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Lipid Peroxidation, Tissue Necrosis, and Metabolic and Mechanical Recovery of Isolated Reperfused Rat Heart as a Function of Increasing Ischemia

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Isolated Langendorff-perfused rat hearts, after 30 min of preperfusion, were submitted to increasing times of global normothermic ischemia (1, 2, 5, 10, 20 and 30 min) or to the same times of ischemia followed by 30 min of reperfusion. Analysis of malondialdehyde, ascorbic acid, oxypurines, nucleosides, nicotinic coenzymes and high-energy phosphates was carried out by HPLC on neutralized perchloric acid extracts of freeze-clamped tissues. In addition, maximum rate of intraventricular pressure development and cardiac output of malondialdehyde, lactate dehydrogenase, oxypurines and nucleosides were monitored during both preperfusion and reperfusion. Besides decreasing energy metabolites and nicotinic coenzyme pool, prolonged ischemia produced oxidation of significant amounts of hypoxanthine and xanthine to uric acid and generation of detectable levels of malondialdehyde (0.002 μmol/g dry weight). After oxygen and substrate readmission, tissue and perfusate malondialdehyde increased only if previous ischemia was longer than 5 min, while lactate dehydrogenase was detected in perfusate of reperfused hearts following 10, 20, and 30 min of ischemia. Highest values of tissue malondialdehyde and total malondialdehyde output were recorded in reperfused hearts subjected to 30 min of ischemia (0.043 μmol/g dry weight and 0.069 μmol/30 min/g dry weight, respectively). Since tissue malondialdehyde was observed without detectable lactate dehydrogenase release in perfusate, it might be stated that malondialdehyde generation (i.e., lipid peroxidation) temporally preceded lactate dehydrogenase release (i.e., tissue necrosis). In reperfused hearts, evaluation of myocardial energy state and of mechanical recovery allowed us to determine times of ischemia beyond which reperfusion did not positively affect these metabolic and functional parameters. Main findings are that, under these experimental conditions, lipid peroxidation might be the cause and not the consequence of tissue necrosis and that duration of ischemia might be the factor deciding effectiveness of reperfusion.

Keywords: Ischemia and reperfusion, malondialdehyde, oxygen radicals, tissue injury, energy metabolism, isolated rat heart.

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INTRODUCTION

Postischemic heart recovery is strictly related to the ability of myocardial cells to resynthesize phosphorylated compounds fundamental for all the energy-requiring reactions, i.e. ATP and creatinephosphate (CrP), degraded during oxygen deprivation.[1] The absence of oxygen and substrate availability produces a rapid ATP decrease and a consequent increase of its catabolites, such as AMP, adenosine (Ado), inosine (Ino) and hypoxanthine (Hyp). Since most of these compounds can freely cross the cell membrane,[2] their loss from the cytoplasm during reperfusion may seriously compromize ATP resynthesis and, therefore, the real effectiveness of oxygen and substrate readmission. In addition, it has been suggested that the further enzymatic degradation of ATP catabolites at up to xanthine (Xan) and uric acid, occurring during reperfusion, may be a possible relevant source of superoxide anion.[3] This molecule, deriving from the partial oxygen reduction, would be the starting point for the production of other more reactive and dangerous oxygen radicals (e.g., hydroxyl radical, singlet oxygen), able to induce irreversible damage to several biologically fundamental molecules, including membrane phospholipids. In the isolated rat heart, it has been demonstrated that oxygen radicals are already produced in a minor extent during ischemia[4] and that a burst of reactive oxygen species is generated from the very early phase of reperfusion.[5] In the same experimental model, conjugated dienes[6] or ascorbyl radical[7] were used as possible valid markers of increased oxidative stress, although recently malondialdehyde (MDA) reliability as biochemical index of oxygen radical-mediated lipid peroxidation has also been demonstrated.[8–9] Connections among duration of ischemia, decrease of energy metabolism during oxygen deprivation, production and loss of ATP catabolites during reperfusion and, finally, onset of oxygen radical damage during reoxygenation are still object of several investigations.[10–11] Moreover, temporal relationship between oxygen radical-induced lipid peroxidation and evidence of tissue necrosis has not yet clearly been established, so that the importance of lipid peroxidation of postischemic tissues is still under evaluation.

Aim of the present study was to monitor the effects of both increasing ischemia times, and of increasing ischemia times followed by a fixed time of reperfusion, on the energy state and oxygen radical damage of the isolated Langendorff-perfused rat heart. By also determining cardiac functions and oxypurines, nucleosides, MDA and lactate dehydrogenase (LDH) output, we sought the respective contribution of ischemia and reperfusion in determining the effectiveness of postischemic myocardial recovery.

MATERIALS AND METHODS

Perfusion Technique

This experimental work was considered and approved by the local institutional ethical committee in accordance with recognized standards on the care and use of laboratory animals.

Adult male Wistar rats of 300–350 g body weight (b.w.) were purchased from Morini (San Polo d' Enza, RE, Italy). They were fed with a standard laboratory diet and water ad libitum in a controlled environment. Rats were anesthetized by intraperitoneal administration of ketamine (50 mg/kg b.w.) and 1500 IU of heparin were intravenously injected into the candal vena cava. Heart was excised and aorta was quickly cannulated for retrograde non-recirculating Langendorff perfusion, using a Krebs-Ringer buffer composed of: 119 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 1.18 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, and 10 mM glucose. The buffer was supplemented with 12 IU/l insulin, adjusted to pH 7.40, filtered through a 0.22 μm Millipore filter, continously gassed with O_2/CO_2 (19:1; v:v) and kept at 37°C, as well as the remainder of the perfusion apparatus, by means of

water-jacketed glassware. Perfusion of spontaneously beating hearts was effected at the constant hydrostatic pressure of 7.95 kPa (81 cm H_2O; 60 mmHg). Ventricular pressure development and maximum rate of intraventricular pressure development (dP/dt) were monitored by introducing a saline-filled latex balloon into the left ventricle via the mitral valve. The ventricular balloon was attached to a pressure transducer (Gould Statham P23DB) via a saline-filled line and ventricular pressure and heart rate were continously recorded on an EasyGraf TA240 recorder (Gould Inc., Valley View, OH, U.S.A.). The diastolic pressure was set at 15–18 mmHg, at which maximal developed pressure was determined. After 30 min of preperfusion, hearts were subjected either to increasing times of global normothermic ischemia (1, 2, 5, 10, 20, 30 min), induced by completely abolishing perfusate inflow through the aorta, or to the same increasing times of ischemia each one followed by 30 min of reperfusion. Control hearts were subjected to 30 min of preperfusion only. Coronary flow was volumetrically measured at regular intervals (2 min) and perfusate samples were collected for 1 min, in ice-cooled tubes, after 1, 2, 5, 10, 20 and 30 min from the beginning of preperfusion or reperfusion. One aliquot was immediately deproteinized by adding 300 µl of 70% $HClO_4$/3 ml perfusate and subsequently used for analyzing the cardiac output of different metabolites. The remaining untreated perfusate aliquot was stored at –20°C until the analysis for determining LDH release was performed. At the end of each step, hearts were freeze-clamped by aluminium tongs (precooled in liquid nitrogen) and immediately immersed in liquid nitrogen. After wet weight determination, about 200 mg of the heart tissue was kept at 150°C/24 h for dry weight (d.w.) determination. The remaining frozen tissue was deproteinized by homogenization in ice-cold 0.6 M $HClO_4$ using an Ultra-Turrax (Janke and Kunkel GmbH & Co. KG, IKA-Werk, Staufen, Germany) as previously described.[8]

Metabolite and Enzyme Analyses

Both acid tissue extracts and perfusates were subjected to a recently described procedure for the optimization of sample preparation for HPLC analysis.[12] Briefly, after neutralization of perchloric acid extracts in the cold by adding 5 M K_2CO_3, samples were immersed for 90 sec in liquid nitrogen for increasing potassium perchlorate precipitation. After centrifugation at 26,980 *g* × 5 min, at 4°C, clean supernatants were extracted with an equal volume of HPLC-grade chloroform for removal of hydrophobic substances. Samples were again centrifuged at 26,980 *g* × 5 min, at 4°C, and aqueous phases were used either for enzymatic analyses of CrP,[13] or for HPLC assay of MDA, ascorbic acid, oxypurines, nucleosides, nicotinic coenzymes and high-energy phosphates, according to Lazzarino et al.[18] It should be underlined that, due to perchloric acid tissue treatment, values of NAD and NADP are comprehensive of their respective reduced forms. Therefore, it must be considered throughout the text that: NAD = NAD + NADH, and NADP = NADP + NADPH. The HPLC apparatus consisted of a Jasco PU-980 dual pump system, connected to a Jasco MD-910 diode array detector (Tokyo, Japan) set up between 200 and 300 nm wavelength for data acquisition. Separations of both standards and samples were performed by using an Alltima C-18, 250 mm × 4.6 mm, 5 µm particle size column (Alltech Associates, Inc., Deerfield, IL, U.S.A.) provided of its own guard column. Due to column length increase, adopted for improving chromatographic resolution with respect to the original method,[8] flow rate was 1.5 ml/min and gradient composition was as follows: 20 min at 100% of buffer A; 3 min at up to 90% of buffer A; 10 min at up to 70% of buffer A; 12 min at up to 55% of buffer A; 15 min at up to 45% of buffer A; 10 min at up to 25% of buffer A; 5 min at up to 0% of buffer A. Data acquisition and chromatogram analysis were performed by a PC-486. Identification of the various compounds in sample runs

was performed by comparing both retention times and absorption spectra of each peak with those obtained in freshly prepared ultrapure standard runs. Concentrations of the different metabolites were determined by calculating areas of each peak at 267 nm wavelength (the maximum of MDA absorption) and comparing them with corresponding peak areas of standard runs. Analysis of LDH release in the perfusate was effected by monitoring NADH oxidation rate in the presence of pyruvate at 340 nm.[14] Spectrophotometric assays were carried out by a Jasco 685 double beam spectrophotometer (Tokyo, Japan).

Data Analysis

The between group comparison was performed by the 1-way analysis of variance. Dependence of myocardial functional recovery (dP/dt) from increasing ischemia duration, as well as correlation between dP/dt and different metabolic parameters, was determined in reperfused hearts by calculating the best fitting curves of experimental values. Subsequently, the first order derivative of each parameter with respect to functional recovery (dparameter/drecovery), was calculated and plotted versus ischemia time. Consequently, it was possible to determine the time of ischemia corresponding to maximal variations of each curve parameter/recovery.

RESULTS

Tissue

Table I reports variations of representative parameters of the cell energy state as a function either of increasing ischemia time or of increasing ischemia time followed by a fixed reperfusion period. Reperfusion effectiveness was strictly related to preceding ischemia. In fact, maintenance of energy metabolism, at sufficient levels to allow myocardial functions (dP/dt) close to preischemic values during reoxygenation (see Fig. 5), did not occur at any ischemia time longer than 5 min. Although ATP was modestly affected by reperfusion, it should be noted that CrP showed higher values at the end of reperfusion than those recorded in the corresponding ischemic only hearts. The marked decline of adenine nucleotides (ΣNT) with respect to ischemia only, particularly evident if previous ischemia was longer than 5 min, indicates the existence of a marked imbalance between phosphorylating and dephosphorylating processes, i.e. between energy production and consumption. Starting from the very early phase of ischemia, significant NAD decrease was observed in ischemic only hearts. The extent of NAD depletion did not vary with increasing ischemia, although the lowest NAD concentration and the only NADP significant decrease were recorded after 30 min of ischemia. Neither NAD nor NADP were resynthesized after reperfusion, but they were vice-versa negatively affected by reperfusion, particularly in hearts after 30 min of previous ischemia.

In Table II are reported variations of oxidative stress representative parameters (MDA, ascorbate) as well as of dephosphorylated ATP catabolites (Hyp, Xan, uric acid, Ino, Ado) of both ischemic and ischemic-reperfused hearts. A small but detectable amount of MDA, produced from peroxidized phospholipid degradation, was determined in ischemic only hearts after 10, 20, or 30 min of ischemia. It is worth underlining that MDA, as already reported,[8–9] was not detectable in control hearts at the end of preperfusion. This result is not in accordance with those published when MDA was assayed by the thiobarbituric acid test.[15–16] The presence of oxidants, despite oxygen absence, was confirmed by ascorbate decrease, which significantly diminished as MDA was produced and, therefore, as ischemia was increased in duration. In accordance with data referring to high-energy phosphate decrease (Table I), both oxypurines (Hyp, Xan, uric acid) and nucleosides (Ino, Ado) progressively increased as a function of oxygen

TABLE I Effect of increasing times of ischemia on tissue concentrations of the main high-energy phosphates and nicotinic coenzymes of control, ischemic and reperfused isolated rat hearts

	TIME (min)	ATP	ADP	AMP	CrP	NAD	NADP	ΣNT
PREPERFUSION	**30**	28.70	7.33	1.66	32.59	9.86	0.60	37.69
		(2.80)	(0.91)	(0.21)	(3.08)	(1.30)	(0.09)	(3.55)
	1	28.27	7.13	1.76	25.70[a]	7.78[a]	0.58	37.16
		(2.91)	(1.08)	(0.26)	(3.86)	(0.11)	(0.08)	(3.15)
	2	27.05	7.87	2.79[a]	18.43[a]	7.87[a]	0.54	37.71
		(3.57)	(1.27)	(0.42)	(1.67)	(0.99)	(0.04)	(2.78)
ISCHEMIA	**5**	26.80	7.09	2.88[a]	13.39[a]	7.64[a]	0.54	36.77
		(2.76)	(2.33)	(0.78)	(2.11)	(1.05)	(0.05)	(4.00)
	10	24.11	6.85	4.04[a]	11.18[a]	7.94[a]	0.46	35.00
		(3.84)	(1.34)	(1.10)	(0.65)	(0.90)	(0.09)	(2.54)
	20	17.54[a]	6.43	4.97[a]	11.61[a]	7.27[a]	0.45	28.94[a]
		(3.98)	(0.88)	(1.24)	(1.18)	(0.99)	(0.09)	(2.05)
	30	10.05[a]	5.27[a]	10.10[a]	10.53[a]	7.06[a]	0.42[a]	25.42[a]
		(2.20)	(0.74)	(0.58)	(1.31)	(1.01)	(0.01)	(1.98)
	1	28.43	7.03	1.35	28.29[a]	7.34[a]	0.55	36.81
		(4.41)	(1.92)	(0.48)	(1.95)	(1.09)	(0.13)	(3.11)
ISCHEMIA	**2**	26.86	6.91	1.72[b]	22.00[ab]	7.69[a]	0.51	35.49
+ 30 min of		(3.66)	(1.68)	(0.43)	(0.72)	(1.32)	(0.11)	(2.75)
REPERFUSION	**5**	21.86[a]	6.69	1.97[b]	23.93[ab]	8.09[a]	0.43	30.52[a]
		(3.66)	(0.88)	(0.23)	(1.76)	(0.98)	(0.08)	(3.04)
	10	19.74[a]	6.12[a]	2.08[b]	23.21[ab]	6.83[a]	0.44	27.94[a]
		(3.13)	(1.16)	(0.26)	(1.58)	(1.43)	(0.16)	(2.74)
	20	16.37[a]	4.97[ab]	2.62[ab]	20.67[ab]	6.10[a]	0.38[a]	23.96[a]
		(2.36)	(0.84)	(0.62)	(3.33)	(1.28)	(0.13)	(2.22)
	30	10.30[a]	2.65[ab]	3.67[ab]	16.69[ab]	4.41[ab]	0.31[ab]	16.62[a]
		(2.43)	(0.46)	(0.94)	(1.16)	(1.27)	(0.07)	(1.89)

Determination of the various compounds, out of CrP (determined enzymatically), was carried out by HPLC on 200 µl of perchloric acid tissue extracts. Perfusion and chromatographic conditions are fully described in Materials and Methods. Each value represents the mean (S.D.) of 6 hearts and is expressed as µmol/g d.w.
ΣNT = ATP + ADP + AMP.
[a]significantly different from preperfusion ($p < 0.05$).
[b]significantly different from ischemia ($p < 0.05$).

deprivation time, again showing the most significant changes after 20 and 30 min of ischemia.

One of the most evident biochemical changes, occurring to reperfused hearts, was represented by MDA increase. The occurrence of lipid peroxidation during reperfusion was induced by at least 5 min of global no-flow ischemia. In these hearts, low but detectable MDA concentration was recorded (0.008 µmol/g d.w.) at the end of 30 min of reflow. Highest MDA concentration was determined in reperfused hearts after 30 min of oxygen deprivation (0.043 µmol/g d.w.). The presence of oxidative stress during myocardial reperfusion is also supported by ascorbate decrease, whose concentration was lower either than preperfusion or than the corresponding times of ischemia only. It is worth recalling that no detectable ascorbate concentration was found in any perfusate sample; this suggests that the evident ascorbate decrease after reperfusion was not due to its outflow from permeabilized cells but rather to its oxidation or, eventually, to decrease in its rate of synthesis.

Perfusate

In Figure 1 is reported MDA cardiac output recorded in the perfusate of hearts subjected to increasing ischemia times followed by 30 min of reperfusion. No MDA release was observed during preperfusion of control hearts, as well as dur-

TABLE II Effect of increasing times of ischemia on tissue concentrations of MDA, ascorbic acid, oxypurines and nucleosides of control, ischemic and reperfused isolated rat hearts.

	TIME (min)	MDA	Ascorbic acid	Hyp	Xan	Uric acid	Ino	Ado
PREPERFUSION	**30**	N.D.	2.94	0.02	0.04	0.07	0.04	0.06
			(0.42)	(0.01)	(0.01)	(0.02)	(0.01)	(0.02)
	1	N.D.	2.61	0.03[a]	0.05	0.07	0.04	0.10
			(0.68)	(0.01)	(0.02)	(0.03)	(0.01)	(0.01)
	2	N.D.	2.86	0.09[a]	0.15[a]	0.23[a]	0.10[a]	0.16[a]
			(0.40)	(0.02)	(0.05)	(0.05)	(0.01)	(0.05)
ISCHEMIA	**5**	N.D.	2.69	0.11[a]	0.19[a]	0.36[a]	0.19[a]	0.21[a]
			(0.17)	(0.02)	(0.06)	(0.11)	(0.04)	(0.08)
	10	0.001[a]	2.14[a]	0.22[a]	0.38[a]	0.54[a]	1.06[a]	1.10[a]
		(0.0003)	(0.36)	(0.08)	(0.09)	(0.11)	(0.36)	(0.14)
	20	0.002[a]	1.88[a]	1.72[a]	1.08[a]	0.607[a]	2.50[a]	3.82[a]
		(0.001)	(0.174)	(0.335)	(0.214)	(0.151)	(0.420)	(0.673)
	30	0.002[a]	1.98[a]	1.72[a]	1.43[a]	0.66[a]	4.45[a]	6.76[a]
		(0.001)	(0.22)	(0.70)	(0.16)	(0.11)	(0.76)	(1.13)
	1	N.D.	2.21	0.02	0.03	0.08	0.03	0.06
			(0.29)	(0.01)	(0.01)	(0.01)	(0.01)	(0.02)
	2	N.D.	1.84[ab]	0.02[b]	0.06[b]	0.11[ab]	0.03[b]	0.08[b]
			(0.23)	(0.01)	(0.02)	(0.02)	(0.01)	(0.02)
ISCHEMIA	**5**	0.008[ab]	1.90[ab]	0.02[b]	0.09[b]	0.12[ab]	0.05[b]	0.11[b]
+ 30 min of		(0.002)	(0.25)	(0.01)	(0.03)	(0.02)	(0.014)	(0.02)
REPERFUSION	**10**	0.015[ab]	1.60[ab]	0.04[b]	0.16[ab]	0.13[ab]	0.05[b]	0.13[ab]
		(0.004)	(0.21)	(0.01)	(0.07)	(0.02)	(0.01)	(0.01)
	20	0.027[ab]	1.33[ab]	0.07[ab]	0.23[ab]	0.24[ab]	0.18[ab]	0.38[ab]
		(0.004)	(0.21)	((0.01)	(0.04)	(0.02)	(0.06)	(0.07)
	30	0.043[ab]	1.05[ab]	0.14[ab]	0.43[ab]	0.30[ab]	1.96[ab]	2.96[ab]
		(0.006)	(0.21)	(0.04)	(0.20)	(0.11)	(0.46)	(0.13)

Determination of the various compounds was carried out by HPLC on 200 µl of perchloric acid tissue extracts. Each value represents the mean (S.D.) of 6 hearts and is expressed as µmol/g d.w.
N.D.= Not Detectable.
[a]Significantly different from preperfusion ($p < 0.05$).
[b]Significantly different from ischemia ($p < 0.05$).

ing reperfusion of hearts previously ischemic for 1, 2 or 5 min. Differently, detectable MDA concentrations were determined throughout the whole duration of reperfusion, if reperfusion itself was preceded by 10, 20 or 30 min of global normothermic ischemia. In all these hearts, MDA was released starting from the 1st min of reperfusion, showing its highest perfusate concentration during the 2nd min of oxygen and substrate readmission. Total MDA cardiac output, determined by calculating areas under each curve reported in Figure 1, showed a strict correlation with the duration of previous ischemia. Interestingly, as reported in Figure 2, LDH release during reperfusion, which is as an index of tissue necrosis, had the same MDA dependence with respect to ischemia duration. In fact, no detectable LDH activity was determined either during preperfusion or during reperfusion of hearts submitted to 1, 2 or 5 min of ischemia; viceversa, 10, 20 or 30 min of previous oxygen and substrate deprivation induced marked LDH release in the perfusate during reperfusion. Total LDH output, calculated as for MDA, correlated to increase ischemia duration. However, a main difference between the time course output of MDA and LDH consisted in LDH release lag time during the first 2 min of reperfusion and in the reperfusion time at which maximal LDH release was observed, i.e. after 30 min of reoxygenation. Figures 3 and 4 report variations of Hyp + Xan + uric acid (Σoxy) and Ado + Ino (ΣN) variations, respectively, in the perfusate of reper-

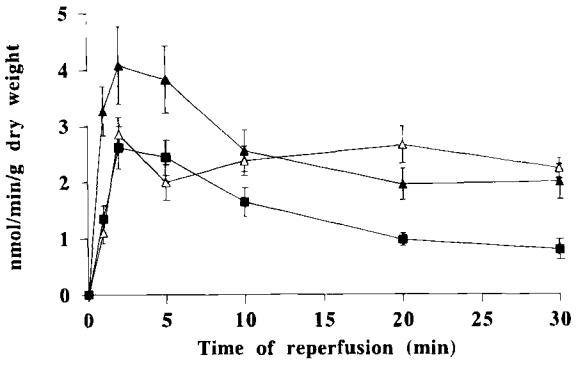

FIGURE 1 Myocardial MDA release of isolated reperfused rat hearts subjected to increasing times of ischemia. Perfusate aliquots were collected at 1, 2, 5, 10, 20 and 30 min from the beginning of myocardial preperfusion or reperfusion, and MDA was directly assayed by HPLC (200 μl), as fully described in Materials and Methods. No detectable MDA release was observed either during preperfusion or reperfusion of hearts previously submitted to 1, 2, or 5 min of ischemia. Each point is the mean of perfusate samples from 6 different hearts. Standard deviations are represented by vertical bars.

(■), hearts subjected to 10 min of ischemia + 30 min of reperfusion.

(△), hearts subjected to 20 min of ischemia + 30 min of reperfusion.

(▲), hearts subjected to 30 min of ischemia + 30 min of reperfusion.

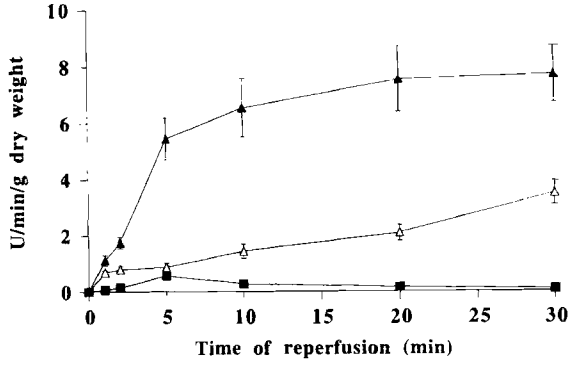

FIGURE 2 Time course of LDH release in perfusate of isolated reperfused rat hearts after increasing times of ischemia. LDH was determined in perfusate collected at 1, 2, 5, 10, 20 and 30 min from the beginning of preperfusion or reperfusion. No detectable LDH release was observed either during preperfusion or reperfusion of hearts previously submitted to 1, 2, or 5 min of ischemia. Each point is the mean of perfusate samples from 6 different hearts. Standard deviations are represented by vertical bars.

(■), hearts subjected to 10 min of ischemia + 30 min of reperfusion.

(△), hearts subjected to 20 min of ischemia + 30 min of reperfusion.

(▲), hearts subjected to 30 min of ischemia + 30 min of reperfusion.

fused hearts after increasing times of ischemia. The highest concentration of both Σoxy and ΣN was observed after 1 min of reperfusion, indicating that flow restoration induced an immediate washout of ATP catabolites which were intracellularly accumulated during ischemia. However, hearts submitted to 10, 20 or 30 min of ischemia, showed relevant cardiac output of Σoxy and ΣN even after 20 or 30 min of reperfusion, thus suggesting the existence of an imbalance between ATP production and consumption.

Myocardial Functional Recovery and Energy Metabolism

Time course functional recovery of reperfused hearts (expressed as percent dP/dt recovery of preischemic value), following different ischemia periods, is reported in Figure 5. The effect of increasing ischemia times on dP/dt recovery, determined at the end of 30 min of reperfusion, as well as the correlation among dP/dt recovery and several metabolic parameters (ATP, ATP + CrP, ΣNT, total Σoxy + total ΣN, LDH, total MDA), was evaluated in detail. In particular, it was initially found F_1 function correlating dP/dt recovery and ischemia duration: dP/dt recovery = F_1 (ischemia time); and F_2 function correlating dP/dt and any of aforementioned metabolic parameters: parameter$_i$ = F_2 (dP/dt recovery). Hence, it was ultimately possible to correlate each parameter as follows: parameter$_i$ = F_2 [F_1(ischemia time)], i.e. the composed dependence. Concerning F_1 function equation, the best fitting analysis revealed that dP/dt recovery and ischemia time had a sigmoidal correlation, according to the following equation: $y = 100 - (x^{m_2}/x^{m_2} + m_1) \times 64$. This curve had a flex point corresponding to an ischemia duration of 4.6 min, as calculated by performing the first order derivative of functional recovery versus ischemia time. By interpolating values of each

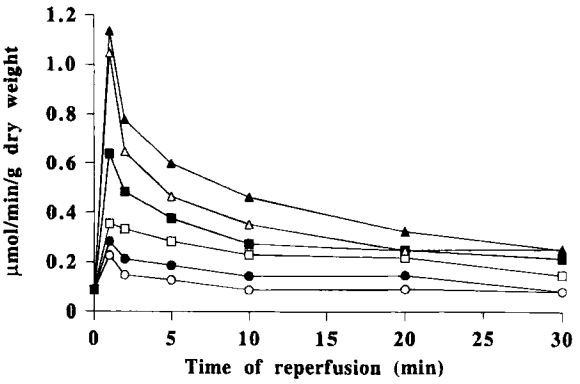

FIGURE 3 Cardiac output of sum of oxypurines (Hyp + Xan + uric acid) during myocardial reperfusion of isolated rat hearts after increasing times of ischemia. Zero time value corresponds to mean value determined in 6 perfusates collected immediately before ischemia, i.e. during the last min of preperfusion. Each point is the mean of perfusate samples from 6 different hearts. Standard deviations have been omitted for the sake of clarity.

(○), hearts subjected to 1 min of ischemia + 30 min of reperfusion.

(●), hearts subjected to 2 min of ischemia + 30 min of reperfusion.

(□), hearts subjected to 5 min of ischemia + 30 min of reperfusion.

(■), hearts subjected to 10 min of ischemia + 30 min of reperfusion.

(△), hearts subjected to 20 min of ischemia + 30 min of reperfusion.

(▲), hearts subjected to 30 min of ischemia + 30 min of reperfusion.

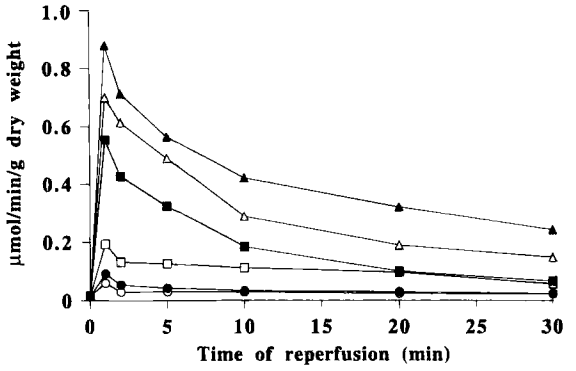

FIGURE 4 Cardiac output of sum of nucleosides (Ino + Ado) during myocardial reperfusion of isolated rat hearts after increasing times of ischemia. Zero time value corresponds to mean value determined in 6 perfusates collected immediately before ischemia, i.e. during the last min of preperfusion. Each point is the mean of perfusate samples from 6 different hearts. Standard deviations have been omitted for the sake of clarity.

(○), hearts subjected to 1 min of ischemia + 30 min of reperfusion.

(●), hearts subjected to 2 min of ischemia + 30 min of reperfusion.

(□), hearts subjected to 5 min of ischemia + 30 min of reperfusion.

(■), hearts subjected to 10 min of ischemia + 30 min of reperfusion.

(△), hearts subjected to 20 min of ischemia + 30 min of reperfusion.

(▲), hearts subjected to 30 min of ischemia + 30 min of reperfusion.

metabolic parameter with dP/dt recovery, equations showing the dependence imposed by F_2 were obtained. Best fitting curves had a sigmoidal trend for ATP, ATP + CrP, ΣNT, total Σoxy + total ΣN. Relationship of ATP, ATP + CrP, ΣNT versus dP/dt recovery could be represented by the same equation found for dP/dt recovery and ischemia time; differently, that of total Σoxy + total ΣN versus dP/dt recovery could be represented according to the following equation: $y = 36 + (x^{m_2}/x^{m_2} + m_1) \times 64$. In order to determine ischemia time at which the maximal variation of a given parameter having a sigmoidal trend occurred, the first order derivative $d\text{parameter}_i/d\text{functional recovery}$ was plotted versus times of previous ischemia. Subsequently, determination of respective minimal and maximal derivative values, obtained by varying ischemia times, was performed. Therefore, it was possible to calculate for each parameter, ischemia duration values corresponding to the flex points of parameter/functional recovery curves; these values are summarized in Table III. It is worth underlining that flex points ranged from a minimum of 5.95 min for total Σoxy + total ΣN, to a maximum of 9.2 min of previous ischemia for ATP, with a mean value of 7.92 min of previous ischemia duration. Concerning dP/dt recovery correlations with total MDA and LDH, they could be described by polynomial equations.

DISCUSSION

The goal of reperfusing postischemic heart is the salvage of the largest number of myocardial cells

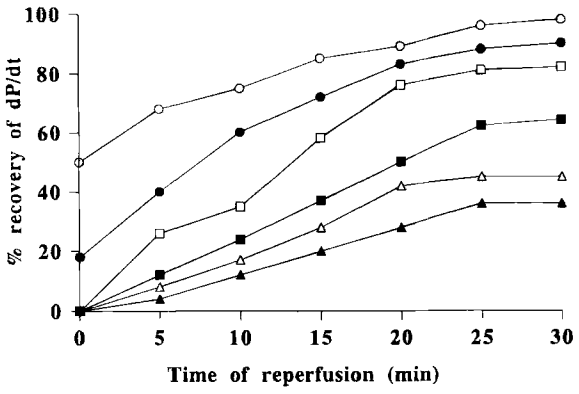

FIGURE 5 Recovery of maximum rate of intraventricular pressure development (dP/dt) of isolated reperfused rat hearts subjected to increasing times of ischemia. Each point represents the mean of 6 hearts and is expressed as percent of preischemic dP/dt. Zero time values correspond to residual dP/dt recorded during the last min of ischemia. Standard deviations have been omitted for the sake of clarity.

(○), hearts subjected to 1 min of ischemia + 30 min of reperfusion.

(●), hearts subjected to 2 min of ischemia + 30 min of reperfusion.

(□), hearts subjected to 5 min of ischemia + 30 min of reperfusion.

(■), hearts subjected to 10 min of ischemia + 30 min of reperfusion.

(△), hearts subjected to 20 min of ischemia + 30 min of reperfusion.

(▲), hearts subjected to 30 min of ischemia + 30 min of reperfusion.

in order to guarantee the tissue functional recovery and hence, the entire organism survival. Although reperfusion restores oxygen and substrate delivery to tissue, its effectiveness in recovering postischemic myocardial metabolism and functions is often limited.[17] In the last years, great attention was given to evaluate mechanisms of oxygen free radical production, because of their potential toxicity towards several important macromolecules; renewed interest was also payed in studying ATP catabolism for its possible connection with oxygen radical formation. Data reported in the present study indicate four major conclusions: i) loss of purines and degradation of NAD and NADP may seriously reduce effectiveness of myocardial reoxygenation after long times of global normothermic ischemia; ii) generation of oxygen-derived free radicals, and therefore of lipid peroxidation, during reperfusion is strictly dependent on the time of previous ischemia; iii) lipid peroxidation is an event that preceds tissue necrosis; iv) in general, duration of ischemia is probably the main event that impedes reperfusion efficacy. Oxypurine and nucleoside loss, as a factor limiting myocardial recovery, has been described by several authors,[18–19] although a direct correlation between their cardiac output and time of oxygen and substrate deprivation has not previously been described. This fact seems particularly interesting since intracellular Ado preservation during myocardial ischemia,[20] as well as exogenously administered Ado,[21] improve functional recovery of postischemic myocardium. Referring to NAD and NADP variations as a function of different times of ischemia and subsequent reperfusion, no data are presently available, albeit NAD and NADP decrease following a fixed ischemia period has already been reported.[8–9–22–23] NAD glycohydrolase activity, the enzyme responsible for NAD degradation into nicotinamide and ADP-ribose, is affected by oxygen deprivation.[24] In addition, oxidative stress induced in different isolated cell lines produced significant reduction of nicotinic coenzyme pool.[25–26] Results obtained in the present experimental conditions, suggest that the remarkable decrease of NAD and NADP after reperfusion, already evident during increasing ischemia, could be considered a critical factor negatively affecting myocyte metabolism and survival. Moreover, this indicates that a degree of oxidative stress capable of depleting nicotinic

TABLE III Flex points of curves obtained by plotting dparameter/drecovery as a function of increasing ischemia duration

Parameter	Time of ischemia corresponding to the flex point
ATP	9.20
ATP + CrP	8.85
Total Σoxy + Total ΣN	5.95
ΣNT	9.01

Different flex points refer to best-fitting sigmoidal waves of experimental data calculated as described under Materials and Methods and Results.

coenzyme concentration, is produced by ischemia and reperfusion in the isolated rat heart. NAD and NADP deficiency might lead to inhibition of enzymatic reactions utilizing these electron transferring cofactors and to alteration of reducing equivalent supply to mitochondria. Modifications of energy metabolism, i.e. loss of oxypurines and nucleosides, decrease of NAD and NADP concentration, lack of adequate ATP resynthesis, might well be in turn responsible for the progressive decrease of reperfusion effectiveness to induce myocardial functional recovery, observed by increasing times of ischemia.

MDA production in ischemic heart tissue could be observed in low amounts only in hearts subjected to at least 10 min of oxygen and substrate deprivation. This paradox, i.e. oxygen radical-mediated lipid peroxidation when oxygen is not supplied to the heart, might take place if part of residual oxygen, still present for a given time into the myocytes and in the extracellular fluid, were transformed into superoxide anion by different possible sources, such as xanthine oxidase activated enzyme[3] or altered mitochondrial capacity to catalyze the tetravalent reduction of molecular oxygen.[27] Propagation of lipid peroxidation might be stopped when remaining tissue oxygen was consumed, so that transformation of conjugated dienes into lipid hydroperoxides was inhibited. It is worth underlining that we found a progressive augmentation of uric acid in hearts after 1, 2, 5, or 10 min of ischemia, while Hyp and Xan significantly increased only after 20 and 30 min of oxygen and substrate deprivation (Table II). This suggests that oxygen concentration inside the tissue was sufficient for allowing oxidoreductive reactions that might be directly involved in superoxide radical generation by xanthine oxidase. It should also be reminded that oxygen-derived free radical generation has been reported to occur during ischemia.[4] Reperfusion was characterized by a marked increase of lipid peroxidation (evaluated in terms of tissue MDA) at any ischemia time longer than 5 min. Hence, it might be conceivable that a given period of oxygen deprivation is requested for inducing those changes leading to a burst of oxygen free radical production during reperfusion, such as the proteolytic transformation of xanthine dehydrogenase into xanthine oxidase.[3–28] For instance, if this phenomenon had occurred in our experimental conditions, it could be calculated as follows: [total Xan (i.e., Xan released in the perfusate + Xan detected in tissue extracts)—total Hyp (calculated as for Xan)] + [total uric acid (calculated as for Xan)—total Xan], that 6.98 μmol/g d.w. of superoxide anions might have been produced. However, data concerning transformation of xanthine dehydrogenase into xanthine oxidase are not unanimous.[29]

In reperfused hearts, comparison between times of previous ischemia necessary for provoking tissue MDA production and that necessary for inducing LDH release in perfusate, attests that MDA generation (i.e., lipid peroxidation) preceded LDH release (i.e., tissue necrosis). In fact, notwithstanding tissue MDA was determined in reperfused hearts subjected to 5 min of ischemia (Fig. 1), same hearts did not show any detectable LDH release in the perfusate throughout the whole duration of reperfusion (Fig. 2). LDH release was only observed in reperfused hearts subjected to at least 10 min of ischemia. Furthermore, time courses of MDA and LDH output during reperfusion of hearts submitted to 10, 20 or 30 min of ischemia (Figs. 1 and 2, respectively) demonstrate that lipid peroxidation (MDA release) temporally preceded an appreciable degree of tissue necrosis (presence of LDH in the perfusate). These findings might answer the question whether lipid peroxidation is the cause or consequence of tissue necrosis[30] and might suggest that: i) once that a given amount of membrane phospholipids are irreversibly altered, probability for loss of membrane integrity and function certainly increases; ii) in this experimental model, lipid peroxidation can occur without tissue necrosis, while tissue necrosis does not exist without lipid peroxidation. Although we determined remarkable perfusate MDA varia-

tions occurring during reperfusion, recent data, obtained by still using the questionable thiobarbituric acid test for assaying MDA, did not show any modification of MDA release in the effluent.[15] Interestingly, authors found about as much MDA in the perfusate during preperfusion (≅ 3.0 nmol/min/g d.w.) as those found by us during reperfusion after 30 min of ischemia. Under the experimental conditions adopted in our study, MDA was not detected either in the tissue or in the perfusate of preperfused only hearts. This discrepancy might, of course, be attributable to the analytical method used which in our case allowed us to determine directly MDA only, instead of unspecifically detecting "thiobarbituric acid reactive substances" (TBARS).[15–16] It should be recalled that results reported in literature, concerning MDA variations in postischemic hearts, are not totally in accordance.[31] Notwithstanding, it was demonstrated that MDA is produced and released into the blood stream also in different pathological conditions of myocardial ischemia and reperfusion in human beings,[32–33–34] thus supporting its validity as an index of oxygen radical-induced lipid peroxidation. In our experiments, if duration of oxygen and substrate deprivation was longer than 5–10 min, energy metabolism and myocardial functional recovery were profoundly depressed. This is clearly evidenced by sigmoidal trend of composed functions correlating metabolic parameters (ATP, ATP + CrP, ΣNT, total Σoxy + total ΣN), dP/dt recovery and ischemia time. First order derivative of metabolic parameters an dP/dt recovery versus ischemia time allowed to calculate flex points of each curve (Table III), whose meaning is to betoken ischemia duration beyond which concentration of each metabolic parameter corresponds to an incomplete dP/dt recovery. Therefore, ischemia times longer than the highest flex point value (9.2 min of previous ischemia) produced such metabolic changes (influencing dP/dt) that were not positively affected by reperfusion, i.e. ischemia induced irreversible alterations of myocardial metabolism and functions. It is worth noting that calculated flex point values closely tied to the time when measured values of corresponding metabolites first became significant (Tables I and II). Interestingly, either phosphorylated compounds (ATP and ATP + CrP) or end products of ATP catabolism (total Σoxy + total ΣN) correlated to myocardial functions of reperfused hearts. Results showing lack of correlation between ATP and cardiac functions either of postischemic myocardium[17–35] or of artificially ATP-depleted normoxic heart,[36–37] have previously been published. Data reported in the present study attest, at least in reperfused hearts, the presence of non linear correlation between ATP and dP/dt recovery. The sigmoidal trend, which was similar to that found by Neely and Grotyohann,[17] suggests that ATP concentrations after reperfusion, higher than value corresponding to the flex point of the curve, slightly influence myocardial functions. Conversely, below the flex point ATP and dP/dt recovery were linearly correlated, so that any ATP decrement was mirrored by reduced rescue of mechanical cardiac functions. The discrepancy between present data and those obtained in artificially ATP-depleted normoxic heart[36–37] might be due to the profound differences existing between the two experimental models. In fact, the latter experimental design does not take in any account the very many changes induced by ischemia, thereby rendering probably incomparable findings odtained in these two models.

The extent of oxygen radical-induced lipid peroxidation of reperfused hearts, seemed strictly related to ischemia-dependent activation of superoxide anion sources. However, in the present experimental study, if ischemia was no longer than 5 min, its negative effects on myocardial metabolism and contractile functions were successfully reverted by reperfusion and were associated to very low levels of lipid peroxidation. This might indicate that reperfusion is not intrinsically negative, but that it is the unavoidable condition allowing to express and/or amplify those

biochemical modifications triggered by previous ischemia. Furthermore, this last observation suggests that ischemia-mediated activation of the various possible sources of oxygen free radicals might represent one of the biochemical molecular events that is macroscopically translated into the lack of myocardial functional recovery during reperfusion. In conclusion, it might be affirmed that ischemia is the main negative event for myocardial metabolism and, if its duration is prolonged, reperfusion without pharmacological interventions, for facilitating recovery of energy metabolism and/or inhibiting oxygen radical production, has a modest efficacy.

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References

[1] L. H. Opie (1992). Cardiac metabolism-emergence, decline, and resurgence. Part II. *Cardiovascular Research*, **26**, 817–830.

[2] M. Van Bilsen, G. J. Van Der Vusse, W. A. Coumans, M. J. M. De Groot, P. H. M. Willemsen and R. S. Reneman (1989). Degradation of adenine nucleotides in ischemic and reperfused rat heart. *American Journal of Physiology*, **257**, H47–H54.

[3] S. L. Thompson-Gorman and J. L. Zweier (1990). Evaluation of the role of xanthine oxidase in myocardial reperfusion injury. *Journal of Biological Chemistry*, **265**, 6656–6663.

[4] S. Pietri, M. Culcasi and P. J. Cozzone (1989). Real-time continous-flow spin trapping of hydroxyl free radical in the ischemic and post-ischemic myocardium. *European Journal of Biochemistry*, **186**, 163–173.

[5] J. L. Zweier, J. T. Flaherty and M. L. Weisfeldt (1987). Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proceedings of the National Academy of Sciences USA*, **84**, 1404–1407.

[6] A. D. Romaschin, I. Rebeyka, G. J. Wilson and D. A. G. Mickle (1987). Conjugated dienes in ischemic and reperfused myocardium: an in vivo chemical signature of oxygen free radical mediated injury. *Journal of Molecular and Cellular Cardiology*, **19**, 289–302.

[7] S. Pietri, M. Culcasi, L. Stella and P. J. Cozzone (1990). Ascorbyl free radical as a reliable indicator of free-radical-mediated myocardial ischemic and post-ischemic injury. *European Journal of Biochemistry*, **193**, 845–854.

[8] G. Lazzarino, D. Di Pierro, B. Tavazzi, L. Cerroni and B. Giardina (1991). Simultaneous separation of malondialdehyde, ascorbic acid, and adenine nucleotide derivatives from biological samples by ion-pairing high-performance liquid chromatography. *Analytical Biochemistry*, **197**, 191–196.

[9] B. Tavazzi, G. Lazzarino, D. Di Pierro and B. Giardina (1992). Malondialdehyde production and ascorbate decrease are associated to the reperfusion of the isolated postischemic rat heart. *Free Radical Biology & Medicine*, **13**, 75–78.

[10] D. J. Hearse and R. Bolli (1992). Reperfusion induced injury: manifestations, mechanisms, and clinical relevance. *Cardiovascular Research*, **26**, 101–108.

[11] A. Manché, S. J. Edmondson and D. J. Hearse (1995). Dynamics of early postischemic myocardial functional recovery. Evidence of reperfusion-induced injury? *Circulation*, **92**, 526–534.

[12] D. Di Pierro, B. Tavazzi, C. F. Perno, M. Bartolini, E. M. Balestra, R. Caliò, B. Giardina and G. Lazzarino (1995). An ion-pairing high-performance liquid chromatographic method for the direct simultaneous determination of nucleotides, deoxynucleotides, nicotinic coenzymes, oxypurines, nucleosides, and bases in perchloric acid cell extracts. *Analytical Biochemistry*, **231**, 407–412.

[13] W. Lamprecht, P. H. Stein, F. Heinz and H. Weisser (1974). Creatine phosphate. In *Methods of Enzymatic Analysis*; (ed. H. U. Bergmeyer), II edit., Vol. 4, Verlag Chemie, Weinheim and Academic Press, London and New York, pp. 1772–1776.

[14] E. Beutler (1971). In *Red Cell Metabolism. A manual of biochemical methods*. (ed. E. Beutler), Grune and Stratton, New York, pp. 59–74.

[15] G. Ambrosio, J. T. Flaherty, C. Duilio, I. Tritto, G. Santoro, P. P. Elia, M. Condorelli and M. Chiariello (1991). Oxygen radicals generated at reflow induce peroxidation of membrane lipids in reperfused hearts. *Journal of Clinical Investigation*, **87**, 2056–2066.

[16] G. Ambrosio, J. L. Zweier, C. Duilio, P. Kuppusamy, G. Santoro, P. P. Elia, I. Tritto, P. Cirillo, M. Condorelli, M. Chiariello and J. T. Flaherty (1993). Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit heart subjected to ischemia and reflow. *Journal of Biological Chemistry*, **268**, 18532–18541.

[17] J. R. Neely and L. W. Grotyohann (1984). Role of glycolytic products in damage to ischemic myocardium: dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic heart. *Circulation Research*, **55**, 816–824.

[18] D. K. Reibel and M. J. Rovetto (1979). Myocardial adenosine salvage rates and restoration of ATP content following ischemia. *American Journal of Physiology*, **237**, H247–H252.

[19] P. A. Uusimaa, K. J. Peuhkurinen, O. Vuolteenaho, H. Ruskoaho and I. E. Hassinen (1992). Role of myocardial redox and energy states in ischemia stimulated release of atrial natriuretic peptide. *Journal of Molecular and Cellular Cardiology*, **24**, 191–205.

[20] F. T. Bunch, J. Thornton, M. V. Cohen and J. M. Downey (1992). Adenosine is an endogenous protectant against stunning during repetitive ischemic episodes in the heart. *American Heart Journal*, **124**, 1440–1446.

[21] R. D. Lasley and R. M. Mentzer (1992). Adenosine improves recovery of postischemic myocardial function via an A_1 receptor mechanism. *American Journal of Physiology*, **263**, H1460–H1465.

[22] G. Lazzarino, M. Nuutinen, B. Tavazzi, L. Cerroni, D. Di Pierro and B. Giardina (1991). Preserving effect of fructose-1,6-bisphosphate on high-energy phosphate compounds during anoxia and reperfusion in isolated Langendorff-perfused rat hearts. *Journal of Molecular and Cellular Cardiology*, **23**, 13–23.

[23] S. M. Humphrey, A. Cartner and D. G. Hollis (1987). Critical early metabolic change associated with myocardial recovery or failure after total ischemia in the rat heart. *Basic Research in Cardiology*, **82**, 304–316.

[24] R. Nuñez, E. Calva, M. Marsch, E. Briones and F. Lopez-Soriano (1976). NAD glycohydrolase activity in hearts with acute experimental infarction. *American Journal of Physiology*, **231**, 1173–1177.

[25] D. Lautier, J. B. Hoflack, J. B. Kirkland, D. Poirier and G. G. Poirier (1994). The role of poly(ADP-ribose) metabolism in response to active oxygen cytotoxicity. *Biochemica Biophysica Acta*, **1221**, 215–220.

[26] R. L. Thies and A. P. Autor (1990). Reactive oxygen injury to cultured pulmonary artery endothelial cells: mediation by poly(ADP-ribose) polymerase activation causing NAD depletion and altered energy balance. *Archives of Biochemistry and Biophysics*, **286**, 353–363.

[27] T. Paraidathathu, H. de Groot and J. P. Kehrer (1992). Production of reactive oxygen by mitochondria from normoxic and hypoxic rat heart tissue. *Free Radical Biology & Medicine*, **13**, 289–297.

[28] B. Tavazzi, L. Cerroni, D. Di Pierro, G. Lazzarino, M. Nuutinen, J. W. Starnes and B. Giardina (1990). Oxygen radical injury and loss of high-energy compounds in anoxic and reperfused rat heart: prevention by exogenous fructose-1,6-bisphosphate. *Free Radical Research Communications*, **10**, 167–176.

[29] A. Bindoli, L. Cavallini, M. P. Rigobello, M. G. Coassin and F. Di Lisa (1988). Modification of the xanthine-converting enzyme of perfused rat heart during ischemia and oxidative stress. *Free Radical Biology & Medicine*, **4**, 163–167.

[30] J. M. C. Gutteridge (1993). Free radicals in disease processes: a compilation of causes and consequences. *Free Radical Research Communications*, **19**, 141–158.

[31] P. L. Barrington, E. Lai and P. B. McCay (1993). Lack of myocardial lipid peroxidation during acute reperfusion injury in perfused guinea pig hearts. *Cardiovascular Research*, **27**, 1339–1345.

[32] B. Giardina, M. Penco, G. Lazzarino, S. Romano, B. Tavazzi, F. Fedele, D. Di Pierro and A. Dagianti (1993). Effectiveness of thrombolysis is associated with a time-dependent increase of malondialdehyde in peripheral blood of patients with acute myocardial infarction. *American Journal of Cardiology*, **71**, 788–793.

[33] G. Lazzarino, P. Raatikainen, M. Nuutinen, J. Nissinen, B. Tavazzi, D. Di Pierro, B. Giardina and K. Peuhkurinen (1994). Myocardial release of malondialdehyde and purine compounds during coronary bypass surgery. *Circulation*, **90**, 291–297.

[34] S. Pucheu, C. Coudray, G. Vanzetto, A. Favier, J. Machecourt and J. de Leiris (1995). Assessment of radical activity during the acute phase of myocardial infarction following fibrinolysis: utility of assaying plasma malondialdehyde. *Free Radical Biology & Medicine*, **19**, 873–881.

[35] V. V. Kupriyanov, V. L. Lakomkin, A. Yu. Steinschneider, M. Ya. Severina, V. I. Kapelko, E. K. Ruuge and V. A. Saks (1988). Relationship between pre-ischemic ATP and glycogen content and post-ischemic recovery of rat heart. *Journal of Molecular and Cellular Cardiology*, **20**, 1151–1162.

[36] V. V. Kupriyanov, V. L. Lakomkin, V. I. Kapelko, A. Yu. Steinschneider, E. K. Ruuge and V. A. Saks (1987). Dissociation of adenosine triphosphate levels and contractile function in isovolumic hearts perfused with 2′-deoxyglucose. *Journal of Molecular and Cellular Cardiology*, **19**, 729–740.

[37] J. A. Hoerter, C. Lauer, G. Vassort and M. Guéron (1988). Sustained function of normoxic hearts depleted in ATP and phosphocreatine: a ^{31}P-NMR study. *American Journal of Physiology*, **255**, C192–C201.