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OXIDATIVE CHANGES IN HYPOXIC-REOXYGENATED RABBIT HEART: A CONSEQUENCE OF HYPOXIA RATHER THAN REOXYGENATION

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Tissue changes consistent with oxidative damage in hypoxic/reoxygenated heart tissue have not been well documented. We recently reported that oxidative perturbations were evident in isolated-perfused rat heart tissue subjected to as little as 10 min hypoxia and that these changes were not exacerbated by reoxygenation. The mechanism and species specificity of this finding is not known. Rabbit hearts, which lack measurable xanthine oxidase activity, were examined for evidence of hypoxia-induced injury. The release of lactate dehydrogenase into the coronary effluent gradually increased during the retrograde perfusion of isolated rabbit hearts with hypoxic medium (containing 10 mM glucose and 2.5 mM calcium), and was slightly enhanced upon reoxygenation after 60 min hypoxia. Cardiac gluthathione content decreased significantly while glutathione disulfide, protein-glutathione mixed disulfides, thiobaribturic acid reactive substances (TBARS), and protein carbonyl contents increased significantly after 60 min of hypoxia, compared to oxygenated controls. These values were unaltered after 4 min of reoxygenation except for a loss of TBARS. The oxidative changes observed in hypoxic rabbit hearts may be caused by energy deficiency impairing normal reductive processes or by the generation of reactive oxygen species as a result of abnormal cell functions, but cannot be related to xanthine oxidase activity.

KEY WORDS: Reactive oxygen species, hypoxia, oxidative stress, reoxygenation, heart, rabbit.

INTRODUCTION

Electron spin resonance techniques have revealed the presence of radicals during myocardial ischemia and upon reperfusion,[1-3] but their source, ability to interact with tissue components, and role in the observed injury has not been determined. Recent work from our laboratory has shown that oxidative changes are evident in isolated-perfused rat hearts subjected to as little as 10 min of hypoxia, and that these changes are not enhanced by reoxygenation.[4] Although reactive oxygen species are not required for the cell lysis that develops upon reoxygenation of hypoxic rat heart tissue,[5,6] it is possible that oxidative reactions contribute to the overall process of hypoxic injury.

Oxidative changes can occur in tissues either as a result of the generation of reactive oxygen species or decreases in tissue protective mechanisms or repair processes. This latter possibility may be particularly important under conditions of oxygen depriva-

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tion where energy levels needed for various antioxidant and repair pathways are diminished. Tissue sources of reactive oxygen species which have been proposed include xanthine oxidase (XO), mitochondria, and leukocytes. Mitochondria have been receiving increasing attention since XO apears not to be a factor in various rat and dog models of cardiac reperfusion or reoxygenation injury, and this phenomenon occurs in species lacking this enzyme.[7,8] However, at least in rat hearts subjected to 30 min ischemia, XO-generated radicals may be responsible for a small portion of the injury[9] and it is possible that XO mediated the oxidative perturbations we observed in hypoxic rat hearts.[4] The aim of the current study was to determine whether oxidative changes occur during hypoxia in rabbit hearts, which lacks XO[8] but in which various studies have supported a role for free radicals in reperfusion injury.[10]

MATERIALS AND METHODS

Glutathione (GSH), glutathione disulfide (GSSG), *t*-butyl hydroperoxide (tBH), 4,4′-bis(dimethylamino) benzhydrol (DAB-OH), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), NAD^+ and NADPH were obtained from Sigma Chemical Company (St. Louis, MO). Brilliant Blue G (60%), malonaldehyde diacetal, 2,4-dinitrophenylhydrazine (DNP), 2,2′-dipyridyl and dithiothreitol, were obtained from Aldrich Chemical Company, (Milwaukee, WI). Bio-beads™ (SM-2,20–50 mesh) were obtained from Bio-Rad Laboratories (Richmond CA).

Male rabbits (New Zealand, 1.5–2 kg) were obtained from Jo Jo's of Seguin Rabbitry (Seguin, TX). Animals were anesthetized with pentobarbital and administered 300 IU of sodium heparin. Hearts were removed, immersed in ice-cold Krebs-Henseleit bicarbonate medium, pH 7.4, containing 2.5 mM $CaCl_2$ and 10 mM glucose, and perfused retrogradely with the same medium at 37°C via the aorta on a non-recirculating apparatus as previously described.[5] Coronary effluents were collected and stored on ice until the end of the experiment. The perfusion medium was gassed continuously with either 95% O_2:5% CO_2 or 95% N_2: 5% CO_2 using an oxygenator device containing 10 meters of gas-permeable silicon tubing (wall thickness 0.024 cm). Upon switching gases, the oxygen content of the medium declined rapidly, reaching 50 μM at 3 min and a minimum of 5 μM at 10 min.

Lactic acid dehydrogenase (LDH) activity in the coronary effluent was measured at 30°C in 100 mM triethanolamine HCl buffer, pH 7.6 containing 0.15 mM NADH, 1 mM EDTA, and 1.5 mM pyruvate. The absorbance change at 340 nm was measured and enzyme activity expressed in units where 1 unit is that which oxidizes 1 mmol NADH per min.

The tissue contents and redox status of various cellular sulfhydryl pools were determined in freeze-clamped heart tissue by procedures described previously.[11] Total GSH + GSSG and protein thiol groups were determined in 100 mg frozen powdered tissue homogenized in 0.3 M perchloric acid containing 5 mM EDTA. A second 100 mg aliquot of frozen tissue powder was used to measure GSSG and protein-glutathione mixed disulfides.[11] This aliquot was homogenized in 30% acetic acid containing 0.6% dipyridyl, 5 mM EDTA and 10 mM DAB-OH which binds to free thiols forming the corresponding sulfides and preventing oxidation. GSH + GSSG and GSSG were measured by the method of Tietze.[12]

Protein carbonyl groups in 50–100 mg samples of frozen tissue power were estimated by the technique of Oliver *et al.*[13] as described previously.[11] The absorbance

spectrum of the 2,4-DNP treated samples was determined using HCl-treated samples as reference. The amount of derivatized carbonyl was calculated from the maximum absorbance at 370 nm using a molar extinction coefficient of 22,000 for DNP derivatives.[13]

Lipid peroxidation was assessed by determining the content of thiobarbituric acid reactive substances (TBARS) in 200 mg aliquots of freeze-clamped heart muscle which were mixed with 20% trichloroacetic acid containing 0.01% butylated hydroxytoluence to stop further peroxidation.[14] TBARS were quantitated at 532 mm using a mM extinction coefficient of 146 determined using acid hydrolyzed malonaldehyde diactal.

Comparisons among multiple groups were made using analysis of variance (ANOVA) program with Student-Newman-Keuls post hoc comparison[15] using a multivariate ANOVA program of the SAS statistical package. All data are expressed as mean ± S.E. A p value of less than 0.05 was considered significant.

RESULTS

The alteration of cardiac cells' integrity was estimated by measuring LDH release after reintroduction of oxygen into rabbit hearts perfused with hypoxic medium for 60 min. LDH release gradually increased with the duration of hypoxia. Upon reoxygenation, enzyme release was enhanced (Figure 1). However, the magnitude of this reoxygenation-induced LDH release was low compared to hypoxic-reoxygenated rat heart.[7] Functionally, all rabbit hearts ceased beating by 60 min of hypoxia, and none resumed any contractile activity at reoxygenation.

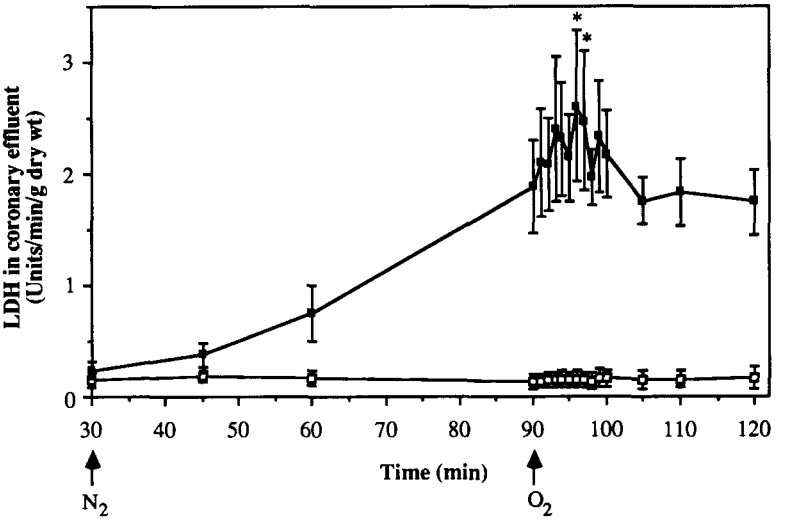

FIGURE 1 LDH released into the coronary effluent from isolated-perfused rabbit heart tissue. Controls were perfused with oxygenated medium for 120 min (open squares). Other hearts received oxygenated medium for 30 min and were then subjected to hypoxia for 60 min following by an additional 30 min of reoxygenation (filled squares). Data are expressed as mean ± S.E. $n = 3$ for all time points. *Significantly different from 90 mins ($p < 0.05$).

TABLE 1
Redox Status of Isolated-Perfused Rabbit Heart Tissue[1]

Index	Control[2]	Hypoxia[3]	Reoxygenation[4]	tBH[5]
GSH (nmol/g wet wt)	858 ± 32	457 ± 26*†	485 ± 11*†	641 ± 40*
GSSG (nmol/g wet wt)	5.4 ± 0.5	7.2 ± 0.1*†	7.5 ± 0.6*†	17.6 ± 2.0*
Mixed disulfides (nmol GSH equivalents/mg protein)	0.69 ± 0.06	0.91 ± 0.05*	0.92 ± 0.04*	1.61 ± 0.35*
Protein carbonyls (nmol/mg protein)	4.0 ± 0.5	8.3 ± 1.2*	7.4 ± 0.3*	7.6 ± 0.5*
TBARS (pmol malondialedhyde/mg protein)	35 ± 2	72 ± 8*†	35 ± 6	48 ± 3*
Protein thiols (nmol GSH equilvalents/mg protein)	107 ± 5	107 ± 6	105 ± 3	116 ± 4

[1]Data are expressed as mean ± S.E. n = 3–4 for all analyses.
[2]Control hearts were perfused with oxygenated medium for 90 min.
[3]Hypoxic hearts were stabilized with oxygenated medium for 30 min followed by 60 min of hypoxia.
[4]Reoxygenated hearts were stabilized with oxygenated medium for 30 min, subjected to 60 min of hypoxia, and reoxygenated for an additional 4 min.
[5]tBH hearts were stabilized with oxygenated medium for 30 min then perfused with 20 μM t-butylhydroperoxide for 10 min.
*Significantly different from Control ($p < 0.05$).
†Significantly different from tBH ($p < 0.05$).

Total cardiac GSH content decreased significantly in isolated-perfused rabbit hearts after 60 min of hypoxia. This decrease was maintained, but not enhanced, after 4 min of reoxygenation compared with oxygenated control hearts (Table 1). Tissue levels of GSSG exhibited a similar percentage increase as GSH declined, although the absolute amount of GSSG formed was not sufficient to explain the loss of GSH (Table 1). Again, reoxygenation did not further alter tissue GSSG levels. The contents of protein-GSH mixed disulfides, protein carbonyl groups and TBARS increased significantly in hypoxic heart tissue compared to oxygenated controls (Table 1). Upon reoxygenation, mixed disulfides and protein carbonyls were not further increased, while TBARS significantly decreased to control levels (Table 1). Cardiac protein thiol contents were unchanged in all groups.

The infusion of 20 μM tBH for 10 min resulted in significant oxidative changes in all parameters measured except protein thiols. The magnitude of the tBH-induced change in protein carbonyls was similar to that induced by hypoxia, while tBH produced larger increases in GSSG and mixed disulfides, and smaller changes in GSH and TBARS.

DISCUSSION

Recently, it was hypothesized that a lack of oxygen results in a "reductive stress"[16] which could lead to radical-mediated tissue injury prior to reoxygenation or reperfusion. In support of this concept, we have found oxidative perturbations after as little

as 10 min of hypoxia in isolated-perfused rat heart tissue.[4] In the current study, a range of indices were studied in rabbit hearts where oxidative changes were again evident during hypoxia and were not enhanced upon reoxygenation.

The unsaturated bonds in membrane lipids are prime targets for free radicals. The content of TBARS, an index of lipid peroxidation, increased after 60 min hypoxia. These data are supported by some reports[17] although conflicting results are also available.[18] The loss of TBARS at reoxygenation is currently unexplained. An identical phenomenon was observed in rat hearts[4] and may be the result of washout or metabolism of TBARS at reoxygenation. TBARS could not be detected in the coronary effluent, but this may have been a result of the sensitivity of the assay which could only measure 1 nmol/ml. Assuming a coronary flow rate of 25 ml/min, nearly the entire cardiac TBARS content of 35 nmol would need to be lost in 1 min before it could be detected.

TBARS are not specific for lipid peroxidation, and the material formed in heart tissue may be unrelated to oxidative processes. Measuring oxidative modifications of amino acid residues within proteins may provide a better index of oxidative injury. Carbonyl groups are present on proteins in normal tissues only at very low levels and their introduction into amino acid residues is a hallmark of oxidative modification. In the present study, the level of protein carbonyls increased during the hypoxic period suggesting either that reactive oxygen species were generated during hypoxia or that repair mechanisms (which may be energy dependent) were impaired. Increases in cardiac GSSG were also found during hypoxia. Cardiac GSSG is exported from the cell by an energy-dependent membrane pump[19] or reacts with protein thiols to produce protein-GSH mixed disulfides at a rate proportional to the increased intracellular GSSG concentration.[20] The presence of glucose and low levels of oxygen in the hypoxic (not anoxic) perfusion medium undoubtedly provided some energy to the heart. Transport of GSSG into the coronary effluent may explain why increases in GSSG and mixed disulfides were not quantitatively sufficient to explain the loss of GSH and why increases have not been consistently observed by others during hypoxia or upon reoxygenation.

Consistent with our previous work in rat hearts,[4] the level of cardiac protein mixed disulfides was increased in rabbit hearts after 60 min of hypoxia. In contrast, the level of cardiac protein thiols was not changed at any point. This finding conflicts with other studies in rat and rabbit[18,21] which demonstrated the decrease of cardiac protein thiols, but matches our findings in the rat using the same model system.[4]

Ferrari *et al.*[21] found that ischemia resulted in a decline of GSH levels in isolated rabbit hearts and that during reperfusion there was no further change in cardiac GSH or GSSG content in unpaced hearts. Decreases in rat heart GSH content during hypoxia have also been reported.[4,18,22] GSH formation is energy dependent[23] and hypoxia impairs the capability of cells to supply NADPH for the reduction of thiols.[24] Thus, the decline in cardiac GSH during oxygen deprivation may be augmented by the diminished availability of energy.

Depending on the index examined, the oxidative modifications induced in rabbit hearts following a 10 min infusion with 20 μM tBH were greater, lesser or the same as those obtained from hypoxic heart. Finding a different pattern of changes with an exogenous peroxide as compared to hypoxia is not surprising. Since the level of oxidative modifications evident after hypoxia were in the range of that produced by an exogenous oxidant, they may be toxicologically significant.

Tissues subjected to both hypoxia and ischemia contain gradually diminishing

levels of oxygen which may be reduced to reactive species at an increased rate due to mitochondrial dysfunction. Our data in the rat heart, where oxidative changes were maximal after 10–15 min hypoxia when oxygen levels reached their minimum,[4] are consistent with this concept. This hypothesis is also supported by electron spin resonance studies finding free radicals during the ischemic period[1-3] and by reports indicating that mitochondria from ischemic heart tissue have excess oxidative injury.[25,26]

The amount of LDH released from rabbit heart tissue gradually increased during hypoxia and was only modestly enhanced upon reoxygenation. These data are similar to those obtained by Hearse *et al.*[27] who found that 20 to 30% of the enzyme release occurred during hypoxia, and reoxygenation had little effect upon the progression of cellular damage. The magnitude of the overall reoxygenation-induced release of LDH by rabbit hearts was small compared to those levels seen in rat hearts perfused under identical conditions. Although this suggests less overall injury in this species, functional recovery was absent in rabbit hearts.

The studies reported here in isolated-perfused rabbit hearts showed changes indicative of oxidative injury during hypoxia. Whether these changes are caused by enhanced radical production or by an energy-dependent shift in the redox status of the cell is unknown. The occurrence of oxidative modifications during hypoxia, while unexpected, may explain why many antioxidants are only protective when given throughout hypoxia and reoxygenation. The cause of these oxidative changes, their contribution to the damage observed in hypoxic tissues, and their relationship to ischemic injury (which may differ), remains to be determined.

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