1); from a heart of group A (b, score 4); from a heart of group C (c, score 2); and from a heart of group D (d, score 3).

switching to the buffer with PO_2 = 67 mmHg. The two stresses were therefore matched for the O_2 supply, which was 1.41 μmoles/min (10% of baseline).

For the purposes of this study, all hearts were exposed to two consecutive stresses (Fig. 1). Groups A and B differed for the sequence of the stresses: hypoxemia/reoxygenation (H/R) followed by ischemia/reperfusion (I/R), and I/R followed by H/R, respectively. Group C hearts were exposed to H/R and then I/R as in group A, but with infusion of 40 IU/ml superoxide dismutase and 104 IU/ml catalase starting at the 5th min of hypoxemia and ending at the 5th min of the recovery from hypoxemia (H/R + I/R [SOD/CAT]). In group D, the hearts were made ischemic



c

d

Fig 3. Continued

right after hypoxemia, eliminating the intermediate reoxygenation step ([H + I]/R).

Statistical tests

The data are expressed as means ± SE. When two groups were compared, the Student's *t*-test for paired or unpaired data was used as appropriate to test the significance of the various differences. When more than two groups were compared, we employed one-way analysis of variance (ANOVA) and the Bonferroni test.

RESULTS

Table 1 reports the myocardial performance at baseline and during hypoxemia or ischemia in

Groups A and B, respectively. No significant difference between hypoxemic and ischemic hearts was evident for LVDP. In contrast, HR, LVDP · HR, and VO_2 were higher in the hypoxemic than the ischemic hearts. Taken together, these data indicate that the demand of energy was higher in hypoxemic than ischemic hearts despite the same supply of O_2.

Figure 2 shows LVDP · HR and EDP in the four groups of hearts at the recoveries from either hypoxemia or ischemia, at the same PO_2 (670 mmHg) and coronary flow (15 ml/min). The changes of LVDP and VO_2 paralleled those of LVDP · HR, and the alterations of CP were consistent with those of EDP (not shown).

We first consider the recoveries from hypoxemia in Group A and from ischemia in Group B. Despite the same O_2 supply, the myocardial depression associated with H/R was more severe than that associated with I/R. LVDP · HR decreased by 12.1 ± 3.4% (p = 0.001) following I/R in Group B vs. 52.1 ± 3.4% ($p <$ 0.0001) following H/R in Group A. The rise of EDP was negligible after I/R in Group B (0.8 ± 0.5 mmHg, p = NS) but dramatic after H/R in Group A (28.4 ± 5.7 mmHg, p = 0.0008). The injuries caused by I/R and H/R were additive, because no significant difference was detected between Groups A and B at the end of the protocols.

Hearts of Group C underwent the same protocol as those of Group A, but with superoxide dismutase and catalase during hypoxemia and the first 5 min of the reoxygenation. The selected enzymatic activity of the two enzymes approached the optimal.[6,7] As expected, the presence of the scavengers did not affect the myocardial function during hypoxemia (LVDP · HR decreased to 17.6 ± 1.5% of baseline in their presence versus 16.8 ± 1.5% in their absence, Group A). Nevertheless, the increase of EDP was almost completely blunted (2.3 ± 0.9 mmHg, p = 0.02). However, LVDP · HR decreased by 35.2 ± 2.7% ($p < 0.0001$). The Bonferroni's test showed that this decrease was less in this Group with respect to the analogous situation in Group A after H/R ($p < 0.05$).

In Group D, the reoxygenation after hypoxemia was eliminated; thus, the myocardial function of this group could be compared with that of the other groups only at the end of the various protocols. The rise of EDP in Group D (6.6 ± 1.8 mmHg, p = 0.07) was less than that observed in Group A ($p < 0.05$ at the Bonferroni's test). Likewise, LVDP · HR decreased by 36.9 ± 4.3% ($p < 0.0001$), but less ($p <$ 0.05, Bonferroni's test) than in Group A. In this group, LVDP · HR was 15 ± 2% of baseline during hypoxemia and 8 ± 1% during ischemia.

Figure 3 shows the microphotographs taken in the various groups of hearts. Only minor alterations at the level of mitochondria were evident in a control heart perfused for 2 h at 15 ml/min flow and PO_2 = 670 mmHg (Fig. 3a, score 1). Group A hearts displayed markedly enlarged mitochondria with broken cristae, light matrix, many amorphous densities, significant myofibrillar contraction bands, distorted Z band, disrupted myofibrils, several invaginations of the nuclear membrane, and margination of chromatin (Fig. 3b, score 4). Group B hearts were similar to those of group A (not shown). The hearts of group C appeared less damaged than those of group A (Fig. 3c, score 2). The hearts of group D were similar to those of group C except for some contraction bands and diffused extracellular edema (Fig. 3d, score 3). Both Groups C and D, however, displayed focal but frequent wrinkling of plasma membranes.

DISCUSSION

In this study, hypoxemia and ischemia were matched for duration, temperature, and O_2 supply. When the O_2 supply was reduced tenfold, the myocardial performance decreased in various patterns according to the way by which the O_2 supply was shortened. The demand of energy was higher in hypoxemic than ischemic hearts, resulting from their higher VO_2, HR, and LVDP · HR. It is difficult to compare this finding with the literature because of the failure to match hypoxemia and ischemia in other studies.[8–10] However, if low-flow ischemia is thought equivalent to hypoxemia with inhibited glycolysis, then the high energy demand in hypoxemia is consistent with the moderate fall of tension observed in hypoxemic hearts with active glycolysis, with respect to the marked fall observed when glycolysis was inhibited.[11] The former was attributed to intracellular acidosis[12] and the latter to the failure of the excitation-contraction coupling.[11]

The recovery of the myocardial function from I/R in Group B was almost complete in contrast to the severe depression observed after H/R in Group A. Three observations indicate that in our model this injury was mediated by the O_2-derived free radicals: (a) the combined effects of superoxide dismutase and catalase partially prevented the injury as already reported;[13] (b) eliminating the reoxygenation step allowed the hearts to recover as with the scavengers; and (c) the ultrastructure derangements observed in Group A were similar to those found in hearts with enzymatically generated O_2-derived free radicals.[14] The morphological damage to myofibrils, mitochondria, and plasma membrane are typical of free radi-

cals,[4,15] and were reversed with the scavengers or by eliminating the reoxygenation.

The increased susceptibility to injury in posthypoxemic hearts with respect to postischemic ones involves different balances between the production of free radicals and the myocardial defences against them. Thus, either H/R enhanced the production of free radicals, or I/R spared the endogenous myocardial protection. The intermediate hypothesis cannot be ruled out.

As for the former hypothesis, the higher energy demand during hypoxemia may have enhanced the catabolism of ATP[16] with associated higher production of hypoxanthine,[17,18] which is a major substrate for the generation of the O_2-derived free radicals in the rat heart.[19–21] This hypothesis is, however, in contrast with the expected faster washout of catabolic products by hypoxemic perfusion and the high membrane permeability for hypoxanthine.[22] The latter hypothesis is consistent with the depletion of the myocardial reserves of superoxide dismutase and glutathione peroxidase observed during hypoxemia.[1,23,24]

Whatever the explanation for the more severe injury in posthypoxemic hearts, neither the scavengers nor eliminating the reoxygenation could ensure full recovery of LVDP · HR from hypoxemia, while EDP and CP were almost completely protected. This implies that the endothelial function, of which EDP and CP are indexes, rather than the contractile one, was a primary target of free radicals in our model, as it was already suggested.[25] This is not unexpected when considering that the O_2-derived free radicals are short-lived species originated in the endothelial cells,[26] and that superoxide dismutase and catalase are large molecules that penetrate slowly into intact cells.[27] Therefore, the free radicals and the radical scavenging effect cannot occur deep within the tissue, but may occur at the endothelium. This implies that a relevant portion of the reoxygenation and reperfusion injuries is related to energy-dependent processes.[17,28–32]

Conclusions

The major determinant of the reperfusion-induced injury was associated to low O_2 supply rather than to low coronary flow. The endothelial part of this injury was mediated by O_2-derived free radicals, but the ventricular portion was associated with the high energy demand during hypoxemia. We believe that distinguishing the effects due to ischemia from those due to hypoxemia, as well as the effects due to the readmission of flow from those due to the readmission of O_2, may contribute to a better understanding ischemic and hypoxemic injuries.

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ABBREVIATIONS

CS—coronary sinus
EDP—end-diastolic pressure
HR—heart rate
H/R—hypoxemia + reoxygenation
I/R—ischemia + reperfusion
LVDP—left ventricle developed pressure
SOD/CAT—super-oxide dismutase + catalase