MOLECULAR ASPECTS OF MYOCARDIAL ISCHEMIA/REPERFUSION INJURY
AND THE PROTECTIVE EFFECTS OF ALLOPURINOL

By

ROBERT K.M. KO

B.Sc., The Chinese University of Hong Kong, 1984
M.Sc., The Chinese University of Hong Kong, 1986

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We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
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Department of Pharmacology & Therapeutics

The University of British Columbia
Vancouver, Canada

Date July 26, 1990
ABSTRACT

A growing body of evidence has now accumulated supporting the involvement of oxygen-derived free radicals in the development of myocardial ischemia/reperfusion (I/R) injury. We have, therefore, undertaken the present study to examine (1) I/R-related alterations in myocardial antioxidant capacity in pentobarbital anesthetized open-chest rabbits subjected to left circumflex coronary artery ligation followed by reperfusion; (2) the protective effects of pretreatment with allopurinol or the 21-aminosteroid U74006F; (3) alternative mechanisms to xanthine oxidase inhibition for allopurinol protection against I/R injury; and (4) the effect of allopurinol treatment on the antioxidant capacity of erythrocytes in pigs used in a heart-lung transplantation study.

In the rabbit myocardium, a marked impairment in myocardial antioxidant capacity developed in association with the onset of irreversible injury, as reflected in the enhancement in glutathione (GSH) depletion and formation of thiobarbituric acid-reactive substances (TBARS) following in vitro incubation of tissue homogenate with tert-butylhydroperoxide (TBHP). During the course of post-ischemic reperfusion, the protracted time-course of alterations in antioxidant capacity dissociated them from the early burst of radical formation known to occur at the onset of post-ischemic reperfusion of the myocardium. When
the time-dependent changes in functional indices of antioxidant status (TBHP-induced GSH depletion and formation of TBARS) were analysed in relation to activities of antioxidant enzymes, evidence suggestive of functionally relevant impairments in Cu,Zn-superoxide dismutase (Cu,Zn-SOD) and glutathione reductase (GRD) activities was found. These results and our demonstration of significant decreases in the activity of GSH-dependent antioxidant enzymes under acidicotic conditions suggest that a transient impairment in the functioning of antioxidant enzymes may be involved in triggering irreversible myocardial I/R injury.

Repetitive brief episodes of I/R produced a progressive decrease in myocardial ATP levels, which was not associated with any detectable changes in myocardial antioxidant capacity. Ischemic preconditioning produced by brief episodes of I/R did not affect the severity of subsequently induced I/R injury. These results suggest that brief episodes of myocardial ischemia do not produce oxidative tissue damage and the ischemia-induced depletion in myocardial ATP level is at least partially dissociable from the I/R-related impairment in tissue antioxidant capacity.

Isolated Langendorff-perfused rabbit hearts subjected to I/R did not show any changes in antioxidant capacity. However, when intact hearts were subjected to ischemia in vivo and a subsequent reperfusion in vitro, an impairment in myocardial antioxidant capacity became apparent. These
results suggest that blood elements, possibly activated neutrophils, may be a crucial factor involved in the development of I/R-induced oxidant injury.

Chronic allopurinol pretreatment (1 mg/ml in drinking water or approximately 75 mg/kg/day) for 7 days prior to ischemia provided significant protection against I/R-induced alterations in myocardial antioxidant capacity, but not the decrease in tissue ATP levels. This chronic allopurinol regimen was found to enhance myocardial GRD activity in non-ischemic tissue. In addition, both allopurinol and oxypurinol inhibited the transition metal ion-catalysed ascorbate oxidation and lipid peroxidation in vitro, likely as a consequence of their metal chelating properties. Similarly, myoglobin-TBHP-catalysed oxidation of uric acid and lipid peroxidation were also suppressed by allopurinol. All these suggest that allopurinol may favorably alter myocardial antioxidant capacity directly by virtue of its transition metal chelating properties and its antioxidant actions in myoglobin-mediated oxidative processes.

The acute administration of 21-aminosteroid U74006F (3 mg/kg, i.v) under conditions comparable to those known to protect against trauma-induced damage in the central nervous system failed to reduce manifestations of oxidative injury in rabbit hearts subjected to ischemia and reperfusion. Although reactive oxy-radicals have been implicated in both types of tissue damage, the observed difference in susceptibility to protection by this steroidal antioxidant
suggests that the molecular mechanisms involved are not identical.

In the heart-lung transplantation study, erythrocytes from allopurinol-treated pigs (given repeatedly at an oral dose of 50 mg/kg) showed a time/dose-dependent increase in antioxidant capacity as reflected in the decrease in malondialdehyde production following in vitro oxidative challenge. The extent of red cell protection in both donor and recipient animals correlated significantly with the functional viability of the transplanted lung tissue, as assessed by tissue water content. These results suggest that the measurement of erythrocyte antioxidant capacity may provide an useful assessment of generalized alterations in tissue antioxidant status produced by pharmacological interventions.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
</tr>
<tr>
<td>DEDICATION</td>
</tr>
</tbody>
</table>

## 1. INTRODUCTION ................................. 1

1.1 Free Radicals in Biological Systems ........ 1

1.1.1 Cellular Defense Mechanisms Against Free Radicals .................. 2
1.1.2 Antioxidant Enzymes ......................... 3
1.1.3 Non-Enzymatic Antioxidants ................. 4
1.1.4 Extracellular Antioxidant System ......... 5
1.1.5 Antioxidant Interactions .................... 6
1.1.6 Cytotoxic Effects of Hydroxyl Radical Other Reactive Oxygen-Derived Species ... 7

1.2 Myocardial Ischemia/Reperfusion Injury ........ 9

1.2.1 Role of Reactive Oxygen-Derived Radicals in Myocardial Ischemia/Reperfusion Injury ................. 10
1.2.2 Cytotoxic Effects of Oxy-Radicals on the Myocardium ......................... 12
1.2.3 Sources of Oxy-Radicals in the Ischemic Myocardium ......................... 13

1.2.3.1 Xanthine Oxidase ......................... 13
1.2.3.2 Activated Neutrophils .................... 14
1.2.3.3 Disruption of the Mitochondrial Electron Transport System ...... 15

1.2.3.4 Metabolism of Arachidonic Acid ........................... 16

1.2.3.5 Oxidation of Catecholamines ........... 16

1.2.3.6 Transition Metal Catalysed Oxy-Radical Production .......... 17

1.2.4 Possible Strategies in the Prevention of Oxy-Radical-Mediated Myocardial Ischemia/Reperfusion Injury ....................... 17

1.3 Rationale and Objectives of the Study .......... 19

1.3.1 Clinical Significance of Ischemia/Reperfusion Injury ............... 19

1.3.2 Protective Effects of Allopurinol Against Ischemia/Reperfusion Injury .... 21

1.3.3 Experimental Approaches ...................... 23

1.3.3.1 Molecular Aspects of Myocardial Ischemia/Reperfusion Injury and the Protective Effects of Allopurinol ...................... 23

1.3.3.2 Ischemia and Reperfusion in a Swine Model of Heart-Lung Transplantation and the Effects of Allopurinol Pretreatment ...... 27

1.3.3.4 Metal Chelating and other Antioxidant Properties of Allopurinol .......... 29

2. MATERIALS AND METHODS ........................................... 32

2.1 Surgical Procedures ............................................ 32

2.1.1 Animal Care .................................................. 32

2.1.2 Experimental Model of Ischemia/Reperfusion Injury ................... 32

2.1.2.1 Pentobarbital Anesthetized Open-Chest Rabbit .................. 32

2.1.2.2 Induction of Regional Ischemia .......................... 33
2.1.2.3 Identification of the Occluded Zone ......................... 33
2.1.2.4 Sham-Operated Animals .................. 33

2.1.3 Isolated Langendorff-Perfused Rabbit Heart ........................................... 34

2.1.3.1 Induction of Regional Ischemia in the Isolated Rabbit Heart .... 34

2.1.3.2 Sham-Control for Isolated Rabbit Heart ....................... 34

2.1.3.3 Induction of Regional Ischemia .. 35
In Vivo Followed by In Vitro Reperfusion

2.1.4 Heart-Lung Transplantation in Pigs ...... 35

2.2 Drug Treatment Protocols ................................. 37

2.2.1 Chronic Allopurinol Treatment in Rabbits ........................................ 37

2.2.2 Acute Allopurinol and Oxypurinol Treatment in Rabbits 37

2.2.3 Acute U74006F Treatment in Rabbits ...... 37

2.3 Biochemical and Chemical Analyses

2.3.1 Tissue Susceptibility to In Vitro Peroxide Challenge ......................... 38

2.3.1.1 Preparation of Tissue Homogenates ............................. 38

2.3.1.2 Susceptibility of Tissue to TBHP-Induced Depletion of GSH ... 38

2.3.1.3 Susceptibility of Tissues to Lipid Peroxidation .. 39

2.3.2 Erythrocyte Susceptibility to In Vitro Peroxide Challenge ................. 40

2.3.2.1 Preparation of Packed Erythrocytes .......................... 40

2.3.2.2 Susceptibility of Erythrocytes to TBHP-Induced Lipid Peroxidation ........ 40
2.3.2.3 Hemoglobin Assay .................. 41

2.3.3 Tissue Antioxidant Enzyme Activities .... 41

2.3.3.1 Preparation of Cytosolic Fractions .................. 42

2.3.3.2 Catalase ................................. 42

2.3.3.3 Cu,Zn-Superoxide Dismutase ...... 43

2.3.3.4 Glutathione Peroxidase ............... 43

2.3.3.5 Glutathione Reductase .................. 44

2.3.3.6 Hemoglobin Assay .......................... 45

2.3.3.7 Correction for Enzyme Activity Contributed by Blood .................. 45

2.3.4 Erythrocyte Antioxidant Enzyme Activities .................. 45

2.3.4.1 Preparation of Hemolysates ...... 45

2.3.4.2 Catalase ................................. 46

2.3.4.3 Cu,Zn-Superoxide Dismutase ...... 46

2.3.4.4 Glutathione Peroxidase ............... 46

2.3.4.5 Glutathione Reductase .................. 46

2.3.4.6 Hemoglobin Assay .......................... 47

2.3.5 Mitochondrial ATPase Activity .................. 47

2.3.5.1 Isolation of Mitochondria ...... 47

2.3.5.2 Mitochondrial Azide-Sensitive ATPase Activity .................. 48

2.3.6 Tissue ATP ................................. 49

2.3.7 Preparation of Erythrocyte Membranes .... 50

2.3.8 Ferric Chloride-Induced Oxidation of Erythrocyte Membrane Lipids .................. 51

2.3.9 Cupric Chloride-TBHP-Induced Oxidation of Erythrocyte Membrane Lipids .................. 52

2.3.10 Myoglobin-TBHP-Induced Oxidation of Erythrocyte Membrane Lipids .................. 53
2.3.11 Transition Metal Ion-Catalysed Oxidation of Ascorbic Acid .......................... 53

2.3.12 UV Absorption Spectroscopy of Ascorbate/Allopurinol/Copper Ion .......... 54

2.3.13 Myoglobin-TBHP-Catalysed Oxidation of Uric Acid ................................. 55

2.3.14 Statistical Analyses .......................... 56

3. RESULTS

3.1 Susceptibility of Tissues to Peroxide Challenge ........................................ 57

3.2 Altered Antioxidant Capacity in Ischemic/Reperfused Myocardial Tissues ........ 62

3.3 Alterations in Myocardial Antioxidant Capacity in Rabbits Subjected to Increasing Periods of Ischemia ................................................. 75

3.3.1 Effects of Allopurinol Pretreatment ...... 80

3.3.2 Effects of Acute Allopurinol and Oxypurinol Pretreatment .......................... 80

3.3.3 Effects of Chronic Allopurinol Treatment on the Activity of Myocardial Antioxidant Enzymes .................................................. 87

3.3.4 Effects of Acute U74006F Treatment on Ischemia/Reperfusion-Induced Alterations in Rabbit Myocardium .......................... 87

3.4 Time-Course of Alterations in Myocardial Antioxidant Capacity During Post-Ischemic Reperfusion .................................................. 91

3.4.1 Alterations in the Activities of Antioxidant Enzymes in Ischemic/Reperfused Myocardial Tissues .................. 100

3.4.2 pH Dependence of Antioxidant Enzyme Activities ...................................... 101

3.5 Brief Episodes of Ischemia .................. 104

3.5.1 Effects of Cumulative Brief Episodes of Ischemia on Myocardial Antioxidant Capacity and ATP Levels .......... 104
3.5.2 Effects of Ischemic Preconditioning on I/R-related Myocardial Alterations ... 109

3.6 I/R-Induced Alterations in Antioxidant Capacity in Isolated Langendorff-Perfused Hearts ................................................. 112

3.7 Heart-Lung Transplantation .............................. 115

3.7.1 Effects of Allopurinol Pretreatment on the Antioxidant Capacity of Pig Erythrocytes .............................................. 115

3.8 Metal Chelating Properties of Allopurinol .... 122

3.8.1 Effects of Allopurinol and Oxypurinol on Transition Metal Ion-Catalysed Oxidation of Ascorbic Acid .................. 123

3.8.2 Transition Metal Ion-Catalysed Oxidation of Erythrocyte Membrane Lipids .............. 128

3.8.2.1 Ferric Chloride-Induced Oxidation of Erythrocyte Membrane Lipids ..................... 129

3.8.2.2 Effects of Allopurinol and Oxypurinol on Ferric Chloride-Stimulated Oxidation of Erythrocyte Membrane Lipids .... 132

3.8.2.3 Effects of Allopurinol and Oxypurinol on Cupric Ion-TBHP-Induced Oxidation of Erythrocyte Membrane Lipids .... 137

3.8.3 UV Absorption Spectra of Allopurinol/Ascorbic Acid/Copper Ion ....................... 140

3.9 Myoglobin-TBHP-Catalysed Oxidation of Uric Acid and Erythrocyte Membrane Lipids .... 145

3.9.1 Effects of Allopurinol and Oxypurinol on Myoglobin-TBHP-Catalysed Oxidation of Uric Acid .............................. 145

3.9.2 Effects of Allopurinol on Myoglobin-TBHP-Induced Oxidation of Erythrocyte Membrane Lipids ....................... 148

4 DISCUSSION ..................................................... 154

4.1 Assessment of Tissue Antioxidant Capacity .... 154
4.2 Alterations in Antioxidant Capacity in Ischemic/Reperfused Rabbit Myocardium .......... 156

4.3 Time-Course of Alterations in Myocardial Antioxidant Capacity and Antioxidant Enzyme Activities During Post-Ischemic Reperfusion ........................................... 159

4.4 The Effects of Cumulative Brief Episodes of Ischemia on Myocardial Antioxidant Capacity and ATP Levels ....................... 162

4.5 I/R-Induced Alterations in Myocardial Antioxidant Capacity in Isolated Langendorff-Perfused Rabbit Hearts ............. 172

4.6 The Effects of Allopurinol Pretreatment on Myocardial I/R Injury ................................ 166

4.7 The Effects of Acute U74006F Treatment on I/R-Induced Alterations in Rabbit Myocardium .............................. 169

4.8 The Effects of Allopurinol Pretreatment on the Antioxidant Capacity of Erythrocytes and the Functional viability of Transplanted Lung Tissue ........................................... 172

4.9 Inhibitory Effects of Allopurinol and Oxypurinol on Transition Metal Ion-Catalysed Ascorbate Oxidation and Lipid Peroxidation ......................................................... 177

4.10 The Effects of Allopurinol on Myoglobin-TBHP-Catalysed Uric Acid Oxidation and Lipid Peroxidation .......................... 184

4.11 Summary and Conclusions ........................................ 187

5. REFERENCES .................................................. 193
<table>
<thead>
<tr>
<th>Table</th>
<th>LIST OF TABLES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Effects of chronic allopurinol treatment on myocardial antioxidant capacity.</td>
<td>88</td>
</tr>
<tr>
<td>II</td>
<td>Effects of acutely administered allopurinol or oxypurinol (50 mg/kg, i.v.) on I/R injury.</td>
<td>89</td>
</tr>
<tr>
<td>III</td>
<td>Effects of chronic allopurinol treatment on the activity of myocardial antioxidant enzymes.</td>
<td>90</td>
</tr>
<tr>
<td>IV</td>
<td>Effects of U74006F pretreatment on I/R-induced alterations in rabbit myocardium.</td>
<td>92</td>
</tr>
<tr>
<td>V</td>
<td>Time-course of post-ischemic reperfusion injury in rabbit myocardium: effects on antioxidant enzymes.</td>
<td>102</td>
</tr>
<tr>
<td>VI</td>
<td>Correlations between the activities of antioxidant enzymes and antioxidant capacity of ischemic/reperfused rabbit myocardium.</td>
<td>103</td>
</tr>
<tr>
<td>VII</td>
<td>Effects of acidotic pH on antioxidant enzyme activities.</td>
<td>105</td>
</tr>
<tr>
<td>VIII</td>
<td>Effects of brief cumulative episodes of ischemia on myocardial antioxidant capacity and ATP levels.</td>
<td>107</td>
</tr>
<tr>
<td>IX</td>
<td>Effects of brief cumulative episodes of ischemia on myocardial antioxidant enzyme activities.</td>
<td>108</td>
</tr>
<tr>
<td>X</td>
<td>Effects of ischemic preconditioning on I/R-induced myocardial alterations.</td>
<td>110</td>
</tr>
<tr>
<td>XI</td>
<td>Effects of ischemic preconditioning on myocardial antioxidant enzyme activities.</td>
<td>111</td>
</tr>
<tr>
<td>XII</td>
<td>I/R-induced alterations in antioxidant capacity in intact or isolated Langendorff-perfused rabbit hearts.</td>
<td>114</td>
</tr>
<tr>
<td>XIII</td>
<td>Inter-animal variation in allopurinol-induced protection against lipid peroxidation in pig erythrocytes.</td>
<td>119</td>
</tr>
<tr>
<td>XIV</td>
<td>Absorbance changes of the allopurinol-ascorbate-cupric chloride mixture: effects of EDTA.</td>
<td>144</td>
</tr>
<tr>
<td>Fig.</td>
<td>LIST OF FIGURES</td>
<td>Page</td>
</tr>
<tr>
<td>------</td>
<td>-----------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>TBHP-induced depletion of GSH in tissue homogenates prepared from non-ischemic tissues from rabbit.</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>TBHP-induced formation of TBARS in tissue homogenates prepared from non-ischemic tissues from rabbit.</td>
<td>61</td>
</tr>
<tr>
<td>3</td>
<td>Catalase activities in rabbit tissues.</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>Cu,Zn-SOD activities in rabbit tissues.</td>
<td>66</td>
</tr>
<tr>
<td>5</td>
<td>Glutathione peroxidase activities in rabbit tissues.</td>
<td>68</td>
</tr>
<tr>
<td>6</td>
<td>Glutathione reductase activities in rabbit tissues.</td>
<td>70</td>
</tr>
<tr>
<td>7</td>
<td>TBHP-induced depletion of GSH in myocardial tissue homogenates following a 40 min period of ischemia with or without subsequent reperfusion for 60 min in rabbits.</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td>TBHP-induced TBARS formation in myocardial tissue homogenates following a 40 min period of ischemia with or without subsequent reperfusion for 60 min in rabbits.</td>
<td>74</td>
</tr>
<tr>
<td>9</td>
<td>Changes in susceptibility of myocardial tissue to TBHP-induced depletion of GSH after varying periods of coronary artery ligation with or without 60 min of reperfusion in rabbits.</td>
<td>77</td>
</tr>
<tr>
<td>10</td>
<td>Changes in susceptibility of myocardial tissue to TBHP-induced formation of TBARS after varying periods of coronary artery ligation with or without 60 min of reperfusion in rabbits.</td>
<td>79</td>
</tr>
<tr>
<td>11</td>
<td>Effect of chronic allopurinol treatment on the susceptibility of myocardial tissue to TBHP-induced depletion of GSH after a 30 min period of coronary artery ligation followed by 60 min of reperfusion in rabbits.</td>
<td>82</td>
</tr>
</tbody>
</table>
12 Effect of chronic allopurinol treatment on the susceptibility of myocardial tissue to TBHP-induced formation of TBARS after a 30 min period of coronary artery ligation followed by 60 min of reperfusion in rabbits. 84

13 Effect of chronic allopurinol treatment on the tissue ATP level after a 30 min period of coronary artery ligation followed by 60 min of reperfusion in rabbits 86

14 Time-course of alterations in myocardial GSH levels during the course of post-ischemic reperfusion in rabbits. 95

15 Time-course of alterations in GSH depletion of myocardial tissue during the course of post-ischemic reperfusion in rabbits. 97

16 Time-course of alterations in formation of TBARS in myocardial tissue during the course of post-ischemic reperfusion in rabbits. 99

17 Effect of allopurinol treatment on susceptibility of pig erythrocytes to lipid peroxidation. 117

18 Correlation between erythrocyte MDA and lung water (LW) levels. 121

19 Cupric ion-catalysed oxidation of ascorbate: effects of allopurinol. 125

20 Cupric and ferric ion - catalysed oxidation of ascorbate in the presence of EDTA. 127

21 Ferric ion - induced formation of TBARS in erythrocyte membranes 131

22 Effects of GSH and ascorbic acid on the time-course of ferric ion - induced formation of erythrocyte membranes. 134

23 Ferric ion - induced formation of TBARS in erythrocyte membranes: effects of allopurinol and oxypurinol. 136

24 Cupric ion-TBHP - induced formation of TBARS in erythrocyte membranes: effects of allopurinol and oxypurinol. 139
25 UV absorption spectrum of the allopurinol-ascorbate-cupric chloride mixture: effects of EDTA.

26 Myoglobin-TBHP-catalysed oxidation of uric acid: effects of allopurinol and oxypurinol.

27 Time-course of myoglobin-TBHP-induced peroxidation of erythrocyte membrane lipids.

28 Myoglobin-TBHP-induced formation of TBARS in erythrocyte membranes: effect of allopurinol.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>allopurinol</td>
</tr>
<tr>
<td>ANS</td>
<td>1-amino-2-naphthol-4-sulfonic acid</td>
</tr>
<tr>
<td>ASC</td>
<td>ascorbic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithio-bis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis-(beta-amino ethyl ether) N,N'-tetraacetic acid</td>
</tr>
<tr>
<td>GPX</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GRD</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione, reduced form</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione disulfide</td>
</tr>
<tr>
<td>HO'</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
</tr>
<tr>
<td>ISC/NON</td>
<td>ischemic/non-reperfused</td>
</tr>
<tr>
<td>ISC/REP</td>
<td>ischemic/reperfused</td>
</tr>
<tr>
<td>LW</td>
<td>lung water</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate, reduced form.</td>
</tr>
<tr>
<td>$O_2\cdot^-$</td>
<td>superoxide anion radical</td>
</tr>
<tr>
<td>OXYP</td>
<td>oxypurinol</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>$pO_2$</td>
<td>arterial partial pressure of oxygen</td>
</tr>
<tr>
<td>PRECON</td>
<td>preconditioning</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
</tr>
<tr>
<td>SHAM-CON</td>
<td>sham-operated control</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TBA</td>
<td>thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>thiobarbituric acid-reactive substances</td>
</tr>
<tr>
<td>TBHP</td>
<td>tert-butylhydroperoxide</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>URC</td>
<td>uric acid</td>
</tr>
</tbody>
</table>
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my parents
and
my sweet heart Tammy
for their passion, patience and unlimited support
1. INTRODUCTION

1.1 Free Radicals in Biological Systems

A free radical is a molecule with an odd (unpaired) electron in its outer orbit; this unpaired electron makes the molecule unstable and highly reactive, especially towards biological molecules such as lipids, proteins and DNA. Free radicals can be produced in biological systems and may be important in a variety of cellular processes such as differentiation [1,2], aging [2,3], mutagenesis [4], carcinogenesis [3] and phagocyte-dependent inflammation [5]. In addition, oxygen-derived free radicals have been implicated in the pathogenesis of many diseases including ischemia/reperfusion (I/R) injury in brain [3], myocardium [6-8], liver [9], kidney [10], intestine [9], pancreas [3,9], skeletal muscle [9] and skin [3], oxygen toxicity of lung [11,12] and retina [3], and rheumatoid arthritis [13]. Furthermore, the toxic effects of some xenobiotics and chemical pollutants, such as paraquat, carbon tetrachloride and cigarette smoke, have been attributed to free radical-mediated tissue damage [14,15]. On the other hand, the free radical generating properties of some xenobiotics, such as chloramphenicol [16], tetracycline [16] and doxorubicin [17], have been exploited for their therapeutic use as anti-microbial and anti-proliferative agents.
1.1.1 Cellular Defense Mechanisms Against Free Radicals

Under normal physiological conditions, the oxygen molecule undergoes tetravalent reduction in aerobic cells via the mitochondrial cytochrome oxidase pathway with resulting formation of water [18]. During this normal course of metabolism, several reactive oxygen intermediates are formed. The first electron transfer to oxygen produces a superoxide anion radical (O$_2$•−) which, in turn, generates hydrogen peroxide (H$_2$O$_2$) following the acceptance of the second electron. With the third electron transfer, a hydroxyl radical (HO•) is formed. Finally, acceptance of the fourth electron generates a H$_2$O molecule. In addition, univalent reduction of O$_2$ also occurs in the mitochondrion; for instance, the semiquinone radical produced from ubiquinone during electron transport can give rise to a superoxide anion radical [19]. In the intact mitochondrion, most of the partially reduced reactive oxygen species are tightly bound to the enzymatic sites involved in their generation. To the extent that small amounts of reactive oxygen species leak into the cytosol, endogenous protective mechanisms, which consist of both enzymatic and non-enzymatic components, are capable of inactivating them, thereby protecting the cell from radical-mediated oxidative damage to membranes and alterations in subcellular organelle structural and functional integrity [20].
1.1.2 Antioxidant Enzymes

Superoxide anion radical (O$_2^-$) is catalytically converted by superoxide dismutase (SOD) into molecular oxygen and H$_2$O$_2$ [21]. Two forms of SOD have been described - a mitochondrial enzyme (Mn-SOD) which is cyanide insensitive and a cytosolic enzyme (Cu,Zn-SOD) which is highly sensitive to inhibition by cyanide [21]. H$_2$O$_2$, although not a free radical, may itself or in the presence of O$_2^-$, give rise to HO$^\cdot$ and singlet oxygen via the transition metal ion-catalysed Fenton or Haber-Weiss reaction, respectively [22]. The decomposition of H$_2$O$_2$ is catalysed by catalase (CAT) whose action, when coupled with that of SOD, can prevent the generation of hydroxyl radicals. Organic hydroperoxides arising from hydroxyl radical-mediated peroxidation reactions are detoxified by the glutathione peroxidase (GPX) [23]. Two forms of GPX exist of which the selenium-containing enzyme (Se-GPX) acts on a variety of substrates, including hydrogen peroxide and organic hydroperoxides [24], while the non-selenium-dependent GPX does not utilize H$_2$O$_2$ and belongs to a family of glutathione transferase enzymes [25]. The catalytic decomposition of hydroperoxides by GPX is associated with the simultaneous oxidation of glutathione (GSH) to its disulfide form (GSSG), which is regenerated by glutathione reductase (GRD) using NADPH derived mainly from the hexose monophosphate shunt enzyme glucose-6-phosphate dehydrogenase as a reducing agent [26]. The aforementioned antioxidant
enzymes are widely distributed in cells of tissues, especially those with high rates of aerobic metabolism, with highest activities often being found in liver cells [26,27].

1.1.3 Non-Enzymatic Antioxidants

In addition to the critical role of antioxidant enzymes in preventing tissue oxidative damage, endogenous molecules, such as α-tocopherol, β-carotene, ascorbic acid, uric acid and GSH or protein thiols etc., are also important in the detoxification of oxy-radicals. Alpha-tocopherol (Vitamin E), a major lipid-soluble antioxidant present in biological membranes, protects against peroxidation of membrane lipids [28]; it can scavenge a variety of reactive oxy-radicals, including O$_2^{'-}$ [29], HO$^*$ and peroxyl radicals [30], as well as singlet oxygen [31]. Beta-carotene, a major carotenoid precursor of Vitamin A, is also present in cellular membranes [32], and likely has antioxidant functions similar to those of α-tocopherol. Thus, lipophilic antioxidants, which are intercalated in biological membranes, are capable of terminating potentially deleterious free radical chain reactions occurring in the lipid bilayers. In aqueous domains, ascorbic acid (Vitamin C) reacts directly with O$_2^{'-}$ [33], HO$^*$ [34] and singlet oxygen [35], while uric acid, a product derived from purine metabolism, not only has an antioxidant profile similar to that of ascorbic acid, but also reduces oxidants produced from the reaction of peroxide with hemoproteins and inactivates catalytically active
transition metal ions by chelation [36]. In addition to serving as a reductant in the GSH-dependent peroxidase system, GSH also reacts directly with a wide variety of free radical species, including carbon-centred, peroxyl, phenoxy and semiquinone radicals [37,38]. Presumably, thiol groups of protein molecules interact with free radical species in a similar manner [39].

1.1.4 Extracellular Antioxidant System

In extracellular fluids, antioxidant enzyme activity is very low [40,41]. However, the iron-binding protein transferrin [42] is present in such a quantity that the amount of reactive iron available in extracellular fluids for catalysing the generation of HO• from O2•− and/or H2O2 is usually negligible. Moreover, the protein ceruloplasmin, in addition to binding copper ions, can catalyse the conversion of ferrous to ferric ion [43], the latter being unreactive towards H2O2. In addition, albumin, a major constituent of the plasma, is capable of binding free copper ions released into the plasma [13]. Therefore, in contrast to the intracellular enzymatic systems, extracellular antioxidant protection is largely directed towards preventing the generation of hydroxyl radicals rather than detoxifying O2•− and H2O2. When the total antioxidant capacity of plasma is assessed in terms of ability to inactivate peroxyl radicals, contributions by various components to the total antioxidant activity of plasma are
as follows: α-tocopherol 5%, ascorbic acid 15%, uric acid 25% and protein thiols 50% [39]. The time-course of changes in concentrations of plasma antioxidants caused by peroxyl radicals indicates that the first line of defense against radical attack is provided by plasma thiols, uric acid (the second vulnerable antioxidant in plasma) being spared during the initial stages of the reaction [39]. Moreover, ascorbic acid seems to play a pivotal role in protecting plasma lipids from peroxidation initiated either by aqueous peroxyl radicals or by activated polymorphonuclear leukocytes; the depletion of plasma ascorbic acid is followed by formation of lipid peroxidation products [44].

1.1.5 Antioxidant Interactions

In enzymatic antioxidant systems, a delicate balance in the activities of these key enzymes must be maintained in order to cope with oxidative challenges. Antioxidant enzymes are protected from free radical-induced inactivation in a co-operative manner; for instance, CAT can prevent the $\text{H}_2\text{O}_2$-induced inactivation of SOD [45], and reciprocally, SOD protects CAT and GPX from $\text{O}_2^{.-}$ mediated inactivation [46]. Moreover, the continuous functioning of GPX requires a sustained supply of GSH regenerated from GSSG via the GRD-catalysed reaction, which, in turn, utilizes hexose monophosphate shunt-derived NADPH as reductant, as mentioned previously. With regard to non-enzymatic antioxidant systems, α-tocopherol can protect β-carotene from
oxidation [47]. On the other hand, α-tocopherol can be continuously regenerated at the expense of ascorbic acid with resulting preservation of lipid-soluble antioxidants [48]. In the presence of transition metals, ascorbic acid can produce free radicals via oxidation reactions [49]; however, uric acid protects this ascorbic acid oxidation by virtue of its metal chelating properties [50,51]. Since superoxide radicals can arise from reaction of GSH with free radicals through the intermediacy of thiyl (GS') radical [52], the antioxidant effect of GSH per se requires the concomitant action of SOD [53]. In addition, α-tocopherol and GPX are believed to act synergistically in the prevention of lipid peroxidation [28]. Furthermore, the redox cycling of myoglobin induced by GSH or ascorbic acid is thought to be an important antioxidant mechanism in protecting muscles against oxidative injury [54,55]. Given the inter-dependence and integration of the entire antioxidant system, an impairment in any one component may upset the whole defense mechanism for protecting against free radical attack, leading to irreversible cellular damage.

1.1.6 Cytotoxic Effects of Hydroxyl Radical and Other Reactive Oxygen - Derived Species

Under conditions that antioxidant processes become impaired and/or overwhelmed by increased production of free radicals, oxidative damage will result from free radical-
mediated reactions which are mainly triggered by HO'. This highly reactive species can cause the peroxidation of membrane lipids by initiating free radical chain reactions, with resulting disruption of cellular or subcellular structural and functional integrity [56,57].

The lipid peroxidation reaction begins with HO' abstraction of a hydrogen atom from the lipid molecule, which then becomes a lipid free radical.

\[ RH + HO' \rightarrow R' + H_2O \]

Following the restructuring of intramolecular unsaturated linkages, the lipid radical reacts with O₂ to form a lipid hydroperoxyl radical.

\[ R' + O_2 \rightarrow R-0-0' \]

The peroxyl radical can react with other lipid molecules in the membrane in the chain propagating phase of the reaction.

\[ R-0-0' + RH \rightarrow R-OOH + R' \]

The lipid hydroperoxide formed can undergo a variety of reactions to yield breakdown products, such as alkanes (e.g. ethane and pentane), malondialdehyde and aldehydes. The presence of transition metals can enhance the reaction cascade by catalysing the degradation of lipid hydroperoxides [58]. On the other hand, the chain reaction will be terminated if two of these free radical molecules combine with each other or if they are inactivated by cellular antioxidants, such as α-tocopherol and β-carotene [28,59]. However, if the lipid bilayer of cell membranes is peroxidized to the extent that the cell becomes incapable of
maintaining electrolyte and volume homeostasis, cell death will ensue. Protein and nucleic acid molecules are also oxidation sensitive [60,61]. Free radical-induced damage to thiol group-containing enzymes and other proteins can culminate in inactivation, cross-linking, or denaturation. Nucleic acid molecules can undergo modification or even scission in the presence of HO· and the resulting damage can give rise to carcinogenesis or mutagenesis. Oxidative damage to carbohydrates can alter any of their cellular receptor functions, including those mediating hormonal and neurotransmitter actions. Furthermore, aldehydes such as malondialdehyde and hydroxynonenal resulting from free radical-induced degradation of polyunsaturated fatty acids can cause cross-linking in lipids, proteins and nucleic acids, leading to marked functional impairment.

1.2 Myocardial Ischemia/Reperfusion Injury

Coronary reperfusion accomplished by thrombolytic or angioplastic procedures has become a standard treatment for patients suffering from acute myocardial infarction [62,63]. An extensive clinical trial which examined intravenous streptokinase treatment showed that early reperfusion, especially within 3 hours following the onset of myocardial infarction, reduced the incidence of sudden death and improved longevity [64]. While the beneficial effects of early coronary reperfusion are undisputable, there are some deleterious consequences of reperfusion, including
intramyocardial hemorrhage, arrhythmias, the "no-reflow" phenomenon, prolonged functional impairment and myocardial cell necrosis [65]. As a result, an increased mortality in patients on the first day following streptokinase treatment was observed [66]. Moreover, large hemorrhagic infarcts have been noted in patients dying after the coronary bypass surgery [67]. During the past few years, a growing body of evidence has accumulated suggesting that reperfusion may indeed have an injurious component, termed "reperfusion injury", which accounts, at least in part, for the adverse sequelae following post-ischemic reperfusion of the myocardium. In this regard, oxygen-derived free radicals have been implicated in the pathogenesis of myocardial ischemia/reperfusion (I/R) injury in various experimental and clinical settings [68,69].

1.2.1 Role of Reactive Oxygen-Derived Radicals in Myocardial Ischemia/Reperfusion Injury

The involvement of endogenous oxy-radicals in the development of myocardial I/R injury was early suggested by the protective effects of anti-free radical interventions in various experimental settings. Many in vitro studies using isolated hearts prepared from several species (including rats, rabbits and pigs) showed that oxy-radical scavengers and iron chelators, when administered at the time of reflow, could blunt the myocardial injury and dysfunction induced by hypoxia with subsequent reoxygenation [70-72]. In studies
using in vivo settings of myocardial ischemia and reperfusion, the administration of oxy-radical scavengers during the period of reperfusion caused a reduction in myocardial infarct size when compared with untreated animals [73,74]. Moreover, oxy-radical scavengers added to preservative solutions could reduce reperfusion injury in hearts following transplantation [75-77]. Recently, earlier difficulties involved in the direct detection and characterization of oxy-radicals in the setting of myocardial ischemia and reperfusion have been overcome by using electron spin resonance measurements with spin traps such as DMPO (5,5-dimethyl-1-pyrolone-N-oxide) [78]. Several in vitro studies with isolated hearts subjected to global ischemia and reperfusion have described a burst of oxy-radical formation within the first minutes of reperfusion [78-80]. Carbon- and oxygen-centered radicals in myocardial tissues harvested from canine hearts subjected to regional ischemia were also observed [81]. Similarly, Bolli et. al. [82] reported an abrupt increase in radical concentration in the coronary venous effluent immediately after the onset of reperfusion in dogs subjected to 15 minutes of coronary occlusion. Taken together, all the foregoing studies provide both indirect and direct evidence of oxy-radical production in the setting of myocardial ischemia and reperfusion.
1.2.2 Cytotoxic Effects of Oxy-Radicals on the Myocardium

Direct cytotoxic effects of oxy-radicals on the myocardium have been demonstrated in studies involving the exposure of in vitro preparations to exogenously generated oxy-radicals. Oxy-radicals generated from a mixture of purine plus xanthine oxidase caused functional impairment of isolated papillary muscles [83,84]. Similarly, the exposure of isolated rat hearts to $O_2^\cdot-$ generated by infusion of hypoxanthine and xanthine oxidase reduced left ventricular developed pressure, depleted high energy phosphate levels, and caused cellular edema [85]. Moreover, exposure of the myocardium to HO$^\cdot$ generated by infusion of xanthine and xanthine oxidase in the presence of iron-loaded transferrin caused severe ultrastructural damage to myocardial cells, including swelling and disruption of mitochondria, blebbing of the sacrolemma and breaks within the sarcolemmal membrane [86]. In in vivo preparations, intracoronary infusion of xanthine oxidase and purine plus iron-loaded transferrin to anesthetized dogs resulted in left ventricular wall-motion abnormalities in the perfused region of the heart [87]. High concentrations of xanthine oxidase/purine/transferrin solution administered into the aortic root through the carotid artery produced myocardial contraction bands and interstitial edema in rat hearts [88]. All these functional, chemical and ultrastructural alterations induced by exogenous oxy-radicals resembled those seen in
ischemic/reperfused hearts and could be prevented by oxy-radical scavengers such as SOD and CAT.

Although the cardiotoxic effects of oxy-radicals are well established, there is still some controversy regarding which of the oxy-radical species is the major culprit. Current opinion seems to suggest that oxy-radical species, such as \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \) and \( \text{HO}^- \), may all contribute to the damage.

1.2.3 Sources of Oxy-Radicals in the Ischemic Myocardium

1.2.3.1 Xanthine Oxidase

One potential source of radical production in ischemia is xanthine oxidase, a purine-degradation enzyme present mainly in endothelial cells [89,90]. In ischemic tissues, degradation of high energy adenine nucleotides can lead to the accumulation of hypoxanthine and xanthine, which are natural substrates for xanthine oxidase. The depletion of cellular energy stores caused by ischemia can impair ion homeostasis, resulting, for example, in an increase of cytosolic calcium and sodium concentrations [91]. The increased intracellular calcium activates a protease [89] which presumably converts xanthine dehydrogenase, a \( \text{NAD}^+ \)-dependent (non-radical producing) form of the enzyme that accounts for about 90% of the total activity in non-ischemic tissue, to the oxygen-dependent (radical producing) xanthine oxidase [92]. Subsequent reperfusion (ie. reoxygenation) of the previously ischemic tissue in the presence of high
concentrations of xanthine oxidase and its substrates can lead to a marked increase in the production of \( O_2^- \) \[93\], with resulting cytotoxic effects mediated by the transition metal-catalysed formation of \( HO^\cdot \) \[94\]. This hypothesis is strongly supported by numerous studies which have shown protective effects of allopurinol (a xanthine oxidase inhibitor \[95\]) on I/R-related damage in a variety of tissues \[90\]. There is no doubt that oxy-radicals can be generated by the xanthine oxidase pathway, but the importance of this pathway in I/R injury may be species dependent. Xanthine oxidase is present in measurable quantities in canine and rat hearts, but has been found to be undetectable in rabbit and human myocardium \[96-98\].

1.2.3.2 Activated Neutrophils

Activated neutrophils are another possible source of oxy-radicals \[68,99\]. If myocardial ischemia produces an increased flux of \( O_2^- \), the \( O_2^- \) sensitive chemotactic factor present in extracellular fluids will be activated \[5\]. The presence of this chemotactic factor would then cause neutrophils to adhere to the endothelium, to extravasate, and eventually enter the interstices \[5\]. Activated neutrophils possess a plasma membrane-associated NADPH-oxidase that reduces \( O_2 \) to \( O_2^- \), with concomitant oxidation of cytosolic NADPH \[100\]. Since the increased production of \( O_2^- \) can attract and activate more neutrophils, a self-perpetuating cycle would ensue. In
addition, myeloperoxidase (located within granules of the neutrophil) can, in the presence of H$_2$O$_2$ (derived from O$_2^{•−}$), lead to formation of hypochlorous acid (HOCl), a highly reactive substance capable of causing extensive cellular damage [101]. In addition to the foregoing actions of circulating neutrophils, mast cells and macrophages resident in the heart may also play an important role in the development of myocardial I/R injury. In this regard, activation of the resident cardiac leukocytes has been demonstrated in isolated rat hearts subjected to hypoxic perfusion with neutrophil-free solution followed by reoxygenation [102].

1.2.3.3 Disruption of the Mitochondrial Electron Transport System

Another potential source of oxy-radicals is the production of O$_2^{•−}$ due to leakage of electrons from the electron transport system within mitochondria [69,103]. During ischemia, the intracellular adenine nucleotide pool is depleted by the increased energy demands and degradative processes, and electron carriers in the mitochondrial respiratory chain are converted to their reduced state. The exposure of O$_2$ to these reduced (i.e., unreoxidized) carriers during reperfusion will lead to the increased formation of O$_2^{•−}$. The impairment in mitochondrial antioxidant capacity during ischemia [104] would enhance the susceptibility of mitochondria to radical attack. This self-induced
mitochondrial damage would progress, leading to greater and greater fluxes of reactive radical species derived from molecular oxygen.

1.2.3.4 Metabolism of Arachidonic Acid

During ischemia, the increase in cytosolic calcium concentration can activate phospholipase A$_2$ [105] which, in turn, releases arachidonic acid from plasma membranes of myocardial cells [106]. Arachidonic acid produces biologically active prostanoids, such as the vasoactive thromboxane A$_2$ and prostacyclin, through the action of cyclooxygenases, which reside mainly in endothelial cells. This cyclooxygenase pathway of arachidonic acid metabolism produces endoperoxides, which are reactive oxidants with the ability to initiate deleterious chain reactions [107]. In addition, arachidonic acid is metabolized by lipoxygenases present in leukocytes to produce leukotrienes and other metabolites that act as chemotaxins to attract and activate more leukocytes [108].

1.2.3.5 Oxidation of Catecholamines

The oxidation of catecholamines may also be a source of oxy-radicals leading to damage in the ischemic/reperfused myocardium [109,110]. The oxidation of epinephrine by microsomal preparations produces O$_2^-$ and other radical intermediates [111,112]. Similar oxidation of epinephrine can occur by other mechanisms involving iron-
containing proteins [113] and iron-heme proteins with H$_2$O$_2$ [114]. In addition, transition metal ions, such as Cu$^{2+}$ and Fe$^{3+}$, catalyse the oxidation of epinephrine, with the generation of oxy-radicals as byproducts [115,116]. Thus, oxidation of catecholamines released from the ischemic hearts could cause a burst of oxy-radical production at the onset of reperfusion [117].

1.2.3.6 Transition Metal Catalysed Oxy-Radical Production

Transition metal ions, particularly certain active forms of iron, can catalyse the production of additional oxy-radicals in ischemic tissue [13]. The iron-loaded transferrin appears to support the catalysis of the Haber-Weiss reaction to produce HO$^\cdot$ [118]. Additional catalytically active forms of iron may be produced from the interaction of hemoproteins with peroxides [119]. In addition, O$_2^\cdot$ interacts with ferritin (iron storage protein) to release iron that can catalyse the formation of reactive oxy-radicals [120].

1.2.4 Possible Strategies in the Prevention of Oxy-Radical-Mediated Myocardial Ischemia/Reperfusion Injury

It now seems quite certain that oxy-radicals play an important role in the development of irreversible I/R injury in the myocardium. Two main strategies could, therefore, be adopted to prevent this clinically relevant form of tissue damage: (1) administration of oxy-radical scavengers or (2)
reduction of oxy-radical production. Pretreatments with SOD and/or CAT have been shown to protect against myocardial I/R injury in various experimental settings [73,77,121], and to prevent the formation of oxy-radicals in ischemic/reperfused myocardium [78]. The conjugated forms of superoxide dismutase (such as the polyethylene glycol conjugates), which have an extended half-life [122], were more effective than the unconjugated form in the protection against I/R-induced damage in vivo [122,123]. Moreover, endotoxin pretreatment, which increases endogenous myocardial catalase activity, was found to decrease the extent of I/R injury in isolated rat hearts [124]. In addition, pretreatment with non-enzymatic antioxidants, such as dihydroquinolone derivatives [125], a-tocopherol [126], ascorbic acid [127] and its lipophilic derivative 2-octadecylascorbic acid [128], and the thiol-containing compound N-acetylcysteine [129], were all found capable of reducing the severity of I/R injury. On the other hand, therapeutic interventions might also be directed towards neutrophil-related oxy-radical formation. In this regard, pretreatment with neutropenic agents or inhibitors of neutrophil activation was shown to reduce infarct size following ischemia and reperfusion in dogs [130,131]. Another potential target for therapeutic intervention is the xanthine oxidase-mediated formation of oxy-radicals. In fact, the administration of allopurinol has been shown to reduce the size of myocardial infarcts induced by coronary artery ligation [132-135] and
reperfusion-induced arrhythmias [134,136]. The catalysis of oxy-radical production in ischemic tissue could also be prevented by the treatment with transition metal ion chelators. This has been demonstrated in the case of deferoxamine which protected against tissue damage in normal or iron-loaded rat hearts subjected to a period of anoxia followed by reoxygenation [137] and the oxy-radical generation during cardiopulmonary bypass in man [138]. In addition, pretreatment with the orally active iron chelator 1,2-dimethy-3-hydroxy-4-pyridone was also able to protect rat hearts from reperfusion injury [139].

1.3 Rationale and Objectives of the Study

1.3.1 Clinical Significance of Ischemia/Reperfusion Injury

Cardiovascular disease remains the major cause of death in North America and Western Europe, and the majority of these deaths are attributable to atherosclerosis-related ischemic heart disease [140]. In acute myocardial infarction resulting from thrombosis, early coronary reperfusion through thrombolysis has been shown to reduce mortality [62,64]. While there is no doubt about the need to reestablish perfusion of the ischemic myocardium, there are some potentially negative aspects of reperfusion which may account for the high incidence of post-infarction mortality and adverse sequelae [141,142]. Timely reperfusion of ischemic myocardium can reduce the amount of tissue necrosis after acute coronary artery occlusion, but
reperfusion, while terminating the ischemia, might also cause further damage to ischemic tissues. Over the past decade, oxygen-derived free radicals generated during the reperfusion phase have been suggested to play an important role in the pathogenesis of irreversible tissue damage following ischemia and reperfusion. A growing body of evidence has now emerged supporting the involvement of oxy-radicals in the development of myocardial I/R injury [68, 143,144].

Reperfusion of the previously ischemic myocardium has become feasible in many clinical situations. Interventions such as the administration of plasminogen activator or streptokinase, and percutaneous transluminal angioplasty have been shown to successfully reestablish coronary flow in patients during myocardial infarction [62,63]. In addition, patients undergoing cardiopulmonary bypass procedures during cardiac surgery are also exposed to periods of ischemia and reperfusion, in which ischemia is induced by aortic cross-clamping with reperfusion being initiated by releasing the cross-clamp. Similarly, in orthotopic heart transplantation, the transplanted heart is reperfused after a prolonged period of storage under conditions of hypothermic global ischemia. Protection against I/R injury has therefore become a crucial factor in optimizing the effectiveness of post-infarction myocardial salvage, thrombolytic or revascularization procedures, cardioplegia and organ transplantation [143].
1.3.2 Protective Effects of Allopurinol Against Ischemia/Reperfusion Injury

The clinically relevant consequences of reperfusion injury have led to intensive investigations of possible therapeutic interventions aimed at reducing the extent of tissue injury. The pretreatment with antioxidant enzymes, such as SOD and CAT, can protect against tissue damage in a variety of experimental models of I/R injury [73,122,145,146]. However, the limited clinical usefulness of these macromolecular substances, which usually have a relatively short in vivo half-life, has provided a strong impetus to devise pharmacological interventions using compounds with more convenient pharmacokinetic properties. One of the more actively investigated agents in this regard is allopurinol, which has been shown to reduce manifestations of ischemic injury in a number of systems, including skeletal muscle [147], liver [148], kidney [149], brain [150,151], intestine [152] and, most notably, the myocardium in several species, including rat [153], dog [133,154], rabbit [155,156] and pig [157].

Allopurinol, a xanthine oxidase inhibitor, was synthesized in the late 1950s and used clinically to inhibit the oxidative degradation of mercaptopurine, an anti-proliferative agent [95]. Moreover, inhibition of xanthine oxidase by allopurinol is particularly useful in managing complications of hyperuricemia, including primary gout and urate stone formation in the kidney [158], allopurinol being
associated with an incidence of adverse reactions of about 3.5% at normal therapeutic dose [159]. The ability of allopurinol to protect against I/R injury was initially attributed to inhibition of xanthine oxidase whose action on hypoxanthine arising from ATP degradation during the course of ischemia and reperfusion leads to the generation of superoxide anion radicals [89]. However, the finding that xanthine oxidase activity is virtually undetectable in rabbit and pig hearts [96,98] has cast considerable doubt on the primary role of xanthine oxidase inhibition in allopurinol protection against I/R injury. This is further strengthened by the finding that xanthine oxidase activity, when present, is found almost exclusively in vascular endothelial cells rather than in cardiac myocytes [160,161].

A number of other possible mechanisms of allopurinol protection have been proposed, including an increased efficiency of ATP salvage and resynthesis [162,163], facilitation of mitochondrial electron transfer [164] and direct inactivation of endogenously formed reactive species, such as hydroxyl radicals or myeloperoxidase-derived hypochlorous acid [165].
1.3.3 Experimental Approaches

1.3.3.1 Molecular Aspects of Myocardial Ischemia/Reperfusion Injury and the Protective Effects of Allopurinol

As noted in previous studies from our laboratory, chronic allopurinol treatment can protect against I/R-induced ultrastructural and biochemical alterations in rabbit myocardium which contains undetectable xanthine oxidase activity [155,156]. We hypothesized that allopurinol pretreatment may decrease tissue susceptibility to oxidant injury (i.e., increase tissue antioxidant capacity) by modifying other processes involved in the generation or scavenging of reactive oxy-radicals which are unrelated to xanthine oxidase inhibition.

As an approach to investigating the time-course of I/R-related alterations in myocardial antioxidant status and determining whether it coincides temporally with those of ultrastructural and biochemical manifestations of irreversible injury observed in the previous studies [155,156], pentobarbital anesthetized open-chest rabbits were subjected to increasing periods of coronary artery ligation, with or without a subsequent 60 min of reperfusion. Antioxidant capacity of myocardial tissue was assessed in terms of susceptibility to in vitro oxidative challenge. The effects of pretreatment with allopurinol (previously found to protect against I/R-induced ultrastructural and biochemical alterations in rabbit myocardium) and U74006F (a novel 21-aminosteroid antioxidant
which has been shown to be markedly protective in various experimental models of brain or spinal cord ischemic injury [166-168]) were examined with regard to their possible effects on I/R-related alterations in myocardial antioxidant capacity.

One possible mechanism of allopurinol protection against myocardial I/R injury might involve alterations in antioxidant enzyme activities. The effects of chronic allopurinol treatment on the activity of myocardial antioxidant enzymes were, therefore, examined in rabbits not subjected to myocardial ischemia and reperfusion. Findings regarding I/R-induced alterations in the activity of myocardial antioxidant enzymes from other investigators have been inconsistent. The activity of myocardial glutathione peroxidase has been reported to be decreased [169], increased [170] or unchanged [104,171,172] following ischemia and/or reperfusion. Peterson et al. [154] have demonstrated an initial decrease in catalase activity in ischemic dog myocardium after 30 min of reflow, with complete restoration of activity after 2 hr of reperfusion. In contrast, evidence that glutathione reductase activity is transiently increased after ischemia suggests that the pattern of changes may vary with the particular enzyme studied [170]. Although a burst of radical formation has been observed during the early phase of post-ischemic reperfusion in the myocardium [78,80], it has been shown in dogs that the maximal degree of myocardial necrosis induced
by a short period of coronary occlusion is not attained until several hours after the initiation of reperfusion [130]. These suggest that secondary oxidative processes occurring during the prolonged period of post-ischemic reperfusion are also important in the development of irreversible I/R injury. Other time-dependent changes in myocardial antioxidant enzyme activities and lipid peroxidation during the course of ischemia and reperfusion have also been observed [173,174]. In the present investigation, we have, therefore, undertaken a time-course study of reperfusion-related alterations in myocardial antioxidant capacity and antioxidant enzyme activities in rabbits subjected to a 40 min period of ischemia to address the following questions: (1) are there alterations in myocardial antioxidant capacity during the course of post-ischemic reperfusion; (2) what is the time-course involved in the foregoing changes in myocardial antioxidant capacity (i.e., does it correlate with the burst of oxy-radical formation occurring within the first few minutes of post-ischemic reperfusion [78,80]); and (3) is there inactivation or possibly a transient impairment in the functioning of myocardial antioxidant enzymes during the course of post-ischemic reperfusion, which is likely related to ischemia-associated acidosis [175,176] and might be crucial in triggering myocardial I/R injury?

In the "stunned myocardium", some early changes, such as contractile impairment and ATP depletion, occurring after
a brief period of ischemia are not readily reversible with reperfusion [177]. It has also been suggested that intermittent brief periods of ischemia may exert a cumulative effect and cause the progressive development of myocardial necrosis and ventricular dysfunction [178]. In contrast, findings reported by Lange et. al. [179] and Swain et. al. [180] have shown that myocardial alterations in coronary blood flow, regional function and ATP content were not cumulative after repetitive brief episodes of ischemia. As an approach to investigating whether I/R-related alterations in myocardial antioxidant capacity can be influenced by the partial depletion of tissue ATP level induced by ischemia, the effects of brief episodes of ischemia and reperfusion on myocardial antioxidant status in relation to tissue ATP levels were examined in pentobarbital anesthetized open-chest rabbits. In addition, the effects of ischemic preconditioning (by repetitive brief episodes of ischemia), which has been shown to reduce infarct size in pigs [181], were also investigated.

As mentioned earlier, oxidants derived from activated neutrophils have been shown to be a crucial factor involved in the development of I/R injury [182,183]. The role of blood elements, mainly neutrophils, in causing I/R-induced oxidative injury was investigated in isolated Langendorff-perfused rabbit hearts subjected to coronary artery ligation followed by reperfusion, and the I/R-related alterations in myocardial antioxidant capacity were examined.
Allopurinol protection against I/R injury has been demonstrated in numerous experimental models of organ transplantation, involving kidney [184], liver [185] and heart [186]. The fact that the protective actions of allopurinol are demonstrable in a variety of tissues, including kidney [187], liver [188] and cerebrum [150], suggests its effects on antioxidant status may be generalized and widespread. This possibility has been explored by examining the effects of chronic allopurinol treatment on the antioxidant capacity of red cells, as reflected in their susceptibility to in vitro oxidative challenge. These experiments were performed in connection with a parallel heart-lung transplantation investigation examining the effects of pretreating donor and recipient animals with allopurinol on the functional viability of transplanted heart and lung tissues as described below.

1.3.3.2 Ischemia and Reperfusion Injury a Swine Model of Heart-Lung Transplantation and the Effects of Allopurinol Pretreatment

Reperfusion of transplanted organs which have been subjected to periods of cold and/or warm ischemia during pre-operative storage may cause deleterious effects, leading to post-transplantation organ failure [9]. Although the exact mechanism responsible for the irreversible damage to transplanted organs has yet to be determined, there is ample evidence from studies of I/R injury in various experimental
and clinical settings [74,189] to suggest the involvement of oxy-radicals.

Heart-lung transplantation has been used successfully in the treatment of end-stage cardiopulmonary disease since 1981, with hundreds of patients now having undergone successful heart-lung transplantation worldwide. The effectiveness of organ preservation for transplantation seems to be a crucial factor in determining the functional viability of transplanted organs [190,191]. Despite the relative success of this most complicated surgical procedure, currently available methods for preservation of the lung from I/R injury following prolonged periods of ischemia are far from optimal [192,193]. Therefore, for several years, it was considered necessary to have donor and recipient at the same centre for performance of the transplantation procedure. Although several methods of preservation have been evaluated, none of them has been successful in preventing highly vulnerable lung tissue from I/R injury [192,193]. In this regard, allopurinol, whose effectiveness in preserving transplanted organs has been well documented [184-186], may be effective in protecting lung tissue against I/R injury. This possibility has been explored by examining the effects of allopurinol pretreatment in a swine model of heart-lung transplantation.
1.3.3.4 Metal Chelating and Other Antioxidant Properties of Allopurinol

The catalytic role of transition metals in mediating oxy-radical induced tissue damage is well established [13,194]. The iron chelator deferoxamine and allopurinol have both been shown to reduce cellular damage in isolated rabbit hearts subjected to a period of hypoxia followed by reoxygenation [195]. In addition, uric acid, whose chemical structure is similar to that of allopurinol, has been shown to be capable of chelating copper and iron ions [50,51]. These findings suggest that another possible mechanism of allopurinol protection against I/R injury might involve chelation of transition metal ions. This idea has been explored by investigating the actions of allopurinol and its metabolite, oxypurinol, on cupric ion- and ferric ion-catalysed oxidation of ascorbate and oxidation of erythrocyte membrane lipids.

Although pharmacological interventions involving pretreatment with allopurinol [155,156] or iron chelators [139,196] have been shown to reduce the extent of I/R-induced tissue damage, the extent of protection is incomplete. This suggests that xanthine oxidase- and iron-dependent systems are likely not the only sources of radical production involved in the development of myocardial I/R injury. Since myoglobin is present in cardiac muscle at a high concentration [197], its pro-oxidant action in ischemic tissue [198-200] might also be an important determinant in
the triggering of uncontrolled oxidative processes. In the ischemic myocardium, increased production of hydrogen peroxide from the enhanced autoxidation of oxy-myoglobin [200] or abnormal metabolism of oxygen [81,201] would stimulate the oxidation of myoglobin, with the concomitant production of reactive ferryl heme oxidants [198,199]. These myoglobin-derived oxidants could result in the peroxidation of biological membrane lipids [202] and oxidative damage to protein molecules [203], subsequently leading to tissue injury. The use of pharmacological agents capable of attenuating the potentially harmful effects of myoglobin would represent a rational approach to protecting against myocardial I/R injury. In order to explore other actions of allopurinol relating to its protective effects on I/R injury, effects of allopurinol and oxypurinol on the myoglobin-t-butylhydroperoxide-catalysed oxidation of uric acid and peroxidation of erythrocyte membrane lipids were examined.

In conclusion, the present investigation was aimed at elucidating the molecular basis in the development of myocardial I/R injury and exploring mechanistic alternatives to xanthine oxidase inhibition in the protection by allopurinol against I/R injury. Information obtained from the aforementioned studies may be useful in devising effective therapy in the prevention of I/R-induced clinically relevant tissue damage. On the basis of observed changes in antioxidant capacity of erythrocytes, we also
intended to explore the possibility of optimizing the effectiveness of therapeutic interventions (including allopurinol) undertaken in patients prior to cardiopulmonary bypass surgery or organ transplantation in order to minimize the risk of damage resulting from post-ischemic tissue reperfusion.
2. MATERIALS AND METHODS

2.1 Surgical Procedures

2.1.1 Animal Care

Animals received food and water ad libitum and were maintained in a constant temperature (22°C) environment with a constant 12-hr light schedule (light on at 0700 hr, off at 1900 hr).

2.1.2 Experimental Model of Ischemia/Reperfusion Injury

2.1.2.1 Pentobarbital Anesthetized Open-Chest Rabbit

Male New Zealand white rabbits (2.5-3.0 kg) were anesthetized with pentobarbital (administered via a marginal ear vein as an initial 30 mg/kg bolus with supplementary doses given perioperatively as required). A tracheotomy was quickly performed and the rabbits were ventilated mechanically using room air supplemented with 100% O₂ for the duration of the experiment. The rate of this artificial ventilation was adjusted to maintain normal values of arterial blood pH and partial pressure of oxygen (pO₂) in rabbits. The chest was opened and the exposed heart was then suspended in a pericardial cradle. The left circumflex coronary artery was identified and a 4-0 silk ligature was passed under it. The ends of the ligature were threaded through a short length of tubing to form a snare. The animal was allowed to stabilize for 15 min before the induction of ischemia.
2.1.2.2 **Induction of Regional Ischemia**

Ischemia was induced by tightening the snare around the coronary artery and maintained by clamping the tubing with a hemostat. After the desired period of ligation, the snare was released to initiate reperfusion. If ventricular fibrillation occurred during the ligation or reperfusion period, sinus rhythm was restored by the application of 0.5 Watt-second countershocks.

2.1.2.3 **Identification of the Occluded Zone**

At the end of the reperfusion period, the heart was excised and put into a cold (4°C) solution of 50 mM Tris-HCl buffer, containing 0.1 mM EDTA, pH 7.6. The snare was pulled tight to reestablish the ligation and the heart was perfused with a solution of Fast Green FCF (0.5 mg/ml in isotonic saline) via the aortic root [204]. The ischemic or ischemic/reperfused left ventricular tissue was identified by the absence of staining and was excised for biochemical analysis.

2.1.2.4 **Sham-Operated Animals**

Sham-operated animals, which served as non-ischemic controls, were subjected to the same surgical procedures except for the tightening of the coronary artery ligature.
2.1.3 Isolated Langendorff-Perfused Rabbit Heart

Male New Zealand white rabbits (2.5-3.0 kg) were anesthetized and subjected to the same surgical procedures as described for open-chest rabbits. After putting the ligature around the left circumflex coronary artery, the heart was excised and placed in Krebs-Henseleit buffer solution (see below for the composition). The heart was quickly mounted on a Langendorff apparatus and perfused retrogradely (100 cm H₂O) through the aortic root with Krebs-Henseleit buffer, containing NaCl (112 mM), NaHCO₃ (20 mM), KCl (5.7 mM), EDTA (0.03 mM), MgCl₂ (1.2 mM) and glucose (11 mM), and gassed with a mixture of O₂/CO₂ (95/5, v/v) at 37°C.

2.1.3.1 Induction of Regional Ischemia in the Isolated Rabbit Heart

Regional ischemia was induced and reperfusion was initiated as described for the open-chest rabbits. At the end of the reperfusion period, the ligature was retightened and the heart was perfused for 1 min with Fast Green FCF solution (0.5 mg/ml in Krebs-Henseleit buffer). The ischemic/reperfused tissue was identified and excised for biochemical analysis.

2.1.3.2 Sham-Control for Isolated Rabbit Heart

Isolated Langendorff-perfused rabbit hearts, which served as non-ischemic controls, were prepared by the same
procedures described in the previous section, but without coronary artery ligation.

2.1.3.3 Induction of Regional Ischemia In Vivo Followed by In Vitro Reperfusion

After a 30 min period of coronary artery ligation in open-chest rabbits, hearts were excised and perfused in the Langendorff apparatus for 10 min, with the ligature still intact. Thereafter, the hearts were reperfused for 60 min with Krebs-Henseleit buffer. The ischemic/reperfused tissue was identified as described previously.

2.1.4 Heart-Lung Transplantation in Pigs

The investigation of effects of chronic allopurinol treatment (in pigs) on the antioxidant capacity of erythrocytes was performed in connection with a parallel heart-lung transplantation study examining the effects of pretreating donor and recipient animals with allopurinol on the post-operative functional viability of transplanted heart and lung tissues. These experiments were carried out in collaboration with a cardiovascular research team at Vancouver General Hospital (VGH). The measurement of erythrocyte susceptibility to oxidative challenge was done by the author. All surgical procedures and cardiorespiratory functional measurements were performed by our collaborators at VGH, as described in detail in a recent report [205]. Briefly, male pigs (20 to 22 kg) were treated with
allopurinol (tablets) given orally at a daily dose of 50 mg/kg for 3 or 5 days. The final dose was given immediately before surgery. The heart-lung transplantation procedures were performed in pigs anesthetized with ketamine (20 mg/kg) and maintained with isoflurane (0.5-2.0%). Harvesting of heart-lung organs from donor animals and the orthotopic transplantation in recipient animals were done using standard techniques. Heart and lung tissues were perfused with cold iso-osmolar cardioplegic solution (8-10°C and 16-18°C, respectively), and placed in hypothermic storage. The total ischemic time, which included the period of orthotopic transplantation, was 6 hours.

Assessments of cardiac and pulmonary function were performed at three time intervals, namely pre-ischemia (T₁), 30 min (T₂) and 2 hr (T₃) post-transplantation. Pre-ischemic (T₁) measurements (which were made in the donor animal) were taken as control for those measured after transplantation (in the recipient animal). Serial measurements of cardiac index (CI), stroke index (SI), lung water content (LW), arterial blood partial pressure of oxygen (pO₂), pulmonary vascular resistance (PVR), alveolar-arterial oxygen gradient (AaG) and alveolar-arterial oxygen tension ratio (AaR) were performed.
2.2 Drug Treatment Protocols

2.2.1 Chronic Allopurinol Treatment in Rabbits

In the chronic treatment regimen, allopurinol (Sigma Chemical Co.) was given orally in alkalinized (with NaOH) drinking water (1 mg/ml at an estimated daily dose of 75 mg/kg) for 7 days. The drinking water of the animals used as controls in these experiments was alkalinized to an equivalent degree as that required to maintain allopurinol in solution (pH 9.0).

2.2.2 Acute Allopurinol and Oxypurinol Treatment in Rabbits

Rabbits were treated with allopurinol or oxypurinol given intravenously at a dose of 50 mg/kg 1 hr prior to coronary artery ligation. Allopurinol or oxypurinol was dissolved in saline (50 mg/ml) by the addition of concentrated NaOH (to pH 11.5). The drug solution was made up to a final volume of 5 ml with saline and administered via the marginal ear vein over 5 min.

2.2.3 Acute U74006F Treatment in Rabbits

Rabbits were treated with the 21-aminosteroid antioxidant U74006F (Upjohn Co.) (3 mg/kg) given as an intravenous bolus (via the marginal ear vein) over 5 min beginning either 15 min prior to coronary artery ligation or 15 min before the onset of reperfusion. Control animals were given drug vehicle.
2.3 Biochemical and Chemical Analyses

2.3.1 Tissue Susceptibility to In Vitro Peroxide Challenge

The antioxidant capacity of tissue was assessed in terms of sensitivity to glutathione (GSH) depletion and lipid peroxidation following incubation of tissue homogenate in vitro with increasing concentrations of t-butylhydroperoxide (TBHP).

2.3.1.1 Preparation of Tissue Homogenates

Anesthetized animals were sacrificed by cardiac excision. Tissue samples from the occluded zone (or a comparable region of left ventricle in control animals) were placed in 50 mM Tris-0.1 mM EDTA, pH 7.6 (10%, w/v) and homogenised using two 15 sec bursts of a Polytron (Brinkmann, Westbury, N.Y.) at 25% maximal speed.

2.3.1.2 Susceptibility of Tissues to TBHP-Induced Depletion of GSH

Aliquots of tissue homogenate (0.3 ml) were combined with an equal volume of saline/azide (isotonic saline with 2 mM sodium azide) containing increasing concentrations of TBHP (final concentrations ranged from 0 to 0.5 mM for heart tissues or 0 to 1.0 mM for kidney or liver tissues). Samples were incubated for 30 min at 37°C and the reaction was terminated with 0.15 ml cold 25% (w/v) TCA solution. Following centrifugation, the supernatants were analysed for GSH (acid soluble-sulfhydryl group) content using 5,5'-
dithio-bis-(2-nitrobenzoic acid) (DTNB). The assay mixture contained 2.4 ml of 0.1 M phosphate buffer (pH 8.0) and 0.3 ml of supernatant, and the reaction was initiated by adding 0.1 ml DTNB solution (3 mM, freshly prepared in phosphate buffer). Absorbance at 412 nm of the reaction mixture was measured at 10 min. The GSH content was estimated using a standard calibration curve. Tissue GSH content (basal GSH level), expressed as nmoles/mg tissue, was measured in the absence of TBHP. TBHP-induced GSH depletion was expressed as % decrease in GSH content when compared with the basal level (ie. in the absence of TBHP).

2.3.1.3 Susceptibility of Tissues to Lipid Peroxidation

Aliquots of tissue homogenate (0.5 ml) were incubated with an equal volume of TBHP in saline/azide (final concentrations ranged from 0 to 10 mM) for 30 min at 37°C. Reactions were terminated by the addition of 0.5 ml cold 28% (w/v) TCA containing 0.1 M sodium arsenite. Following centrifugation, a one-milliter aliquot of the supernatant was combined with 0.5 ml TBA solution (0.5% (w/v) 2-TBA in 0.025 M NaOH) and boiled for 15 min. The formation of thiobarbituric acid-reactive substances (TBARS) was estimated from the absorbance at 532 nm, as described by Tappel & Zalkin [206].
2.3.2 Erythrocyte Susceptibility to In Vitro Peroxide Challenge

The antioxidant capacity of erythrocytes was assessed in term of sensitivity to lipid peroxidation following in vitro incubation with increasing concentrations of TBHP.

2.3.2.1 Preparation of Packed Erythrocytes

Heparinized blood samples were obtained from animals and erythrocytes were separated by centrifugation at 3,000 xg for 5 min at 4°C using a clinical centrifuge (Int. Equip. Co, Needham, Mass.), with removal of the plasma and white cells. Red cells were then washed twice with isotonic saline containing 2.0 mM sodium azide.

2.3.2.2 Susceptibility of Erythrocytes to TBHP-Induced Lipid Peroxidation

Aliquots (50 μl) of erythrocytes were weighed and combined with 0.45 ml of saline-azide solution (to give a 10% suspension), and preincubated for 5 min at 37°C. Peroxidative challenge was induced by the addition of an equal volume (0.5 ml) of TBHP in saline-azide (final TBHP concentrations ranged from 0.25 to 10 mM). After a 30 min incubation at 37°C, the reaction was terminated by addition of 0.5 ml 28% TCA-0.1 M sodium arsenite. The reaction mixture was centrifuged and a 1.0 ml aliquot of the supernatant was combined with 0.5 ml TBA solution (0.5% (w/v) 2-TBA in 0.025 M NaOH) and boiled for 15 min. The
extent of malondialdehyde (MDA) production, expressed as nmoles MDA/mg Hb, was estimated from absorbances at 532 nm and 453 nm of the reaction mixture using acid-hydrolysed malondialdehyde bis(diethylacetal) (Aldrich) as a standard, according to the method of Stocks & Dormandy [207] and Gilbert et al. [208].

2.3.2.3 Hemoglobin Assay

Hemoglobin was measured using the cyanomethemoglobin method of Drabkin & Austin [209]. An aliquot (50 µl) of erythrocytes was weighed and diluted with 4.95 ml H₂O, and the resulting hemolysate was centrifuged to remove the membrane debris. An aliquot of the supernatant was mixed with 0.5 ml of 1.8 mM K₃Fe(CN)₆, 0.5 ml of 2.5 mM KCN and sufficient H₂O to make up a final volume of 1.5 ml. Absorbance at 540 nm of the reaction mixture was measured at 30 min and hemoglobin concentration (mg/ml) was estimated using a standard calibration curve. The hemoglobin concentration of erythrocytes was expressed as mg Hb/g RBC.

2.3.3 Tissue Antioxidant Enzyme Activities

Tissue antioxidant enzyme activities were measured using cytosolic fractions prepared from tissue homogenates. Spectrophotometric measurements were performed using a Perkin-Elmer model Lambda 6B spectrophotometer or a Beckman ACTA C2 spectrophotometer. All assays were carried out at room temperature (22°C - 25°C) unless otherwise indicated.
2.3.3.1 Preparation of Cytosolic Fractions

Tissue homogenates (prepared as described in the previous section) were diluted 1:3 with the homogenising buffer and centrifuged for 15 min, either at 16,000 xg in an Eppendorf 5415 microcentrifuge (heart tissues) or at 105,000 xg in a Beckman L2-65 ultracentrifuge (liver or kidney tissues), to obtain the cytosolic fraction.

2.3.3.2 Catalase

The activity of catalase (CAT) was measured according to the method described by Aebi [210]. An aliquot (0.9 ml) of cytosolic fraction was mixed with 18 μl of diluted ethanol (95% ethanol/H₂O, 1:1 (v/v)). After incubating for 30 min on ice, 0.1 ml of cold Triton X-100 solution (10% (v/v) in 50 mM Tris-0.1 mM EDTA, pH 7.6) was added and 0.4 ml of this mixture was diluted with 9.6 ml of 50 mM phosphate buffer (pH 7.0) immediately prior to assay. A two milliliter-aliquot of this diluted sample was put into a cuvette and the reaction was initiated by adding 1 ml of 30 mM H₂O₂ (freshly prepared in 50 mM phosphate buffer, pH 7.0). After mixing, absorbance at 240 nm was measured at 15 sec and 30 sec, and enzyme activity was expressed as K/mg tissue, where K is the rate constant [210].
2.3.3.3 Cu,Zn-Superoxide Dismutase

The activity of Cu,Zn-superoxide dismutase (Cu,Zn-SOD) was measured according to the method described by Winterbourne et al. [211]. An aliquot (2.0 ml for heart or 0.5 ml for liver and kidney tissues) of cytosolic fraction was combined with sufficient H$_2$O to make up a volume of 2.0 ml. Following addition of 0.5 ml ethanol and 0.3 ml chloroform, the mixture was vortexed thoroughly and centrifuged at 3,000 xg for 5 min using a clinical centrifuge. The resulting supernatant was again centrifuged in an Eppendorf centrifuge (at maximal speed) for 5 min to obtain a clear extract. Assay mixtures contained 1.0 ml of 75 mM phosphate buffer (pH 7.8), 0.2 ml of 0.1 M Na$_2$EDTA-1.5 mg% NaCN, 0.1 ml of 1.5 mM Nitro blue tetrazolium, varying aliquots of the clear extract (0 - 500 μl) and sufficient H$_2$O to make up a final volume of 2.95 ml. The reaction was initiated by adding 50 μl of 0.12 mM riboflavin and reaction mixtures were mixed and illuminated with fluorescent light for 2.5 min twice (with vortexing between and at the end of the illumination). Absorbance at 560 nm of the reaction mixtures was measured and enzyme activity was expressed as Units/mg tissue [211].

2.3.3.4 Glutathione Peroxidase

The activity of glutathione peroxidase (GPX) was measured using the method of Paglia & Valentine [212] as modified by Lawrence & Burke [213]. An aliquot (0.5 ml) of
cytosolic fraction was combined with an equal volume of double-strength Drabkin's reagent (0.0016 M KCN-0.0012 M K₃Fe(CN)₆-0.0238 M NaHCO₃), and the mixture was kept on ice until used for the assay. Reaction mixtures contained 2.0 ml of 75 mM phosphate buffer (pH 7.0), 50 μl of 60 mM GSH, 100 μl of glutathione reductase enzyme solution (30 Units/ml, Sigma Chemical Co), 50 μl of 0.12 M sodium azide, 100 μl of 15 mM Na₂EDTA, 100 μl of 3 mM NADPH, varying aliquots of sample (100-300 μl for heart or 50-100 μl for liver and kidney tissues) and sufficient H₂O₂ to make up a final volume of 2.9 ml. The reaction was initiated by addition of 100 μl 7.5 mM H₂O₂ solution and absorbance changes of the reaction mixtures were monitored spectrophotometrically at 340 nm for 5 min. Enzyme activity was calculated using an extinction coefficient for NADPH at 340 nm of 6.22 x 10⁶/M/cm and expressed as nmols NADPH/min/mg tissue.

2.3.3.5 Glutathione Reductase

The activity of glutathione reductase (GRD) was measured using the method of Long & Carson [214]. Reaction mixtures contained 0.5 ml of 18 mM glutathione disulfide (GSSG), 1 ml of 0.45 M Tris-90 mM EDTA (pH 7.6), an aliquot (0.4 ml for heart or 0.1 ml for liver and kidney tissues) of cytosolic fraction and sufficient H₂O₂ to make up a final volume of 2.9 ml. The reaction was initiated by addition of 100 μl 3 mM NADPH solution and absorbance changes at 340 nm
of the reaction mixtures were monitored spectrophotometrically for 5 min. Enzyme activity was calculated using an extinction coefficient for NADPH at 340 nm of $6.22 \times 10^6$/M/cm and expressed as nmoles NADPH/min/mg tissue.

2.3.3.6 Hemoglobin Assay
The hemoglobin content of cytosolic fractions was measured by the method described previously.

2.3.3.7 Correction for Enzyme Activity Contributed by Blood
Activities of tissue cytosolic antioxidant enzymes were corrected for the corresponding enzyme activities contributed by the blood contaminants. The correction was estimated from the hemoglobin content of the cytosolic fraction and the activity of enzymes measured in erythrocytes obtained from the same animal.

2.3.4 Erythrocyte Antioxidant Enzyme Activities
Activities of antioxidant enzymes in erythrocytes were measured in hemolysates prepared as described below.

2.3.4.1 Preparation of Hemolysates
A 0.4 ml aliquot of packed erythrocytes (prepared as described previously) was combined with 3.6 ml $H_2O$ and the mixture was subjected to three freeze and thaw cycles using dry ice/acetone to obtain the hemolysate.
2.3.4.2 Catalase

An aliquot (20 μl) of hemolysate was added to 10 ml phosphate buffer (50 mM, pH 7.0) immediately prior to assay. This diluted hemolysate was assayed as described for the tissue cytosolic fractions. Catalase activity was expressed as K/mg Hb.

2.3.4.3 Cu,Zn-Superoxide Dismutase

An aliquot (0.5 ml) of hemolysate was extracted and assayed as described for the tissue cytosolic fractions. Cu,Zn-SOD activity was expressed as Units/mg Hb.

2.3.4.4 Glutathione Peroxidase

An aliquot (0.4 ml) of hemolysate was mixed with 3.6 ml H₂O, and 0.5 ml of this diluted hemolysate was then combined with an equal volume of double-strength Drabkin's solution (prepared as described previously), and the mixture was kept on ice until used for the assay. Varying aliquots (50 - 150 μl) of sample were assayed as described for the tissue cytosolic fractions. Glutathione peroxidase activity was expressed as nmoles NADPH/min/mg Hb.

2.3.4.5 Glutathione Reductase

Diluted hemolysate (prepared as described for the GPX assay) was used for this determination. Reaction mixtures contained the same constituents as outlined for the tissue
cytosolic fractions except that 100, 300 and 500 µl aliquots of hemolysate were assayed. After adding 100 µl of 3 mM NADPH, reaction components were quickly mixed, and absorbances at 340 nm were measured. Reaction mixtures were incubated at 37°C for 15 min and absorbances at 340 nm were again measured. Glutathione reductase activity, expressed as nmoles NADPH/min/mg Hb, was determined from absorbance changes during the incubation period, using an extinction coefficient for NADPH at 340 nm of 6.22 x 10⁶/M/cm.

2.3.4.6 Hemoglobin Assay

An aliquot of diluted hemolysate (prepared as described for the GPX assay) was centrifuged and the supernatant was analysed for hemoglobin content as described previously section.

2.3.5 Mitochondrial ATPase Activity

2.3.5.1 Isolation of Mitochondria

Samples of control or ischemic left ventricular tissue (0.8 g) were homogenised in 8 ml buffer (0.25 M sucrose-50 mM Tris, pH 7.4) using a Polytron (with one 5 sec burst at 25% maximal speed). The homogenate was centrifuged at 1,200 xg for 10 min. The supernatant was decanted and centrifuged at 11,000 xg for 15 min. The pellet was resuspended in 8 ml homogenising buffer and centrifuged again at 11,000 xg for 15 min. The final pellet was resuspended in 0.6 ml homogenisation buffer. The protein
content of this mitochondrial fraction was determined by the Lowry method using bovine serum albumin as a standard [215].

### 2.3.5.2 Mitochondrial Azide-Sensitive ATPase Activity

ATPase activity of the mitochondrial fraction was determined using the procedure described by Moore & Godin [216] with only slight modification. Assay mixtures, in a final volume of 2.8 ml, contained the following at the final concentrations given in the brackets: Tris-HCl buffer, pH 7.4 (55 mM), MgCl₂ (3 mM), EGTA (0.3 mM), ATP (3 mM) and Triton X-100 (0.005%, v/v) and were incubated at 37°C for 5 min. The reaction was initiated by adding 0.2 ml of membrane suspension (containing 75 µg membrane protein in the presence of sodium azide (5 mM) or 7.5 µg membrane protein in the absence of sodium azide). After a 15 min incubation at 37°C, the reaction was terminated by adding 1 ml of cold 10% TCA, followed by centrifugation using a clinical centrifuge. The resulting supernatant was assayed for inorganic phosphate (Pi) using the method described by Fiske & Subbarow [217]. A 3.0 ml aliquot of the supernatant was mixed with 1.4 ml H₂O and 0.4 ml of 5% ammonium molybdate, and the reaction was initiated by adding 0.2 ml ANS solution prepared as follows. A mixture of 1-amino-2-naphthol-4-sulfonic acid (0.125 g) and sodium sulfite (0.25 g) in ~30 ml of freshly prepared sodium bisulfite solution (15% (w/v) final concentration) was heated until the solution turned yellow, and was made up to
a final volume of 50 ml. After an incubation for 15 min at room temperature, absorbance at 660 nm was measured. The Pi content was estimated with reference to a phosphate standard. ATPase activities were expressed as μmoles Pi/mg membrane protein/hr. The azide-sensitive mitochondrial ATPase activity was estimated by subtracting the value measured in the presence of sodium azide from that measured in the absence of sodium azide.

2.3.6 Tissue ATP

Transmural samples of control or ischemic ventricular tissue (approximately 100 mg) were quick-frozen in liquid nitrogen and stored at -70°C for periods not exceeding 1 week. Tissues were ground with 6% perchloric acid (4 μl/mg tissue) under liquid nitrogen and then thawed on ice. After centrifugation, an aliquot (80 μl) of the supernatant was neutralized with 60 μl of 1.4 M KHCO₃, let stand on ice for 15 min and recentrifuged. The resulting supernatant was analysed for ATP content as described by Jaworek et al. [218]. An aliquot (100 μl) of the supernatant was mixed with 1 ml of NADH-containing phosphoglyceric acid buffer (prepared by adding 1 ml of 3-phosphoglyceric acid buffer (Sigma) to a vial containing 0.3 mg NADH (Sigma) and mixing with 2 ml of H₂O) and 80 μl of H₂O. Absorbance at 340 nm was measured after 3 min, 20 μl of phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase enzyme solution (Sigma Chemical Co.) was added
and the absorbance was measured again 5 min later. The ATP content was estimated from the change in absorbance of the reaction mixture using a standard calibration curve.

2.3.7 **Preparation of Erythrocyte Membranes**

Erythrocyte membranes were prepared from outdated human blood by stepwise hypotonic lysis as described by Godin & Schrier [219]. One unit of outdated human blood (obtained from the Red Cross) was diluted to 1,200 ml with cold saline, and centrifuged at 600 xg, followed by the careful removal of plasma and buffy coat material. Red cells were washed (with centrifugation at 15,000 xg) with isotonic saline and an aliquot (100 ml) of these washed erythrocytes was diluted to 1,200 ml with cold 0.8 M NaCl solution. The mixture was stirred for 10 min at 4°C, and centrifuged at 15,000 xg for 5 min. The resulting pellet which included light-colored material (erythrocyte membranes) was resuspended and diluted to 1,200 ml with cold 0.06 M NaCl. This procedure was repeated three times using NaCl solutions of decreasing concentrations (0.04 M, 0.02 M and 0.009 M, respectively). When 0.02 M or 0.009 M NaCl was used, the pH of mixture was carefully adjusted to 7.4 with 1 M Tris (pH 7.4), and the non-lysed erythrocytes (which formed a discreet pellet underneath the membrane layer) were removed after each centrifugation. After the stepwise hypotonic lysis, the resulting membrane preparation was resuspended in 10 mM Tris-HCl (pH 7.4), with final volumes ranging from
600 to 1,200 ml, depending on the extent of visible hemoglobin contamination. After stirring for 10 min at 4°C, the mixture was centrifuged at 30,000 xg for 10 min, and the membranes were removed and pooled. An aliquot of this membrane preparation was used for protein determination using the Lowry method [215], and the remainder was quick-frozen in dry ice/acetone prior to storage at -20°C and used within one week.

2.3.8 Ferric Chloride-Induced Oxidation of Erythrocyte Membrane Lipids

The ferric chloride-induced oxidation of erythrocyte membrane lipids was measured in isotonic saline adjusted to pH 7.4 with 1 M Tris, pH 7.4. Reactions were performed either in the absence or presence of various concentrations of the test compounds. All chemicals were dissolved in buffered saline except butylated hydroxytoluene (BHT), which was dissolved in isopropanol; 10 μl of this isopropanol solution was used in the reaction mixture. The reaction was initiated by adding 250 μl of ferric chloride solution in a mixture containing 0.3 mg membrane protein in a final volume of 1 ml. Reaction mixtures were incubated for 30 min at 37°C. When time-course studies were performed, GSH or ascorbic acid, at a final concentration of 50 μM, was added (in 10 μl) before the reaction was initiated. Following increasing periods of incubation at 37°C, the reactions were terminated by adding 0.5 ml cold 28% TCA, containing 0.1 M
sodium arsenite. Following centrifugation, the supernatant was assayed for TBARS by mixing 1 ml supernatant with 0.5 ml TBA reagent (0.5% (w/v) 2-TBA in 0.025 M NaOH), heating in a boiling water bath for 15 min and measuring the absorbance at 532 nm. None of the test compounds was found to interfere with the TBA color reaction.

2.3.9 *Cupric Chloride-TBHP-Induced Oxidation of Erythrocyte Membrane Lipids*

The cupric chloride-TBHP-induced oxidation of erythrocyte membrane lipids was measured in isotonic saline adjusted to pH 7.4 (with 1 M Tris, pH 7.4). The reaction was initiated by adding 250 μl cupric chloride-TBHP solution (0.2 mM - 10 mM, final concentrations) to a mixture containing 0.75 mg membrane protein, in a final volume of 1.0 ml, followed by a 30 min incubation at 37°C. Reactions were terminated by adding 3 ml cold 1% phosphoric acid. Following the addition of 1 ml TBA reagent (0.6% (w/v) 2-TBA in 0.05 M NaOH), the mixture was boiled for 45 min. After cooling, the samples were extracted with 5 ml of n-butanol/pyridine (15/2, v/v) and centrifuged to achieve phase separation. The TBARS content of the butanol layer was determined by measuring the absorbance at 532 nm.
2.3.10 Myoglobin-TBHP-Induced Oxidation of Erythrocyte Membrane Lipids

The myoglobin-TBHP-induced oxidation of erythrocyte membrane lipids was measured in 50 mM phosphate buffer, pH 7.0. Reaction mixtures, in a final volume of 1 ml, contained 26 μM myoglobin and 0.3 mg membrane protein, either in the absence or presence of the test compounds at increasing concentrations. All chemicals were dissolved in phosphate buffer, except BHT, which was dissolved in isopropanol; 10 μl of this isopropanol solution was used in the reaction mixture. The reaction was initiated by adding 100 μl TBHP at a final concentration of 1.0 mM. Reaction mixtures were incubated for 30 min or varying periods (10 - 110 min) at 37°C and the reactions were terminated by adding 0.5 ml cold 28% (w/v) TCA, containing 0.1 M sodium arsenite. The content of TBARS in the reaction mixtures was assayed as described for ferric chloride-stimulated oxidative reactions.

2.3.11 Transition Metal Ion-Catalysed Oxidation of Ascorbic Acid

Ascorbate oxidation was monitored spectrophotometrically by the decrease in absorbance at 280 nm. This wavelength was chosen rather than 265 nm, the absorption maximum for ascorbate, in order to minimize the contribution by allopurinol to the absorption. The cupric or ferric ion-catalysed oxidation of ascorbate was assayed
in 10 mM phosphate buffer, pH 7.4, either in the absence or presence of 0.1 mM EDTA used to reduce the basal oxidation of ascorbate catalysed by transition metal contaminants present in the reaction medium. The reaction mixture, in a final volume of 3 ml, contained 100 μM ascorbate in the absence or presence of the test compounds. The reaction was initiated by adding 100 μl of cupric or ferric chloride solution at final concentrations ranging from 10 to 100 μM. The change in absorbance at 280 nm of the reaction mixture was monitored continuously when the assay was performed in the absence of EDTA. When 0.1 mM EDTA was present in the reaction mixture, the absorbance at 280 nm was measured 1 min after the addition of cupric or ferric chloride. After a period of incubation at room temperature (15 or 40 min for cupric or ferric ion-catalysed reactions, respectively), the absorbance was again measured. The rate of ascorbate oxidation, expressed as nmoles/min, was calculated using a standard calibration curve and was constant over the incubation period. The rate of cupric or ferric ion-catalysed ascorbate oxidation was corrected for the basal oxidation rate in the absence of exogenous added metal ions.

2.3.12 UV Absorption Spectroscopy of Ascorbate/Allopurinol/
Copper Ion

UV absorption spectra of reaction mixtures were recorded with a Perkin-Elmer model Lambda 6B
Various combinations of allopurinol/ascorbic acid/cupric chloride or EDTA were mixed in the following manner: 1.0 ml of 250 μM allopurinol; 1.0 ml of 125 μM ascorbic acid; 0.5 ml of 500 μM EDTA; 0.5 ml of 500 μM cupric chloride and sufficient H2O to make up a final volume of 3 ml. Spectra of the reaction mixtures were recorded 2 min after the constituents had been mixed.

2.3.13 Myoglobin-TBHP-Catalysed Oxidation of Uric Acid

Oxidation of uric acid was monitored spectrophotometrically by the decrease in absorbance at 292 nm, the absorption maximum of uric acid, as described by Howell & Wyngaarden [220]. The myoglobin-TBHP-catalysed oxidation of uric acid was assayed in 50 mM phosphate buffer, pH 7.0, containing 2.0 mM sodium azide. Reaction mixtures, in a final volume of 3 ml, contained 100 μM uric acid and 26 μM myoglobin, either in the absence or presence of the test compounds at increasing concentrations. The reaction was initiated by adding 100 μl TBHP at a final concentration of 0.5 mM. Absorbance of the reaction mixture at 292 nm was measured 1 min after the addition of TBHP. After 40 min of incubation at room temperature, the absorbance was measured again. The rate of uric acid oxidation, expressed as nmoles/min, was calculated using a standard calibration curve and was constant over the incubation period.
2.3.14 Statistical Analyses

Comparisons between two means were performed using a 2-tailed unpaired Student's t test. Multiple comparisons among groups were done using one-way analysis of variance (ANOVA) followed by Duncan's test to assess specific group difference at $P < 0.05$.

Correlation between parameters was assessed by correlation analysis. Alterations in pig erythrocyte MDA over the treatment period were analysed by ANOVA with repeated measures followed by Duncan's test to assess specific group difference at $P < 0.05$. 
3. RESULTS

3.1 Susceptibility of Tissues to Peroxide Challenge

The susceptibility of tissues (heart, liver and kidney) to peroxide challenge was assessed in terms of sensitivity to GSH depletion and the formation of TBARS, an indirect index of lipid peroxidation, following in vitro exposure of tissue homogenates to increasing concentrations of TBHP. As shown in Figs. 1 and 2, the concentration dependence of these two processes, regardless of the tissue being examined, differed markedly, with GSH depletion being more sensitive and formation of TBARS occurring at concentrations of TBHP higher than those inducing maximal GSH depletion. Homogenates prepared from liver tissues showed significantly higher GSH levels than those prepared from kidney or myocardial tissues, with the latter having the lowest values either in the absence or presence of TBHP at all concentrations tested (Fig. 1). The susceptibility of tissue homogenates to TBHP-induced formation of TBARS also differed markedly among tissues; levels of TBARS produced by liver homogenates were greater than those of kidney or myocardial tissue, with the latter having the lowest values at final concentrations of TBHP greater than 0.5 mM (Fig. 2).

Activities of antioxidant enzymes, namely catalase (CAT), Cu,Zn-superoxide dismutase (Cu,Zn-SOD), glutathione peroxidase (GPX) and glutathione reductase (GRD)
Fig. 1  TBHP-induced depletion of GSH in tissue homogenates prepared from non-ischemic tissues from rabbit.

Assays were performed by *in vitro* incubation of tissue homogenate (10%, w/v) with increasing concentrations of TBHP, as described in Materials and Methods. Values were expressed as nmoles GSH/mg tissue. Each point represents mean ± SEM, with n = 5 animals for each tissue. The three tissues showed statistically significant (P < 0.05) differences from each other at all TBHP concentrations tested.
Fig. 2 TBHP-induced formation of TBARS in tissue homogenates prepared from non-ischemic tissues from rabbit.

Assays were performed by in vitro incubation of tissue homogenate (10%, w/v) with increasing concentrations of TBHP, as described in Materials and Methods. TBARS content was expressed as absorbance at 532 nm. Each point represents mean ± SEM, with n = 5 animals for each tissue. The three tissues showed statistically significant (P < 0.05), differences from each other at all TBHP concentrations tested with the exception of the liver and kidney at the lowest concentration of TBHP.
were measured in rabbit tissues. All the activities of myocardial antioxidant enzymes were significantly lower than those of liver or kidney (Fig. 3-6). The activities of CAT and Cu,Zn-SOD in liver and kidney were 7- and 2-fold, respectively, higher than those of myocardial tissue (Fig. 3,4). The highest activities of GPX and GRD were found in liver and kidney tissues, respectively, with the values significantly differing from those of other tissues examined (Fig. 5,6).

3.2 Altered Antioxidant Capacity in Ischemic/Reperfused Myocardial Tissues

The antioxidant capacity of myocardial tissue homogenates prepared from control, ischemic or ischemic/reperfused tissues, was assessed in terms of susceptibility to GSH depletion and the formation of TBARS following in vitro oxidative challenge with increasing concentrations of TBHP. Myocardial tissue from animals subjected to a 40 min period of ischemia induced by left circumflex coronary artery ligation showed significant decreases in GSH levels at all concentrations of TBHP tested (Fig. 7). The small increases (relative to SHAM-CON samples) in formation of TBARS were statistically significant at the highest concentrations of TBHP tested (i.e., 5 and 10 mM) (Fig. 8). The foregoing ischemia-induced changes in GSH and TBARS were greatly intensified by a subsequent 60 min period of reperfusion, with all values
Fig. 3 Catalase activities in rabbit tissues.

The activity of catalase (CAT) was measured using cytosolic fraction prepared from tissue homogenate, as described in Materials and Methods. CAT activity was expressed as K/mg tissue, where K is the rate constant. Values given are mean ± SEM, with n = 5 animals for each tissue. The heart showed a statistically significant (P < 0.05) difference from the liver and kidney.
Fig. 4 Cu,Zn-Superoxide dismutase activities in rabbit tissues.

The activity of Cu,Zn-superoxide dismutase (Cu,Zn-SOD) was measured using cytosolic fraction prepared from tissue homogenate, as described in Materials and Methods. Cu,Zn-SOD activity was expressed as Units/mg tissue. Values given are mean ± SEM, with n = 5 animals for each tissue. The heart showed a statistically significant (P < 0.05) difference from the liver and kidney.
Cu,Zn-SOD

Units / mg tissue

Heart  Liver  Kidney
Fig. 5 Glutathione peroxidase activities in rabbit tissues.

The activity of glutathione peroxidase (GPX) was measured using cytosolic fraction prepared from tissue homogenate, as described in Materials and Methods. GPX activity was expressed as nmoles/NADPH/min/mg tissue. Values given are mean ± SEM, with n = 5 animals for each tissue. All the three tissues showed statistically significant (P < 0.05) differences from each other.
GPX

nmoles NADPH / min / mg tissue

Heart
Liver
Kidney
**Fig. 6 Glutathione reductase activities in rabbit tissues.**

The activity of glutathione reductase (GRD) was measured using cytosolic fraction prepared from tissue homogenate. GRD activity was expressed as nmoles NADPH/min/mg tissue. Values given are mean ± SEM, with n = 5 animals for each tissue. All the three tissues showed statistically significant (P < 0.05) differences from each other.
Fig. 7 TBHP-induced depletion of GSH in myocardial tissue homogenates following a 40 min period of ischemia with or without subsequent reperfusion for 60 min in rabbits.

Assays were performed by in vitro incubation of tissue homogenate (10%, w/v) prepared from control (non-ischemic, sham-operated), ischemic and/or reperfused myocardial tissue with increasing concentrations of TBHP. Values were expressed as nmoles GSH/mg tissue. Each point represents mean ± SEM, with n = 5 animals in each case. The three experimental groups showed statistically significant (P < 0.05) differences from each other at all TBHP concentrations tested with the exception of the SHAM-CON (non-ischemic) and ISC/NON (ischemic/non-reperfused) samples in the absence of TBHP.
Fig. 8 TBHP-induced TBARS formation in myocardial tissue homogenates following a 40 min period of ischemia with or without subsequent reperfusion for 60 min in rabbits.

Assays were performed by in vitro incubation of tissue homogenate (10%, w/v) prepared from control (non-ischemic, sham-operated), ischemic and/or reperfused myocardial tissue with increasing concentrations of TBHP, as described in Materials and Methods. TBARS content was expressed as absorbance at 532 nm. Each point represents mean ± SEM, with n = 5 animals in each case. The three experimental groups showed statistically significant (P < 0.05) differences from each other at all TBHP concentrations tested with the exception of the SHAM-CON and ISC/NON samples at the three lowest concentrations of TBHP.
SHAM/ISC/CON NON REP

TBHP Concentration (mM)

Absorbance at 532 nm

0.80

0.40

0.00

0 5 10

TBHP Concentration (mM)
differing significantly from those for non-ischemic and ischemic, non-reperfused tissues both in the absence and presence of TBHP challenge (Fig. 7,8).

3.3 Alterations in Myocardial Antioxidant Capacity in Rabbits Subjected to Increasing Periods of Ischemia

The time-course of alterations in myocardial antioxidant capacity was examined in animals subjected to increasing periods of coronary artery ligation with or without subsequent reperfusion (60 min). Fifty-five animals were randomly assigned to one of the following groups consisting of 5 animals each: **SHAM-CON**: sham-operated animals, no ischemia; **ISC/NON** (5 groups): 5, 10, 20, 40 and 60 min period of ischemia, respectively, without reperfusion; **ISC/REP** (5 groups): 5, 10, 20, 40 and 60 min of ischemia, respectively, followed by 60 min of reperfusion. GSH depletion and formation of TBARS were estimated following *in vitro* exposure of tissue homogenates to TBHP at final concentrations of 0.05 and 2.5 mM, respectively. During the course of ischemia (Fig. 9, solid lines), an enhanced GSH susceptibility to oxidation was present from the earliest time point examined (5 min), while the increase in the formation of TBARS did not become significant until 60 min post-ligation (Fig. 10, solid line). These effects, suggestive of an impairment in myocardial antioxidant capacity, were greatly intensified by post-ischemic reperfusion (Fig. 9,10, dotted lines). After
Fig. 9 Changes in susceptibility of myocardial tissue to TBHP-induced depletion of GSH after varying periods of coronary artery ligation with or without 60 min of reperfusion in rabbits.

Assays were performed by *in vitro* incubation of tissue homogenate (10%, w/v) prepared from ischemic and/or reperfused myocardial tissue, as described in Materials and Methods. GSH depletion was expressed as % decrease when compared with control incubation (i.e., in the absence of TBHP). Values given are %GSH depletion measured at a final concentration of TBHP of 0.05 mM. Each point represents mean ± SEM, with n = 5 animals in each case. * denotes significant (P < 0.05) difference when compared with the corresponding ISC/NON animals.
GSH

% decrease

Ligation time (min)

ISC/NON

ISC/REP

*
Fig. 10 Changes in susceptibility of myocardial tissue to TBHP-induced formation of TBARS after varying periods of coronary artery ligation with or without 60 min of reperfusion in rabbits.

Assays were performed by _in vitro_ incubation of tissue homogenate (10%, w/v) prepared from ischemic and/or reperfused myocardial tissue with increasing concentrations of TBHP. TBARS content was expressed as absorbance at 532 nm. Values given are TBARS formation measured at a final concentration of TBHP of 2.5 mM. Each point represents mean ± SEM, with n = 5 animals in each case. * denotes significant (P < 0.05) difference when compared with the corresponding ISC/NON animals.
TBARS

○ ISC/NON  ● ISC/REP

Absorbance at 532 nm

Ligation time (min)
an initial period suggesting an enhancement of tissue antioxidant capacity, TBHP-induced GSH depletion showed a striking increase above the value in the presence of ischemia alone between 20 and 40 min of ischemia (Fig. 9). In this same time interval, the formation of TBARS also increased dramatically (Fig. 10).

3.3.1 Effects of Chronic Allopurinol Pretreatment

The effects of chronic allopurinol pretreatment (1 mg/ml in drinking water at an estimated daily dose of 75 mg/kg) on I/R-induced alterations in myocardial susceptibility to in vitro oxidative challenge with TBHP were examined. Significant protection against GSH depletion (Fig. 11) and lipid peroxidation (Fig. 12) was seen in the absence of any detectable alterations in tissue levels of ATP (Fig. 13) and GSH (data not shown). Allopurinol treatment did not alter the myocardial GSH levels of control (i.e., non-ischemic) tissue or its sensitivity to TBHP-induced GSH depletion and formation of TBARS (Table I).

3.3.2 Effects of Acute Allopurinol and Oxypurinol Pretreatment

Allopurinol administered acutely as a single intravenous bolus (50 mg/kg) 1 hr prior to the induction of ischemia was found to have no significant protective effect on the enhanced GSH depletion or formation of TBARS resulting from 30 min of coronary artery ligation followed
Fig. 11 Effect of chronic allopurinol treatment on the susceptibility of myocardial tissue to TBHP-induced depletion of GSH after a 30 min period of coronary artery ligation followed by 60 min of reperfusion in rabbits.

Animals were treated with allopurinol (given orally 1 mg/ml in drinking water for 7 days at an estimated daily dose of 75 mg/kg) or vehicle prior to coronary artery ligation. GSH depletion, expressed as % decrease, was measured at a final concentration of TBHP of 0.05 mM, as described in Materials and Methods. Values are given as mean ± SEM, with n = 8 animals in each case. * denotes significant (P < 0.05) difference when compared with the corresponding ISC/REP animals in the absence of drug treatment.
Fig. 12 Effect of chronic allopurinol treatment on the susceptibility of myocardial tissue to TBHP-induced formation of TBARS after a 30 min period of coronary artery ligation followed by 60 min of reperfusion in rabbits.

Animals were treated with allopurinol or vehicle as described in Fig. 11. TBARS formation, expressed as absorbance at 532 nm, was measured at a final concentration of TBHP of 2.5 mM. Values are given as mean ± SEM, with n = 8 animals in each case. * denotes significant (P < 0.05) difference when
TBARS

Absorbance at 532 nm

- **SHAM-CON**
- **ISC/REP**
- **ISC/REP ALP-Treated**

* indicates a significant difference.
Fig. 13 Effect of chronic allopurinol treatment on the tissue ATP level after a 30 min period of coronary artery ligation followed by 60 min of reperfusion in rabbits

Animals were treated with allopurinol or vehicle as described in Fig. 11. ATP content, expressed in nmoles/mg tissue, was measured in transmural myocardial tissue, as described in Materials and Methods. Values are given as mean ± SEM, with n = 8 animals in each case.
by a 60 min period of reperfusion. Oxypurinol, the active metabolite of allopurinol, was also devoid of protective activity under these conditions (Table II).

3.3.3 Effects of Chronic Allopurinol Treatment on the Activity of Myocardial Antioxidant Enzymes

As an approach to elucidating the molecular basis of the protective effects of chronic allopurinol pretreatment, the effects of this treatment regimen on the activity of myocardial antioxidant enzymes, namely CAT, Cu,Zn-SOD, GPX and GRD, were examined. As shown in Table III, no significant changes were apparent except for GRD. The activity of myocardial GRD in the allopurinol-treated animals was significantly increased (by approximately 30% relative to vehicle-treated controls).

3.3.4 Effects of Acute U74006F Treatment on Ischemia/Reperfusion-Induced Alterations in Rabbit Myocardium

Twenty-five rabbits were randomly assigned to one of the following groups consisting of 5 animals each: Group 1: no ischemia, treated with drug vehicle only; Group 2: no ischemia, treated with U74006F; Group 3: ischemia (40 min) and reperfusion (60 min), treated with drug vehicle only; Group 4: ischemia and reperfusion, treated with U74006F prior to coronary ligation; Group 5: ischemia and reperfusion, treated with U74006F prior to the onset of reperfusion.
Table I  Effects of chronic allopurinol treatment on myocardial antioxidant capacity.

<table>
<thead>
<tr>
<th></th>
<th>Basal GSH (nmole/mg tissue)</th>
<th>% GSH Depletion</th>
<th>TBARS Formation (OD532)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>2.18 ± 0.02</td>
<td>67 ± 5.0</td>
<td>0.23 ± 0.006</td>
</tr>
<tr>
<td>ALP-treated</td>
<td>2.08 ± 0.06</td>
<td>63 ± 4.7</td>
<td>0.24 ± 0.013</td>
</tr>
</tbody>
</table>

Animals were treated with allopurinol or vehicle, as described in Fig. 11. GSH depletion and TBARS formation were measured at final concentrations of TBHP of 0.15 and 2.5 mM, respectively. All values are given as mean ± SEM, with n = 5 in each group.
Table II  Effects of acutely administered allopurinol or oxypurinol (50 mg/kg, i.v.) on I/R injury.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Basal GSH (nmoles/mg tissue)</th>
<th>% GSH Depletion</th>
<th>TBARS Formation (OD$_{532}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-control (no ischemia)</td>
<td>2.39 ± 0.12</td>
<td>23 ± 6.0</td>
<td>0.21 ± 0.009</td>
</tr>
<tr>
<td>none</td>
<td>1.56 ± 0.10</td>
<td>51 ± 8.7</td>
<td>0.38 ± 0.047</td>
</tr>
<tr>
<td>allopurinol</td>
<td>1.67 ± 0.10</td>
<td>45 ± 12.5</td>
<td>0.31 ± 0.058</td>
</tr>
<tr>
<td>oxypurinol</td>
<td>1.84 ± 0.12</td>
<td>54 ± 8.9</td>
<td>0.32 ± 0.046</td>
</tr>
</tbody>
</table>

Drugs were administered as a single dose 1 hr prior to a 30 min period of coronary artery ligation followed by 60 min of reperfusion. Measurement of GSH depletion and TBARS formation used final TBHP concentrations of 0.075 and 2.5 mM, respectively. All values are given as mean ± SEM, with n = 9 in all experimental groups except for sham-control with n = 5.
Table III  Effects of chronic allopurinol treatment on the activity of myocardial antioxidant enzymes.

<table>
<thead>
<tr>
<th></th>
<th>CAT</th>
<th>Cu, Zn-SOD</th>
<th>GPX</th>
<th>GRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.0006</td>
<td>0.59</td>
<td>2.7</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>±0.0002</td>
<td>±0.03</td>
<td>±0.2</td>
<td>±0.04</td>
</tr>
<tr>
<td>ALP-treated</td>
<td>0.0004</td>
<td>0.62</td>
<td>3.0</td>
<td>1.9*</td>
</tr>
<tr>
<td></td>
<td>±0.0001</td>
<td>±0.15</td>
<td>±0.1</td>
<td>±0.2</td>
</tr>
</tbody>
</table>

The activity of CAT (K/mg tissue), Cu, Zn-SOD (units/mg tissue), GPX (nmoles NADPH/min/mg tissue) and GRD (nmoles NADPH/min/mg tissue) were measured in left ventricular tissues. All values are given as mean ± SEM, with n = 5 in each group.

* significantly different from control.
In the absence of coronary artery ligation, treatment with U74006F had no discernible effects on any of the biochemical characteristics examined (Table IV). A 40 min period of ischemia followed by 60 min of reperfusion produced significant decreases in mitochondrial ATPase activity and in tissue levels of ATP and GSH as well as a marked reduction in myocardial antioxidant capacity (i.e., enhanced susceptibility to TBHP-induced GSH depletion and formation of TBARS). With the exception of mitochondrial ATPase inactivation, none of these I/R-induced alterations were influenced by U74006F pretreatment. When U74006F was administered prior to the induction of ischemia, the mitochondrial ATPase showed a small degree of protection which did not quite attain statistical significance. However, when U74006F was given shortly before the onset of reperfusion, the activity of this subcellular marker enzyme did not differ from that in non-ischemic samples (Table IV).

3.4 Time-Course of Alterations in Myocardial Antioxidant Capacity During Post-Ischemic Reperfusion

In order to investigate whether or not I/R-related alterations in myocardial antioxidant capacity correlate temporally with the burst of oxygen radical formation which occurs within the first few minutes of post-ischemic reperfusion [78,80], the time-course of alterations in myocardial antioxidant capacity was examined in rabbits subjected to a fixed (40 min) period of coronary artery
### Table IV  Effects of U74006F pretreatment on I/R-induced alterations in rabbit myocardium.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>M-ATPase (umol Pi/hr/mg Protein)</th>
<th>ATP (nmol/mg tissue)</th>
<th>GSH Depletion</th>
<th>TBARS Formation (OD$_{532}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>266±20</td>
<td>3.83±0.21</td>
<td>2.07±0.04</td>
<td>19±4.5</td>
</tr>
<tr>
<td>Group 2</td>
<td>275±9</td>
<td>4.06±0.16</td>
<td>2.13±0.08</td>
<td>22±5.8</td>
</tr>
<tr>
<td>Group 3</td>
<td>206±11$^a$</td>
<td>0.54±0.11$^a$</td>
<td>1.06±0.06$^a$</td>
<td>72±5.2$^a$</td>
</tr>
<tr>
<td>Group 4</td>
<td>230±11$^a$</td>
<td>0.49±0.08$^a$</td>
<td>1.06±0.06$^a$</td>
<td>72±1.8$^a$</td>
</tr>
<tr>
<td>Group 5</td>
<td>252±7</td>
<td>0.67±0.07$^a$</td>
<td>1.23±0.09$^a$</td>
<td>66±6.2$^a$</td>
</tr>
</tbody>
</table>

Measurements of GSH depletion and TBARS formation in vitro used final TBHP concentrations of 0.075 and 2.5 mM, respectively. Values are given as mean ± SEM for $n = 5$ in each group. See Materials and Methods for identifications of Group 1 to Group 5.

$^a$ - significantly different from corresponding non-ischemic value (i.e. Group 1 and Group 2)


ligation followed by increasing periods of reperfusion. Fifty-four rabbits were randomly assigned to one of the following groups consisting of 6 animals each: **SHAM-CON**: sham-operated, no ischemia; **ISC/NON**: ischemia without reperfusion; **ISC/REP**: (7 groups) - ischemia followed by reperfusion for 1, 5, 15, 30, 60, 90 and 120 min, respectively. Myocardial antioxidant capacity was not altered with time in sham-operated animals, even though the duration of the experiment was between 2 and 3 hours (data not shown). Figures 14, 15 and 16 show the basal tissue GSH levels, GSH depletion at 0.075 mM (final concentration) TBHP and TBARS formation at 2.5 mM TBHP as a function of reperfusion time. As seen in these figures, myocardial GSH levels and susceptibility to TBHP-induced GSH depletion and formation of TBARS changed only slightly after 40 min of ischemia and none of these alterations attained statistical significance when compared with non-ischemic (SHAM-CON) samples. During the initial 5 min of reperfusion, basal GSH levels remained unchanged (relative to SHAM-CON or ischemic/non-reperfused (ISC/NON) samples), but decreased significantly below those of SHAM-CON and ISC/NON tissues after 15 min of reperfusion (Fig. 14). A progressive decrease in basal glutathione levels occurred after periods of reperfusion greater than 15 min, with all values being significantly different from those of SHAM-CON and ISC/NON tissues (Fig. 14). Moreover, during the initial 15 min of reperfusion, the susceptibility of myocardial tissue to
Fig. 14  Time-course of alterations in myocardial GSH levels during the course of post-ischemic reperfusion in rabbits.

Animals were subjected to increasing periods of reperfusion following a 40 min period of coronary artery ligation. Myocardial GSH level, expressed as nanomoles/mg tissue, was measured as described in Materials and Methods. Each point represents the mean ± SEM of values from 6 animals.

+ - significantly different from SHAM-CON or ISC/NON animals

* - significantly different from both SHAM-CON and ISC/NON animals.
Fig. 15 Time-course of alterations in GSH depletion of myocardial tissue during the course of post-ischemic reperfusion in rabbits.

Animals were subjected to increasing periods of reperfusion following a 40 min period of coronary artery ligation. GSH depletion, expressed as % depletion, was measured as described in Materials and Methods. Measurement of GSH depletion used a final concentration of TBHP of 0.075 mM. Each point represents the mean ± SEM of values from 6 animals. + and * denote are as described in Fig. 14.
Fig. 16 Time-course of alterations in formation of TBARS in myocardial tissue during the course of post-ischemic reperfusion in rabbits.

Animals were subjected to increasing periods of reperfusion following a 40 min period of coronary artery ligation. TBARS formation, expressed as absorbance at 532 nm, was measured as described in Materials and Methods. Measurement of TBARS formation used a final concentration of TBHP of 2.5 mM. Each point represents the mean ± SEM of values from 6 animals. + and * are as described in Fig. 14.
TBHP-induced GSH depletion was altered in a complex manner (Fig. 15). After reperfusion for 5 min, myocardial tissues became less susceptible to GSH depletion (relative to ISC/NON samples); however, an abrupt increase in susceptibility occurred 15 min after the initiation of reperfusion (Fig. 15). Reperfusion for periods longer than 15 min resulted in a further enhancement in the susceptibility to GSH depletion; maximal depletion was not attained until 90 min of reperfusion, with all values being significantly different from those of SHAM-CON and ISC/NON tissues (Fig. 15). Finally, the initial 5 min of reperfusion had minimal effects on the formation of TBARS induced by TBHP, but reperfusion for periods longer than 5 min resulted in a drastic increase; the maximal level of TBARS formed was not attained until 60 min after the initiation of reperfusion, with all values differing significantly from those of SHAM-CON and ISC/NON tissues (Fig. 16).

3.4.1 Alterations in the Activities of Antioxidant Enzymes in Ischemic/Reperfused Myocardial Tissues

As an approach to investigating the possibility of inactivation or a transient impairment in the functioning of antioxidant enzymes during post-ischemic reperfusion, the activities of three antioxidant enzymes, namely, Cu,Zn-SOD, GPX and GRD were examined in ischemic/reperfused myocardial tissue in connection with the reperfusion time-course study.
Myocardial CAT was not examined because its activity in rabbit hearts (when correction is made for contamination by blood-derived catalase) is virtually undetectable under the assay conditions used. As shown in Table V, mean activities of Cu,Zn-SOD, GPX and GRD did not show any significant change (relative to SHAM-CON samples) following a 40 min period of ischemia, with or without subsequent reperfusion for periods up to 120 min. However, when correlation analysis was performed using data from all the ischemic/reperfused rabbits (7 ISC/REP groups, n=42), the activities of Cu,Zn-SOD and GRD were found to correlate positively with basal GSH levels, but negatively with TBHP-induced GSH depletion and the formation of TBARS. No significant correlations were found for GPX (Table VI).

3.4.2 pH Dependence of Antioxidant Enzyme Activities

The intracellular pH of rabbit myocardium has been reported to decrease from 7.0 to 6.0 after 30 min of ischemia [177]. This might be an important factor in causing I/R injury, if transient functional impairment in the activity of antioxidant enzymes were to occur under these conditions. In order to explore this possibility, the effects of acidotic pH on antioxidant enzyme activities were examined. The activities of CAT, GPX and GRD were measured in hemolysates and homogenates of myocardial and liver tissue, using buffers adjusted to pH 6.0 and pH 7.0, respectively. Because the measurement of Cu,Zn-SOD activity
Table V  Time-course of post-ischemic reperfusion injury in rabbit myocardium: effects on antioxidant enzymes.

<table>
<thead>
<tr>
<th>Reperfusion time (min)</th>
<th>SHAM-CON</th>
<th>ISC/NON</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>SOD</td>
<td>0.64</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>±0.03</td>
<td>±0.05</td>
</tr>
<tr>
<td>GPX</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>±0.2</td>
<td>±0.1</td>
</tr>
<tr>
<td>GRD</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>±0.2</td>
<td>±0.1</td>
</tr>
</tbody>
</table>

The activities of Cu,Zn-SOD (Units/mg tissue), GPX (nmoles NADPH/min/mg tissue) and GRD (nmoles NADPH.min/mg tissue) were measured as described in Materials and Methods. Values are given as mean ± SEM for n = 6. SHAM-CON and ISC/NON are the non-ischemic and ischemic/non-reperfused groups, respectively.
Table VI  Correlations between the activities of antioxidant enzymes and antioxidant capacity of ischemic/reperfused rabbit myocardium.

<table>
<thead>
<tr>
<th></th>
<th>Basal GSH levels</th>
<th>TBHP-induced</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GSH depletion</td>
<td>Formation of TBARS</td>
</tr>
<tr>
<td>Cu,Zn-SOD</td>
<td>+0.48</td>
<td>-0.50</td>
<td>-0.43</td>
</tr>
<tr>
<td></td>
<td>($P &lt; 0.001)$</td>
<td>($P &lt; 0.001$)</td>
<td>($P &lt; 0.004$)</td>
</tr>
<tr>
<td>GRD</td>
<td>+0.54</td>
<td>-0.51</td>
<td>-0.55</td>
</tr>
<tr>
<td></td>
<td>($P &lt; 0.0005$)</td>
<td>($P &lt; 0.001$)</td>
<td>($P &lt; 0.0005$)</td>
</tr>
<tr>
<td>GPX</td>
<td>+0.05</td>
<td>-0.09</td>
<td>-0.01</td>
</tr>
<tr>
<td></td>
<td>($P &lt; 0.750$)</td>
<td>($P &lt; 0.600$)</td>
<td>($P &lt; 0.970$)</td>
</tr>
</tbody>
</table>

The relationship between activities of antioxidant enzymes and antioxidant status was quantitatively assessed by correlation analysis. The antioxidant capacity of ischemic/reperfused myocardial tissues was assessed in terms of susceptibility to TBHP-induced GSH depletion and formation of TBARS (at final concentrations of TBHP of 0.075 and 2.5 mM, respectively), and antioxidant enzyme activities were measured by in vitro assays, as described in Materials and Methods. Data given are correlation coefficients with corresponding probabilities in parentheses.
was not feasible under conditions of low pH, the effect of acidic pH on the activity of this enzyme was not examined. As shown in Table VII, the activities of antioxidant enzymes (except erythrocyte CAT) were suppressed to varying degrees.

3.5 Brief Episodes of Ischemia

It has been shown that brief episodes of ischemia can cause prolonged alterations in myocardial ultrastructure, high energy phosphates and regional function [221]. Experimental and clinical evidence concerning the occurrence of myocardial necrosis following such brief episodes of ischemia (less than 20 min in duration) has been inconsistent [178-180, 221]. The effects of brief episodes of ischemia on myocardial antioxidant capacity was examined in rabbits subjected to coronary artery ligation.

3.5.1 Effects of Cumulative Brief Episodes of Ischemia on Myocardial Antioxidant Capacity and ATP Levels

Twenty-four rabbits were randomly assigned to one of the following groups consisting 6 animals each: SHAM-CON: sham-operated animals, no ischemia; Brief ISC/REP (1 cycle): 10 min period of coronary artery ligation followed by 15 min of reperfusion; Brief ISC/REP (4 cycles): 10 min period of coronary artery ligation followed by 15 min of reperfusion, 4 times; Data for ISC/REP (40 min period of coronary artery ligation followed by 60 min of reperfusion)
Table VII  Effects of acidotic pH on antioxidant enzyme activities.

<table>
<thead>
<tr>
<th></th>
<th>Erythrocyte</th>
<th></th>
<th>Liver</th>
<th></th>
<th>Heart</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.0</td>
<td>6.0</td>
<td>% decr.</td>
<td>7.0</td>
<td>6.0</td>
<td>% decr.</td>
</tr>
<tr>
<td>CAT</td>
<td>0.080</td>
<td>0.081</td>
<td>-1.9</td>
<td>0.0085</td>
<td>0.0074*</td>
<td>-13.4</td>
</tr>
<tr>
<td></td>
<td>±0.004</td>
<td>±0.004</td>
<td>±0.8</td>
<td>±0.0004</td>
<td>±0.0004</td>
<td>±2.3</td>
</tr>
<tr>
<td>GPX</td>
<td>38</td>
<td>14**</td>
<td>64</td>
<td>22</td>
<td>4.1**</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>±2.7</td>
<td>±1.2</td>
<td>±1.5</td>
<td>±1.3</td>
<td>±0.1</td>
<td>±1.0</td>
</tr>
<tr>
<td>GRD</td>
<td>4.8</td>
<td>2.8**</td>
<td>42</td>
<td>5.9</td>
<td>3.6**</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>±0.3</td>
<td>±0.2</td>
<td>±3.0</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±2.0</td>
</tr>
</tbody>
</table>

The activities of CAT (K/mg Hb or mg tissue), GPX and GRD (nmole NADPH/min/mg Hb or mg tissue) were measured in buffers adjusted to pH 7.0 and pH 6.0, respectively, using hemolysates, non-ischemic liver and left ventricular tissues. Values are given as mean ± SEM for n = 5. The effect of acidotic pH on enzyme activity is expressed as a percentage decrease relative to that at pH 7.0. N.D. = "Not-Determined" - CAT activity in the heart was undetectable under the assay conditions used.

* = P < 0.01, ** = P < 0.001 when compared with the values at pH 7.0
animals were obtained from the previous study (see Table IV) Repetitive brief episodes of ischemia (up to 4 cycles) had no discernible effect on myocardial GSH levels or on susceptibility to TBHP challenge (relative to SHAM-CON), except for a small but significant decrease in susceptibility to GSH depletion after one ischemic episode (Table VIII), while a prolonged ischemic insult followed by 60 min of reperfusion caused a significant decrease in myocardial GSH levels, and an enhanced susceptibility to TBHP-induced depletion of GSH and formation of TBARS. However, myocardial ATP levels were significantly decreased (relative to SHAM-CON samples) after repeated exposure to brief episodes of ischemia, with the extent of depletion depending on the number of exposures (Table VIII). Moreover, myocardial ATP levels measured following the prolonged ischemic insult were significantly lower than those measured after repetitive brief episodes of ischemia, although the total duration of time during the period of ischemia and reperfusion was the same in all cases. No significant changes in the activities of myocardial Cu,Zn-SOD, GPX or GRD were seen following either brief or prolonged episodes of ischemia followed by reperfusion (Table IX).
Table VIII  Effects of brief cumulative episodes of ischemia on myocardial antioxidant capacity and ATP levels.

<table>
<thead>
<tr>
<th></th>
<th>Basal GSH level (nmoles/mg tissue)</th>
<th>% GSH Depletion</th>
<th>TBARS Formation (OD532)</th>
<th>ATP (nmoles/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM-CON</td>
<td>2.13±0.10</td>
<td>21±3.5</td>
<td>0.23±0.007</td>
<td>3.96±0.01</td>
</tr>
<tr>
<td>Brief ISC/REP (1 cycle)</td>
<td>1.93±0.09</td>
<td>7±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.24±0.016</td>
<td>2.71±0.23</td>
</tr>
<tr>
<td>Brief ISC/REP (4 cycles)</td>
<td>1.87±0.12</td>
<td>15±5.7</td>
<td>0.25±0.018</td>
<td>1.64±0.48&lt;sup&gt;a, b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISC/REP</td>
<td>1.06±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>73±4.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.57±0.062&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.54±0.11&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Rabbits were subjected either to a 10 min period of coronary artery ligation followed by 15 min of reperfusion (Brief ISC/REP, 1 or 4 cycles) or a 40 min period of ligation with 60 min of reperfusion (ISC/REP). GSH depletion and TBARS formation were measured at final concentrations of TBHP of 0.075 and 2.5 mM, respectively. Values are given as mean ± SEM for n = 6 in each group.

<sup>a</sup> - significantly different from SHAM-CON.
<sup>b</sup> - significantly different from Brief ISC/REP (1 cycle).
<sup>c</sup> - significantly different from all groups.
Table IX  Effects of brief cumulative episodes of ischemia on myocardial antioxidant enzyme activities.

<table>
<thead>
<tr>
<th></th>
<th>Cu,Zn-SOD (Units/mg tissue)</th>
<th>GPX (nmole NADPH/min/mg tissue)</th>
<th>GRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM-CON</td>
<td>0.83±0.02</td>
<td>2.4±0.1</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>Brief ISC/REP (1 cycle)</td>
<td>0.76±0.03</td>
<td>2.6±0.2</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>Brief ISC/REP (4 cycles)</td>
<td>0.74±0.02</td>
<td>3.1±0.4</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>ISC/REP</td>
<td>0.79±0.02</td>
<td>2.5±0.1</td>
<td>1.8±0.1</td>
</tr>
</tbody>
</table>

Myocardial activities of Cu,Zn-superoxide dismutase (Cu,Zn-SOD), glutathione peroxidase (GPX) and glutathione reductase (GRD) were measured as described in Materials and Methods. Values are given as mean ± SEM for n = 6 in each group.
3.5.2 Effects of Ischemic Preconditioning on I/R-related Myocardial Alterations

Preconditioning of pig myocardium by brief episodes of ischemia has been shown to reduce the extent of I/R injury following a subsequent prolonged period of ischemia and reperfusion [181]. In order to investigate whether ischemic preconditioning produces any effects on the I/R-related alteration in myocardial antioxidant capacity, the following study was undertaken. Eighteen rabbits were randomly assigned to one of the following groups consisting of 6 animals each: SHAM-CON: sham-operated animals, no ischemia; PRECON: repetitive brief episodes of ischemia (5 min period of coronary artery ligation followed by 5 min of reperfusion), 4 times; PRECON+ISC/REP: repetitive brief episodes of ischemia (as in the preceding group) followed by a 40 min period of coronary artery ligation with 60 min of reperfusion. ISC/REP: 40 min period of coronary artery ligation followed by 60 min of reperfusion. Repetitive episodes of ischemia produced no detectable effects on myocardial GSH levels or on susceptibility to TBHP challenge; however, tissue ATP levels were significantly decreased (relative to SHAM-CON samples) (Table X). When the ischemic preconditioning was followed by a 40 min period of coronary artery ligation with 60 min of reperfusion, a significant decrease (relative to SHAM-CON and PRECON samples) in myocardial GSH levels and an enhanced
<table>
<thead>
<tr>
<th></th>
<th>Basal GSH level (nmoles/mg tissue)</th>
<th>% GSH Depletion</th>
<th>TBARS Formation (OD₅₃₂)</th>
<th>ATP (nmoles/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM-CON</td>
<td>2.19 ± 0.10</td>
<td>10 ± 4.7</td>
<td>0.23 ± 0.007</td>
<td>3.96 ± 0.01</td>
</tr>
<tr>
<td>PRECON</td>
<td>1.95 ± 0.11</td>
<td>10 ± 3.0</td>
<td>0.23 ± 0.009</td>
<td>2.47ᵃ</td>
</tr>
<tr>
<td>PRECON + ISC/REP</td>
<td>1.57ᵃᵇ ± 0.16</td>
<td>58ᵃᵇ ± 12.0</td>
<td>0.50ᵃᵇ ± 0.102</td>
<td>0.43ᵃᵇ ± 0.21</td>
</tr>
<tr>
<td>ISC/REP</td>
<td>1.44ᵃᵇ ± 0.05</td>
<td>55ᵃᵇ ± 11.8</td>
<td>0.34 ± 0.040</td>
<td>0.82ᵃᵇ ± 0.11</td>
</tr>
</tbody>
</table>

GSH depletion and TBARS formation were measured at final concentrations of TBHP of 0.1 and 2.5 mM, respectively. Values are given as mean ± SEM for n = 6 in each group.

ᵃ - significantly different from SHAM-CON.
ᵇ - significantly different from PRECON.
Table XI  Effects of ischemic preconditioning on myocardial antioxidant enzyme activities.

<table>
<thead>
<tr>
<th></th>
<th>Cu,Zn-SOD (Units/mg tissue)</th>
<th>GPX (nmoles NADPH/min/mg tissue)</th>
<th>GRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM-CON</td>
<td>0.855±0.032</td>
<td>2.42±0.06</td>
<td>2.18±0.25</td>
</tr>
<tr>
<td>PRECON</td>
<td>0.880±0.032</td>
<td>2.63±0.07</td>
<td>1.73±0.22</td>
</tr>
<tr>
<td>PRECON+ISC/REP</td>
<td>0.809±0.034</td>
<td>2.37±0.09</td>
<td>2.10±0.24</td>
</tr>
<tr>
<td>ISC/REP</td>
<td>0.861±0.067</td>
<td>2.51±0.12</td>
<td>1.75±0.11</td>
</tr>
</tbody>
</table>

Myocardial activities of Cu,Zn-superoxide dismutase (Cu,Zn-SOD), glutathione peroxidase (GPX) and glutathione reductase (GRD) were measured as described in Materials and Methods. Values are given as mean ± SEM for n=6 in each group.
susceptibility to TBHP-induced depletion of GSH and formation of TBARS occurred (Table X). Myocardial ATP levels were further reduced, with all values being significantly lower than those of SHAM-CON and PRECON samples. Ischemic preconditioning did not seem to affect the I/R-induced myocardial alterations when compared with those of non-preconditioned animals (Table X). No significant changes in myocardial antioxidant enzyme activities were seen in PRECON or PRECON+ISC/REP tissues (Table XI).

3.6 I/R-Induced Alterations in Antioxidant Capacity in Isolated Langendorff-Perfused Hearts

As an approach to investigating the role of blood elements involved in causing impairment in myocardial antioxidant capacity during ischemia and reperfusion, isolated buffered-perfused rabbit hearts were subjected to a 40 min period of coronary artery ligation followed by 60 min of reperfusion. Eighteen rabbits were randomly assigned to one of the following groups consisting of 6 animals each: SHAM-CON (IN VITRO): sham-operated Langendorff-perfused hearts, no ischemia; ISC/REP (IN VITRO): Langendorff-perfused hearts, a 40 min period of coronary artery ligation followed by 60 min of reperfusion; ISC/REP (IN VIVO/IN VITRO): animals subjected to a 30 min period of coronary artery ligation in vivo, with an additional 10 min of ischemia during Langendorff perfusion (i.e., in vitro) that was followed by 60 min of reperfusion in vitro. Data
for SHAM-CON (IN VIVO) and ISC/REP (IN VIVO) (40 min period of coronary ligation followed by 60 min of reperfusion) were obtained from the ischemia time-course studies (see Figs. 9 and 10). In isolated and Langendorff-perfused hearts, tissue GSH levels were significantly decreased by 40% (relative to SHAM-CON (IN VIVO) samples). This was associated with an enhancement in tissue susceptibility to TBHP-induced depletion of GSH but not to the formation of TBARS (Table XII). Following a 40 min period of ischemia with 60 min of reperfusion in vitro, myocardial GSH levels as well as tissue susceptibility to oxidative challenge remained unchanged (relative to SHAM-CON (IN VITRO) samples). In contrast, a significant decrease in myocardial GSH levels and an increase in tissue susceptibility to TBHP challenge occurred following ischemia and reperfusion in vivo (i.e., ISC/REP (IN VIVO) samples). When the hearts were subjected to a 30 min period of ischemia in vivo followed by an additional 10 min of ischemia in vitro, reperfusion with buffer for 60 min in vitro caused a significant increase (relative to SHAM-CON (IN VITRO) samples) in tissue susceptibility to TBHP-induced formation of TBARS, while the tissue GSH levels and their susceptibility to TBHP-induced depletion remained relatively unchanged when compared with those of SHAM-CON (IN VITRO) samples (Table XII).
Table XII  I/R-induced alterations in antioxidant capacity in intact or isolated Langendorff-perfused rabbit hearts.

<table>
<thead>
<tr>
<th></th>
<th>Basal GSH (nmoles/mg tissue)</th>
<th>% GSH Depletion</th>
<th>TBARS Formation (OD532)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SHAM-CON</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(IN VIVO)</td>
<td>2.39</td>
<td>13</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>±0.12</td>
<td>± 3.9</td>
<td>±0.009</td>
</tr>
<tr>
<td><strong>SHAM-CON</strong></td>
<td>1.44⁴</td>
<td>59⁴</td>
<td>0.20</td>
</tr>
<tr>
<td>(IN VITRO)</td>
<td>±0.06</td>
<td>± 2.1</td>
<td>±0.007</td>
</tr>
<tr>
<td><strong>ISC/REP</strong></td>
<td>1.33</td>
<td>51</td>
<td>0.20⁵</td>
</tr>
<tr>
<td>(IN VITRO)</td>
<td>±0.10</td>
<td>± 5.9</td>
<td>±0.009</td>
</tr>
<tr>
<td><strong>ISC/REP</strong></td>
<td>1.14</td>
<td>58</td>
<td>0.27⁴, ⁵</td>
</tr>
<tr>
<td>(IN VIVO/IN VITRO)</td>
<td>±0.13</td>
<td>± 1.6</td>
<td>±0.022</td>
</tr>
<tr>
<td><strong>ISC/REP</strong></td>
<td>1.00⁴</td>
<td>62⁴</td>
<td>0.53⁴</td>
</tr>
<tr>
<td>(IN VIVO)</td>
<td>±0.08</td>
<td>± 6.3</td>
<td>±0.043</td>
</tr>
</tbody>
</table>

Measurements of GSH depletion and TBARS formation used final TBHP concentrations of 0.05 and 2.5 mM, respectively. Values are given as mean ± SEM, with n = 6 in SHAM-CON, ISC/REP (IN VITRO) and ISC/REP (IN VIVO/IN VITRO) groups.

⁴ - significantly different from SHAM-CON (IN VIVO).
⁵ - significantly different from SHAM-CON (IN VITRO).
⁶ - significantly different from ISC/REP (IN VIVO).
⁷ - significantly different from ISC/REP (IN VITRO).
3.7 Heart-Lung Transplantation

As shown in the previous section, chronic allopurinol treatment protected against I/R-induced impairment in myocardial antioxidant capacity in rabbits. This observation and the fact that protective actions of allopurinol are demonstrable in a variety of tissues [150,187,188] suggest its effects on antioxidant status may be generalized and widespread. The effects of chronic allopurinol treatment on the antioxidant capacity of erythrocytes and the functional consequences of ischemia and reperfusion were examined using a swine model of heart-lung transplantation.

3.7.1 Effects of Allopurinol Pretreatment on the Antioxidant Capacity of Pig Erythrocytes

Donor and recipient pigs were treated with allopurinol given orally at a daily dose of 50 mg/kg for 5 days. The last dose was given on the day of surgery. The susceptibility of pig erythrocytes to in vitro oxidative challenge, as shown by the decrease in TBHP-induced MDA production, was significantly reduced by allopurinol pretreatment (Fig. 17). The pooled data shown in this figure indicate that the protective effect of allopurinol was time- (or dose-) dependent, so that the mean reduction in MDA production (induced by TBHP at a final concentration of 0.5 mM) did not attain statistical significance until day 3 (P < 0.02). Inhibition of lipid peroxidation by
Fig. 17 Effect of allopurinol treatment on susceptibility of pig erythrocytes to lipid peroxidation.

Measurement of erythrocyte MDA used a final concentration of TBHP of 0.5 mM. Each point represents the mean ± SEM of values from 12 animals. * $P < 0.02$ relative to control (i.e. prior to initiation of drug administration).
The graph illustrates the change in nmoles MDA / mg Hb × 10 over time. The x-axis represents the days (Day 1 to Day 5) and the y-axis represents the nmoles MDA / mg Hb × 10. There are error bars indicating variability. Significant differences are marked with asterisks (*) on Day 3 and Day 4.
allopurinol reached a maximal value of 33% by day 4 (P < 0.02 relative to baseline, but not significantly different from value at day 3). By day 5, erythrocyte MDA levels were no longer significantly different from baseline levels (i.e., at day 1). When patterns from individual experimental animals are examined, however, the considerable degree of inter-animal variability in the time-course of allopurinol-induced protection against lipid peroxidation becomes immediately apparent (Table XIII). Thus, although maximal protection was usually attained by day 3 or 4 (7 out of 12 animals), some (3 out of 12) responded maximally on day 2 (i.e., after a single dose of allopurinol) while substantial decreases in red cell lipid peroxidation were observed in 2 animals on day 5. The differences observed could not be accounted for by variations in the forced peroxidation assay, since replicate determinations agreed within ± 5%.

On the fifth day following the initiation of allopurinol treatment, pigs were allocated to donor or recipient categories for the heart-lung transplantation study. A convenient measure of lung functional integrity assessed 2 hr post-transplantation was the estimation of lung water content. This index of functional viability correlated significantly with the extent of allopurinol protection against TBHP-induced lipid peroxidation in red cells from individual donor (P < 0.005) and recipient (P < 0.05) animals (Fig. 18). The lung water contents were
Table XIII  Inter-animal variation in allopurinol-induced protection against lipid peroxidation in pig erythrocytes. [Heparinized venous blood samples were drawn before oral allopurinol administration at a daily dose of 50 mg/kg. The susceptibility of erythrocytes to TBHP-induced lipid peroxidation was measured. Values given are percentage of control (i.e. values at Day 1)].

<table>
<thead>
<tr>
<th>Pig</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56.3</td>
<td>22.9</td>
<td>68.8</td>
<td>47.9</td>
</tr>
<tr>
<td>2</td>
<td>109.5</td>
<td>71.4</td>
<td>95.5</td>
<td>63.5</td>
</tr>
<tr>
<td>3</td>
<td>87.1</td>
<td>85.7</td>
<td>34.3</td>
<td>75.7</td>
</tr>
<tr>
<td>4</td>
<td>78.0</td>
<td>60.0</td>
<td>32.0</td>
<td>74.0</td>
</tr>
<tr>
<td>5</td>
<td>60.0</td>
<td>80.0</td>
<td>85.5</td>
<td>49.3</td>
</tr>
<tr>
<td>6</td>
<td>76.2</td>
<td>104.8</td>
<td>100.0</td>
<td>90.5</td>
</tr>
<tr>
<td>7</td>
<td>87.2</td>
<td>76.6</td>
<td>29.8</td>
<td>74.5</td>
</tr>
<tr>
<td>8</td>
<td>97.2</td>
<td>93.0</td>
<td>32.4</td>
<td>83.1</td>
</tr>
<tr>
<td>9</td>
<td>66.2</td>
<td>73.5</td>
<td>95.6</td>
<td>108.8</td>
</tr>
<tr>
<td>10</td>
<td>63.5</td>
<td>71.4</td>
<td>95.2</td>
<td>103.2</td>
</tr>
<tr>
<td>11</td>
<td>122.4</td>
<td>70.1</td>
<td>53.7</td>
<td>108.9</td>
</tr>
<tr>
<td>12</td>
<td>116.9</td>
<td>77.9</td>
<td>84.9</td>
<td>105.1</td>
</tr>
</tbody>
</table>

Susceptibility of erythrocytes to TBHP-induced lipid peroxidation was assessed as described in Materials and Methods. Underlined figures represent maximal protection.
Fig. 18 Correlation between erythrocyte MDA and lung water (LW) levels.

The regression lines of donor (●—●) and recipient pigs (○—○) erythrocyte MDA levels against LW levels. Red cell MDA was measured prior to surgery (i.e., on day 5) and LW was measured at 2 hr following post-transplantation reperfusion.
Donors \( r = 0.95, P < 0.005 \)

Recipients \( r = 0.82, P < 0.05 \)
found to correlate well with other indices of pulmonary function, such as arterial blood $pO_2$, alveolar-arterial gradient ($AaG$) and alveolar-arterial ratio ($AaR$) (data not shown).

3.8 Metal Chelating Properties of Allopurinol

A number of alternative mechanisms to xanthine oxidase inhibition in the protection by allopurinol against I/R injury have been proposed [162,164,165]. However, the chelation of transition metal ions, whose catalytic actions in oxy-radical-mediated reactions are also important determinants in the pathogenesis of oxidative tissue damage [13,194], has not yet been considered. The metal chelating actions of allopurinol and its active metabolite oxypurinol were, therefore, examined in in vitro systems measuring the transition metal ion-catalysed oxidation of ascorbic acid and oxidation of erythrocyte membrane lipids.
3.8.1 Effects of Allopurinol and Oxypurinol on Transition Metal Ion-Catalysed Oxidation of Ascorbic acid

Since the mechanism of transition metal ion-catalysed ascorbate oxidation involves formation of an ascorbate-transition metal ion complex [225], transition metal chelators would be expected to interfere with this interaction, thereby decreasing the reaction rate.

Ascorbate oxidized spontaneously in phosphate buffer in the absence of added transition metal ions (Fig. 19a). This basal ascorbate oxidation was decreased by allopurinol (100 μM final concentration). Cupric chloride, at a final concentration of 10 μM, dramatically increased the rate of ascorbate oxidation (Fig. 19b). Allopurinol also inhibited this cupric ion-catalysed oxidation, in a concentration-dependent manner, with almost complete suppression at a final concentration of 100 μM. On the other hand, the addition of ferric chloride, up to a final concentration of 100 μM, did not significantly alter the rate of ascorbate oxidation (data not shown).

When the oxidation of ascorbate was measured in the presence of 100 μM EDTA, the oxidation rate was decreased from the basal value of 3.40 ± 0.11 (SEM) nmoles/min (Fig. 19a) to 1.01 ± 0.12 nmoles/min (Fig. 20). The addition of cupric chloride, at a final concentration of 10 and 30 μM, did not stimulate ascorbate oxidation under these conditions (data not shown), but when present at a final concentration of 100 μM, cupric chloride significantly
Fig. 19 Cupric ion-catalysed oxidation of ascorbate: effects of allopurinol.

Ascorbate oxidation, as indicated by the decrease in absorbance at 280 nm, was monitored for 5 min in reaction mixtures containing 100 μM ascorbate in 10 mM phosphate buffer (pH 7.4), either in the absence or presence of allopurinol, as described in Materials and Methods. Each point represents the mean value of three experiments, with the SEM < 5% of the mean.
Fig. 20 Cupric and ferric ion-catalysed oxidation of ascorbate in the presence of EDTA.

Assays were performed as described in Materials and Methods. Rates of ascorbate oxidation, expressed in nanomoles/min, are given as mean ± SEM of three experiments. *, ** and *** denote significant (P < 0.05, P < 0.01 and P < 0.001, respectively) differences when compared with appropriate controls.
increased the rate of ascorbate oxidation to $17.2 \pm 1.05$ nmoles/min (Fig. 20). Both allopurinol and oxypurinol (500 μM final concentrations) significantly inhibited this cupric ion-catalysed oxidation of ascorbate, reducing the oxidation rate to 22% and 16% of control, respectively. However, when allopurinol and oxypurinol were added at a final concentration of 50 μM, only oxypurinol produced a significant degree of inhibition. Ferric chloride, when added in the presence of 100 μM EDTA, stimulated the oxidation of ascorbate in a concentration-dependent manner to a maximum of $5.88 \pm 0.49$ nmoles/min (Fig. 20). Both allopurinol and oxypurinol at final concentrations of 500 μM slightly inhibited the oxidation of ascorbate catalysed by 10 μM ferric chloride, but at increased concentrations of ferric chloride, this inhibitory effect of allopurinol and oxypurinol was abolished. Uric acid, at a final concentration of 50 μM, inhibited the cupric ion-catalysed oxidation of ascorbate, reducing the rate of oxidation to 63% of control, but had no significant inhibitory effect on ferric ion-catalysed oxidation.

3.8.2 **Transition Metal Ion-Catalysed Oxidation of Erythrocyte Membrane Lipids**

Recently, considerable evidence has emerged suggesting the important role of lipid peroxidation in the pathogenesis of many diseases, drug toxicities and I/R injury [3]. In the heart, lipid peroxidation of cardiomyocyte membranes induced
by reactive oxy-radicals generated during the reperfusion of ischemic tissue may be responsible for the development of irreversible damage [169]. The involvement of iron in both initiation [222] and propagation [223] phases of lipid peroxidation reactions within biological membranes is well established. Moreover, iron-dependent lipid peroxidation is thought to play a crucial role in pathologically relevant oxy-radical-induced tissue damage in vivo [224]. Therapeutic interventions involving the use of agents that inhibit iron-dependent lipid peroxidation may therefore represent a rational approach to the management of oxy-radical-related diseases. The ferric ion or cupric ion-TBHP-stimulated lipid peroxidation was examined in vitro using erythrocyte membranes as a lipid source. The effects of allopurinol and oxypurinol on the transition metal ion-catalysed oxidation of membrane lipids were also investigated.

3.8.2.1 Ferric Chloride-Induced Oxidation of Erythrocyte Membrane Lipids

When human erythrocyte membranes were incubated with increasing concentrations of ferric chloride, an enhancement of lipid peroxidation was observed, as indicated by the formation of TBARS, an indirect measure of lipid peroxidation (Fig. 21). The concentration-dependent increase reached a maximum value at 0.3 mM, which was
Fig. 21 Ferric ion-induced formation of TBARS in erythrocyte membranes.

Assays were performed as described in Materials and Methods. TBARS content was expressed as absorbance at 532 nm. Each point represents the mean ± SD of triplicate samples from one representative experiment.
followed by a progressive decrease at higher concentrations of ferric ion.

The time-course of Fe\(^{3+}\)-stimulated production of TBARS showed a typical autocatalytic pattern, wherein the initiation of lipid peroxidation was preceded by a 10 min lag phase after which levels of TBARS increased rapidly, attaining maximal levels after approximately 90 min of incubation (Fig. 22). When GSH or ascorbic acid, at a final concentration of 50 µM, was added to the reaction mixture, an increased rate of TBARS formation was observed, with a shortening of the lag phase to less than 5 min (Fig. 22). The stimulatory effect of ascorbic acid on the Fe\(^{3+}\)-induced lipid peroxidation was greater than that of GSH, with the former causing a significant increase in maximal TBARS production.

3.8.2.2 Effects of Allopurinol and Oxypurinol on Ferric Chloride-Stimulated Oxidation of Erythrocyte Membrane Lipids

Incubation of erythrocyte membranes with ferric chloride increased the production of TBARS in a concentration-dependent manner (Fig. 23). Butylated hydroxytoluene (BHT) reduced the extent of ferric ion-induced lipid oxidation, with almost complete inhibition at a final concentration of 4 µM at all ferric ion concentrations tested. Isopropanol, the solvent in which the BHT was dissolved, had no effect in this system (data
Fig. 22  Effects of GSH and ascorbic acid on the time-course of ferric ion-induced formation of TBARS in erythrocyte membranes.

Assays were performed as described in Materials and Methods. TBARS content was expressed as absorbance at 532 nm. GSH or ascorbic acid (ASC) was added to the reaction mixture at a final concentration of 50 μM.
- ● - CON  - ■ - +GSH  - ▲ - +ASC

Absorbance at 532 nm

Incubation time ( min )
Fig. 23 Ferric ion-induced formation of TBARS in erythrocyte membranes: effects of allopurinol and oxypurinol.

Assays were performed as described in Materials and Methods. TBARS content was expressed as absorbance at 532 nm. Values are given as mean ± SEM of at least three experiments. * and *** denote significant (P < 0.05 and P < 0.001, respectively) differences when compared with the control.
not shown). EDTA at a final concentration of 0.4 mM strongly suppressed lipid oxidation induced by 0.2 mM ferric chloride, but the inhibitory action was almost completely overcome by increasing concentration of ferric chloride to 0.4 mM (Fig. 23). Uric acid, when added at a final concentration of 2 mM, had a comparable inhibitory effect to EDTA, but the higher concentration of ferric chloride only partly overcame the inhibition (Fig. 23). Allopurinol (4 mM) or oxypurinol (3 mM) significantly inhibited lipid peroxidation induced by 0.2 mM ferric chloride; however, at the higher concentration of ferric chloride, the inhibitory effects of both these compounds were no longer significant (Fig. 23). None of these compounds interfered with the TBA color reaction (data not shown).

3.8.2.3 Effects of Allopurinol and Oxypurinol on Cupric Ion-TBHP-Induced Oxidation of Erythrocyte Membrane Lipids

The peroxidation of erythrocyte membrane lipids was stimulated by TBHP, as indicated by the production of TBARS (Fig. 24). This peroxidation was not affected by either allopurinol or oxypurinol. When TBHP was combined with cupric chloride, there was a marked increase in the extent of lipid peroxidation, even though cupric chloride alone produced no significant effect on peroxidation (Fig. 24). Allopurinol and oxypurinol at final concentrations of 500 μM reduced this enhanced peroxidation to 87% and 88% of
Fig. 24  Cupric ion-TBHP - induced formation of TBARS in erythrocyte membranes: effects of allopurinol and oxypurinol.

Assays were performed as described in Materials and Methods. Cupric chloride and TBHP were added at final concentrations of 0.2 mM and 10 mM, respectively. TBARS content was expressed as absorbance at 532 nm. The basal level of TBARS production in the reaction mixture was measured in the absence of both cupric chloride and TBHP. Values are mean ± SEM of at least three experiments. *, ** and *** denote significant (P < 0.05, P < 0.01 and P < 0.001, respectively) differences when compared with the control.
control, respectively (Fig. 24), but no further decreases in TBARS level were observed at concentrations of allopurinol up to 2 mM. BHT strongly inhibited lipid peroxidation induced by the cupric chloride-TBHP mixture, in a concentration-dependent manner, reducing the TBARS level in the presence of 100 μM BHT to below that produced by TBHP alone (Fig. 24). Uric acid also significantly inhibited the cupric chloride-TBHP-induced peroxidation in a concentration-dependent manner, with the production of TBARS being reduced to 72% of control at a final concentration of 500 μM (Fig. 24).

3.8.3 UV Absorption Spectra of Allopurinol/Ascorbic Acid/Copper ion

UV spectral analysis has been used to demonstrate the formation of a complex between uric acid and copper ion involved in the inhibition by uric acid of the cupric ion-catalysed oxidation of ascorbate [50]. The possible complexation among allopurinol, transition metal ions and ascorbic acid was, therefore, examined in the present study using UV spectral analysis.

The spectrum generated from a mixture of allopurinol and ascorbate (spectrum B in Fig. 25a) was very similar to the sum of the individual spectra of allopurinol and ascorbic acid (data not shown). Cupric chloride, although it did not appreciably alter the spectrum of allopurinol (data not shown), generated a distinctive spectrum in the
Fig. 25 UV absorption spectrum of the allopurinol-ascorbate-cupric chloride mixture: effects of EDTA. Spectra were recorded as described in Materials and Methods. Spectra in (a) were recorded from the allopurinol-ascorbate-cupric chloride (A, —— ), allopurinol-ascorbate (B, ----- ) and cupric chloride (C, — — — ) mixtures, respectively. Spectra in (b) represent the allopurinol-ascorbate-cupric chloride-EDTA (A', —— ), allopurinol (B', —— —— ) and ascorbate-cupric chloride-EDTA (C', —— —— — ) mixtures, respectively.
Absorbance vs. Wavelength (nm)

Graph a:
- Curve A with peaks at 206 and 231 nm
- Curve B with peak at 206 nm
- Curve C with peak at 300 nm

Graph b:
- Curve A' with peaks at 206 and 231 nm
- Curve B' with peak at 206 nm
- Curve C' with peak at 300 nm
presence of allopurinol and ascorbate (spectrum A in Fig. 25a). This spectrum showed a decrease in absorption between 250 nm and 280 nm, presumably resulting from the oxidation of ascorbate. Moreover, there was a slight decrease and a moderate increase in absorbances at 206 nm and 231 nm, respectively, and a slight increase in absorption between 280 nm and 340 nm, which became apparent when absorbances of the allopurinol-ascorbate and cupric chloride spectra were subtracted from those of the allopurinol-ascorbate-cupric chloride spectrum (see Table XIV). The addition of EDTA to the allopurinol-ascorbate-cupric chloride mixture abolished these spectral changes (Table XIV), with the production of a spectrum (labelled A' in Fig. 25b) comparable to that expected for the simple addition individual absorbances for allopurinol and an ascorbate-cupric chloride-EDTA mixture (spectra B' and C' in Fig. 25b). Similar changes in spectral characteristics were observed following the addition of cupric chloride to an oxypurinol-ascorbate mixture (data not shown). In contrast, the addition of ferric chloride to an allopurinol-ascorbate mixture merely resulted in a spectrum similar to that produced by a mixture of allopurinol and ferric chloride (data not shown).
Table XIV  Absorbance changes of the allopurinol - ascorbate - cupric chloride mixture: effects of EDTA.

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<th>206 nm</th>
<th>231 nm</th>
<th>300 nm</th>
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<tr>
<td>ALP-ASC-Cu</td>
<td>-0.1339</td>
<td>+0.0947</td>
<td>+0.0494</td>
</tr>
<tr>
<td></td>
<td>(±0.0370)</td>
<td>(±0.0091)</td>
<td>(±0.0126)</td>
</tr>
<tr>
<td>ALP-ASC-Cu-EDTA</td>
<td>+0.0189*</td>
<td>-0.0060*</td>
<td>+0.0011*</td>
</tr>
<tr>
<td></td>
<td>(±0.0610)</td>
<td>(±0.0032)</td>
<td>(±0.0012)</td>
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* changes in absorbances were calculated by subtracting the absorbances both of B or B' and C or C' spectra from those of A or A' spectrum, respectively (see also Fig. 31). Values are given as mean ± SD for n = 3.

* - P < 0.005 when compared with the ALP-ASC-Cu mixture.
3.9 Myoglobin-TBHP-Catalysed Oxidation of Uric Acid and Erythrocyte Membrane lipids

Since myoglobin is present in cardiac muscle at a high concentration [197], its pro-oxidant action in ischemic tissue [198-200] might also be important in triggering uncontrolled oxidative processes. The use of pharmacological agents capable of attenuating the potentially harmful effects of myoglobin might represent a rational approach to protecting against myocardial I/R injury. Myoglobin reacts with hydroperoxides with resulting formation of reactive oxidants which can oxidize uric acid [220] and biological membrane lipids [202]. These myoglobin-dependent oxidative reactions were examined in vitro by measuring the oxidation of uric acid and erythrocyte membrane lipids, and the effects of allopurinol and oxypurinol on these oxidative reactions were also investigated.

3.9.1 Effects of Allopurinol and Oxypurinol on Myoglobin-TBHP-Catalysed Oxidation of Uric Acid

In the presence of myoglobin (26 μM final concentration), TBHP (0.5 mM) stimulated the rate of uric acid oxidation to 1.73 ± 0.07 (SEM) nmoles/min (Fig. 26). No significant oxidation of uric acid was observed when TBHP was added in the absence of myoglobin, and vice versa (data not shown). This myoglobin-TBHP-catalysed oxidation of uric acid was strongly inhibited by ascorbic acid and GSH in a
Fig. 26 Myoglobin-TBHP - catalysed oxidation of uric acid: effects of allopurinol and oxypurinol.

Assays were performed as described in Materials and Methods. Rate of uric acid oxidation was expressed as nmol/min. Values are given as mean ± SEM for n = 3. *, ** and *** denote significant (P < 0.05, P < 0.01 and P < 0.001, respectively) differences when compared with the control.

ASC - ascorbic acid
GSH - reduced glutathione
ALP - allopurinol
OXYP - oxypurinol
concentration-dependent manner (Fig. 26). Both allopurinol
and oxypurinol inhibited the rate of uric acid oxidation,
reducing the oxidation rate to 80% and 82% of control,
respectively, at final concentrations of 1 mM (Fig. 26).

3.9.2 Effects of Allopurinol on Myoglobin-TBHP-Induced
Oxidation of Erythrocyte Membrane Lipids

Incubation of erythrocyte membranes with the myoglobin-
TBHP mixture (26 μM and 1.0 mM final concentrations,
respectively) resulted in a time-dependent increase in the
formation of TBARS, an indirect index of lipid peroxidation
(Fig. 27). This myoglobin-TBHP-induced formation of TBARS
approached a maximum after approximately 110 min of
incubation. Neither myoglobin nor TBHP, when added alone,
caused any detectable formation of TBARS (data not shown).

The inhibitory effects of the test compounds on the
myoglobin-TBHP-induced peroxidation of erythrocyte membrane
lipids were examined in reaction mixtures incubated for a
period of 30 min (during which time period the rate of
TBARS formation in control samples was linear, see Fig. 27).
BHT inhibited the peroxidation of membrane lipids in a
concentration-dependent manner, significantly reducing the
TBARS produced to 21% of control at a final concentration of
0.1 mM (Fig. 28). Allopurinol, when added at a final
concentration of 2 mM, significantly reduced the formation
of TBARS to 90% of control, but the inhibition was not
Fig. 27  Time-course of myoglobin-TBHP-induced formation of TBARS in erythrocyte membranes.

Assays were performed as described in Materials and Methods. TBARS content was expressed as absorbance at 532 nm. Each point represents the mean for n = 3, with the SD < 5% of the mean.
Absorbance at 532 nm

Incubation time (min)
Fig. 28 Myoglobin-TBHP-induced formation of TBARS in erythrocyte membranes: effect of allopurinol.

Assays were performed as described in Materials and Methods. TBARS content was expressed as absorbance at 532 nm. Values are given as mean ± SEM from three experiments using different membrane preparations. *, ** and *** denote significant (P < 0.05, P < 0.01 and P < 0.001, respectively) differences when compared with the control.

BHT - butylated hydroxytoluene
ALP - allopurinol
URC - uric acid
Absorbance at 532 nm

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<th>0.00</th>
<th>0.25</th>
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<tbody>
<tr>
<td>CON</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHT</td>
<td>0.01mM</td>
<td>0.1mM</td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>1.0mM</td>
<td>2.0mM</td>
<td></td>
</tr>
<tr>
<td>URC</td>
<td>0.1mM</td>
<td>0.1mM</td>
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significant when added at a final concentration of 1 mM (Fig. 28). Uric acid also inhibited the lipid peroxidation at a final concentration of 0.1 mM, reducing the TBARS produced to 83% of control (Fig. 28).
4. DISCUSSION

4.1 Assessment of Tissue Antioxidant Capacity

Tert-butylhydroperoxide (TBHP) has previously been used as a model hydroperoxide in studying the effects of oxidative stress in intact cells or organs, such as heart, liver and kidney [226]. Its stability during storage and high solubility in aqueous phases have permitted its application in a variety of in vitro systems involving the study of free radical-mediated processes [227-229]. One such application was exploited in the present investigation in that the antioxidant capacity of tissues was assessed by measuring sensitivity of tissue homogenates to glutathione (GSH) depletion and formation of thiobarbituric acid-reactive substances (TBARS) following in vitro incubation with TBHP. Presumably, oxy-radicals generated from the hemoprotein-catalysed decomposition of TBHP molecules [229,230] can react with antioxidant molecules present in the tissue homogenate, and initiate free radical-chain reactions, such as the peroxidation of polyunsaturated lipid components of biological membranes.

The oxidative reactions initiated by TBHP could induce GSH oxidation, as indicated by the concentration-dependent decrease in GSH content in tissue homogenates prepared from heart, liver and kidney tissues (Fig. 1). Increasing concentrations of TBHP, which presumably overwhelmed the antioxidant capacity of tissue homogenates, caused an
increase in formation of TBARS, an indirect index of lipid peroxidation (Fig. 2). Under the conditions employed in this in vitro assay, glutathione peroxidase (GPX) present in the tissue homogenate would probably be inactivated in the presence of TBHP [300]. It is, therefore, unlikely that the depletion of GSH is a result of GPX-dependent enzymatic degradation of TBHP. This is strengthened by the finding that liver tissue homogenates, although possessing the highest GPX activity among the tissues studied (Fig. 5), did not show a greater extent of TBHP-induced GSH depletion (Fig. 1). In contrast, TBARS production in liver homogenates was greater than those in other tissues at all TBHP concentrations tested (Fig. 2). This might indicate that liver, in fact, is more susceptible to lipid damage than heart and kidney. It has been shown that hepatotoxicity induced by environmental pollutants (eg. carbon tetrachloride) and drug metabolites (eg. acetaminophen) likely involves oxy-radical-mediated lipid peroxidation processes [301,302]. Both high tissue GSH levels and activities of antioxidant enzymes in liver, especially catalase (CAT) and GPX, may suggest a compensatory mechanism for protecting against oxidative injury induced by endogenously generated oxidants in this vulnerable tissue. In this regard, the depletion of liver GSH content by dimethyl maleate in mice was associated with an enhanced susceptibility to hepatotoxicity produced by agents such as acetaminophen and carbon tetrachloride [302,303]. Thus,
measurements of susceptibility of tissue homogenates to TBHP-induced GSH depletion and formation of TBARS may be able to reflect, to a certain extent, the antioxidant capacity of tissue with regard to GSH protection and susceptibility of membrane lipids to oxidative challenge. A similar approach has been used to assess the antioxidant capacity of plasma by measuring the decomposition of various non-enzymatic antioxidants using a water-soluble and heat-labile compound, 2,2'-azo-bis (2-amidinopropane) as a free radical generating agent [39].

4.2 Alterations in Antioxidant Capacity in Ischemic/Reperfused Rabbit Myocardium

Changes in the sensitivity of tissue homogenates to in vitro oxidative challenge have been used as an indication of altered tissue susceptibility to oxidative injury in vivo [227,228]. A recent study by Ferreira et al. [189] has shown that myocardial biopsies from patients undergoing revascularization surgery exhibited significant increases in oxidative stress, as measured in vitro by hydroperoxide-induced chemiluminescence, the magnitude of which paralleled biochemical and ultrastructural indices of myocardial damage. Our finding that an abrupt increase in sensitivity of myocardial tissue homogenates to TBHP-induced oxidative challenge (indicative of impairment in antioxidant capacity) occurred after 20 - 40 min period of ischemia with 60 min of reperfusion which paralleled the onset of irreversible
injury [155,156] suggests the involvement of oxidative processes in these pathological changes.

GSH is a crucial determinant of tissue susceptibility to oxidative damage [231-233] and impaired GSH homeostasis has been shown to be a feature of myocardial I/R injury, particularly during the post-ischemic reperfusion phase [129,171]. The decrease in GSH levels of tissues subjected to oxidative stress is believed to reflect the formation of mixed disulfides with protein sulfhydryls and/or of glutathione disulfide (GSSG), the latter being capable in some systems of undergoing ATP-dependent extracellular translocation [171], thereby diminishing the cellular supply of GSH from the NADPH-dependent reduction of GSSG by glutathione reductase (GRD) [234]. The results presented here suggest the existence of a cellular GSH pool showing increased susceptibility to oxidative depletion which becomes maximal early in the course of ischemia (about 10 min) and remains constant (in the absence of reperfusion) with periods of ischemia up to 60 min (Fig. 9). Under these conditions, minimal increases in lipid susceptibility to oxidative challenge are observed (Fig. 10). Reperfusion following ischemia of short duration (i.e., 5 min) revealed evidence for a transient enhancement of tissue antioxidant capacity as reflected in a significant decrease in susceptibility of GSH to oxidative depletion (Fig. 9). As also noted in the reperfusion time-course study, an increased stability of myocardial GSH to peroxide-induced
depletion was observed 5 min after the initiation of post-ischemic reperfusion (see Fig. 15). One possible explanation is suggested by results of a study reported by Zimmer et al. [235] who showed that oxidative stress produced by peroxide challenge can cause stimulation of the NADPH-generating hexose monophosphate shunt in isolated perfused rat hearts. An increased production of NADPH, which is likely to be limiting in the GRD-mediated regeneration of GSH from the disulfide form, might therefore explain the transient increase in antioxidant capacity seen in our study. The enhanced GSH status may be beneficial for scavenging reactive oxidants by redox cycling of myoglobin in the myocardium, thereby protecting against oxidative damage in cardiac muscle [54]. However, this early protective effect showed a striking reversal with increasing duration of ischemia or reperfusion which was characterized by a marked increase in susceptibility of tissue GSH and lipids to oxidation.

The greater sensitivity of GSH to TBHP-induced depletion (when compared with TBHP-induced peroxidation of lipids, see Fig. 7,8) and the higher susceptibility of myocardial GSH to I/R-related alterations (Fig. 9) than that of TBARS formation during the course of ischemia and reperfusion suggest that GSH may play a pivotal role in protecting against tissue oxidative injury. Our demonstration of the high GSH level in liver which is a major site of reactive oxidant production in vivo [304] is consistent with this
hypothesis. This view is further strengthened by results of recent studies by Ceconi et al. [129] who showed that N-acetyl-cysteine pretreatment leading to a preservation of GSH levels in rabbit hearts subjected to ischemia and reperfusion also decreases the extent of cellular damage as characterized by creatine phosphokinase release and impaired mitochondrial function. Moreover, Chatham et al. [236] and Singh et al. [237] have recently demonstrated that the depletion of myocardial GSH by buthionine sulfoximine pretreatment can exacerbate the extent of I/R injury in rats and pigs.

4.3 **Time-Course of Alterations in Myocardial Antioxidant Capacity and Antioxidant Enzyme Activities During Post-Ischemic Reperfusion**

A progressive impairment in myocardial antioxidant capacity was observed in rabbits during the course of post-ischemic reperfusion. The protracted time-course of these changes dissociated them from the early burst of radical formation shown to occur at the onset of post-ischemic reperfusion of the myocardium [78,80]. Mullane et al. [130] have shown that the maximal degree of myocardial necrosis induced by a short period of coronary occlusion in dogs is not attained until several hours after the initiation of reperfusion. This observation and our results, as presented here, suggest that secondary oxidative processes occurring during the prolonged period of post-ischemic reperfusion are
involved in the development of irreversible I/R injury. Oxidants derived from the activated neutrophils that infiltrate the previously ischemic myocardium [182,183] may be one factor in causing such oxidative damage. This is further supported by results obtained in our study using isolated Langendorff-perfused rabbit hearts subjected to ischemia and reperfusion, namely that I/R-induced alterations in myocardial antioxidant capacity seen in intact hearts were not discernible in blood-free-perfused hearts (see Table XII).

The high susceptibility of the myocardium to damage by ischemia and reperfusion and by free radical-generating drugs such as adriamycin [238] may relate, in part, to the relatively low activity of antioxidant enzymes in this tissue [27]. As noted in preliminary studies from our laboratory, a 40 min period of ischemia with 60 min of reperfusion, despite causing irreversible tissue damage, did not produce any detectable changes in the activity of myocardial antioxidant enzymes. The results obtained in the present study, which are in agreement with this early observation, indicate that mean antioxidant enzyme activities in ischemic/reperfused myocardial tissue did not change significantly during the course of post-ischemic reperfusion, when compared with those of non-ischemic or ischemic/non-reperfused tissue (Table V). When the activities of antioxidant enzymes (Cu,Zn-superoxide dismutase (Cu,Zn-SOD) and GRD) were viewed in relation to
functional indices of myocardial antioxidant status in animals undergoing ischemia and reperfusion, significant correlations which suggested functional impairment of antioxidant enzymes became apparent. The use of multiple correlation analyses with a large number of animals has allowed the emergence of information which was not apparent from simple group averaging. The lack of such a correlation for GPX may indicate that its activity in the myocardium is not limiting. In addition, GPX of both myocardial and brain tissue has been reported to be less susceptible than Cu,Zn-SOD to ischemia-induced inactivation [239,240]. While the assignment of cause/consequence relationship to the I/R-induced suppression of antioxidant enzyme activities and alterations in antioxidant capacity still remains to be determined, it seems unlikely, in the absence of any detectable changes in GPX activities, that the suppression of enzyme activities is caused by I/R-induced irreversible tissue necrosis whose effects on enzyme inactivation would likely be non-specific. This is further strengthened by the finding in rat myocardium that while the activity of SOD was decreased immediately following the induction of ischemia, significant reduction in the activity of GPX was not seen until tissue necrosis was apparent [239]. Therefore, changes in Cu,Zn-SOD and GRD activities may precede, rather than accompany, irreversible tissue injury. Finally, a reversible impairment in the functioning of antioxidant enzymes, as suggested by both pH-dependence studies and
correlation analyses, may occur during the early phase of reperfusion when the intracellular pH has not returned to normal. In this regard, the intracellular pH of rabbit myocardium has been reported to decrease from 7.0 to 6.0 after a 30 min period of ischemia [177]. If such transient enzyme inhibition coincided with the burst of oxy-radicals in the early post-ischemic reperfusion phase [78,80], it could be critical in the triggering of irreversible myocardial I/R injury.

4.4 The Effects of Cumulative Brief Episodes of Ischemia on Myocardial Antioxidant Capacity and ATP Levels

Much evidence has accumulated supporting the involvement of oxy-radicals in causing contractile dysfunction in myocardial "stunning" [6]. Pretreatment with oxy-radical scavengers and other antioxidants have consistently been shown to improve post-ischemic dysfunction following a brief period of ischemia [241-246]. However, the protection afforded by oxy-radical scavenger pretreatment is often not associated with any improvement in myocardial high-energy phosphate stores [241-243]. This suggests that the oxy-radical-mediated contractile dysfunction may be independent of high-energy phosphate status in the ischemic myocardium. Our demonstration that the partial depletion of myocardial ATP content after repetitive brief episodes of ischemia was not associated with any detectable changes in myocardial antioxidant
capacity is consistent with this hypothesis. The inability of cells to maintain adequate levels of ATP is a characteristic feature of irreversible damaged cells [247]. The impairment of mitochondrial function and/or the loss of total adenine nucleotides in the cells have been attributed to the failure of ATP resynthesis, thereby causing irreversible cell damage [247-249]. Our results which demonstrated the inability of hearts to restore the normal ATP levels even after one brief episode of ischemia might be attributable to the ischemia-induced mitochondrial dysfunction and adenine nucleotide depletion. The absence of detectable changes in myocardial antioxidant capacity following 4 cycles of brief episodes of ischemia suggest that the depletion of ATP (to 40% of the control level) by ischemia could not trigger irreversible tissue damage. However, reperfusion following a prolonged period of ischemia (40 min) which reduced tissue ATP level by 75% could lead to irreversible damage [50]. It has also been shown in isolated rat hearts that the abrupt reduction in ATP levels (to 40% of control values) following a 20 min period of global ischemia was associated with depletion of adenine nucleotides which paralleled the onset of irreversible damage [247]. Since the preservation of adenine nucleotides arising from ischemia-induced ATP degradation is important for post-ischemic ATP resynthesis [254], the failure of repetitive brief episodes of ischemia, but not the prolonged period of ischemia and reperfusion, to produce
irreversible tissue damage might be related to the severity of adenine nucleotide depletion in the ischemic tissue. An extended period of ischemia and reperfusion likely resulted in a greater degree of tissue wash-out of adenine nucleotides, thereby causing irreversible injury. The increased stability of GSH to TBHP-induced depletion (Table VIII) was probably caused by oxidative stress arising from one brief period of ischemia. This concurs with results obtained in studies involving rabbits subjected to 5 min period of ischemia with 60 min of reperfusion (Fig. 9) or a 40 min period of ischemia followed by 5 min of reperfusion (Fig. 14). In both cases, a stimulation of the hexose monophosphate shunt in response to oxidative stress may be involved.

Although the repetitive brief episodes of ischemia decreased myocardial ATP levels, the severity of subsequently induced I/R injury, as indicated by the increased tissue susceptibility to TBHP-induced GSH depletion and formation of TBARS, did not appear to be altered by this ischemic preconditioning process (Table X). These results suggest that changes in myocardial antioxidant capacity and ATP levels can occur independently, although ATP depletion usually accompanies the development of irreversible I/R-induced injury [156,247].
4.5 I/R-Induced Alterations in Myocardial Antioxidant Capacity in Isolated Langendorff-perfused Rabbit Hearts

The involvement of neutrophils in the pathogenesis of myocardial I/R injury now seems well established [130,162,183]. Under the influence of chemotactic substances produced from ischemic tissues, neutrophils infiltrate into the ischemic myocardium and become activated [162]. The generation of cytotoxic oxidants and the release of proteolytic enzymes from activated neutrophils can cause damage to the vascular endothelium and to the cardiac myocytes [183]. These processes are greatly intensified when oxygen is reintroduced to the ischemic tissue during the reperfusion phase. In addition to the deleterious effects produced by neutrophil-derived oxidants and proteolytic enzymes, the aggregation of activated neutrophils in myocardial capillaries can impair coronary blood flow, thereby further exacerbating the ischemic injury and resulting in the "no-reflow" phenomenon after the initiation of reperfusion [162]. Results obtained in the present study using isolated Langendorff-perfused rabbit hearts subjected to ischemia and reperfusion suggest that blood elements, possibly activated neutrophils, may be a crucial factor in causing I/R-related oxidative injury in the myocardium. Although the antioxidant capacity was depressed in buffer-perfused hearts (as indicated by the decrease in tissue GSH levels and an enhanced susceptibility to GSH depletion), manifestations of oxidative injury
(i.e., increased susceptibility to TBHP-induced GSH depletion and formation of TBARS) were not observed following a 40 min period of ischemia with 60 min of reperfusion under these neutrophil-free perfusion conditions (Table XII).

When the induction of myocardial ischemia was performed in vivo (i.e., with blood components present in the ischemic tissue), reperfusion with buffer solution could also cause oxidative injury, as indicated by an enhancement in susceptibility to TBHP-induced formation of TBARS, although the extent of oxidative damage was less than that observed in animals undergoing both ischemia and reperfusion in vivo (Table XII). This suggests that oxy-radical-mediated processes involved in the development of I/R injury may, at least in part, be initiated by oxidants produced from activated neutrophils which accumulate in the ischemic tissue. In this regard, influx of neutrophils into the myocardium has been observed after a 45 min to 3 hour period of transient coronary occlusion in the canine myocardium [305,306].

4.6 The Effects of Allopurinol Pretreatment on Myocardial I/R Injury

The I/R-induced impairment in myocardial antioxidant capacity was attenuated by a chronic allopurinol treatment regimen previously shown to prevent ultrastructural and biochemical alterations in ischemic/reperfused rabbit
myocardium [155,156]. In contrast to N-acetyl-cysteine [129], which can act as a direct precursor in cellular glutathione synthesis, the protective actions of allopurinol, as described here, were not associated with any significant effect on the depressed GSH content of myocardial tissues following ischemia and reperfusion (data not shown). This result differs from that reported by Peterson et. al. [154] who showed that the markedly reduced myocardial GSH levels in dogs subjected to coronary artery ligation (60 min) followed by reperfusion (30 or 120 min) are fully restored to control levels after 120 min of reperfusion in animals treated orally with allopurinol (50 mg/kg) for 2 days prior to the experiment and as an intravenous infusion beginning 30 min prior to coronary occlusion. Although a direct comparison between the present study and the foregoing one by Peterson et. al. [154] is precluded by the multiplicity of differences in experimental conditions, an effect of chronic allopurinol treatment on myocardial antioxidant capacity is apparent in both cases.

While the exact role of antioxidant enzymes in the development of myocardial I/R injury is yet to be determined, the reperfusion time-course studies suggest the functionally relevant impairment in myocardial Cu,Zn-SOD and GRD activities during the course of post-ischemic reperfusion. The protection against myocardial I/R injury afforded by exogenously administered SOD and CAT [73,146] also indicates that the activity of these enzymes may be
inadequate under conditions of increased oxidative stress. In this regard, the ability of certain substances such as acetyl-homocysteine-thiolactone [251], the antioxidant 6,6'-methylene-bis 2,2'-dimethyl-4-methane sulfonic acid sodium 1,2-dihydroquinoline [252] and bacterial endotoxin [253] to protect tissues against oxidative injury has been attributed to the enhancement or preservation of antioxidant enzyme activities. Our finding that myocardial GRD activity was increased by a chronic allopurinol treatment regimen which can reduce the extent of I/R injury suggests that such a mechanism might be involved in allopurinol protection against myocardial I/R injury.

The protection afforded by allopurinol pretreatment in ischemic/reperfused rabbit myocardium was not associated with any detectable preservation of tissue ATP levels (Fig. 13). The ATP-sparing effect of allopurinol observed in some models of I/R injury has been attributed to a reduction in the degradation of nucleotide precursors, thereby allowing their more efficient reutilization for ATP synthesis [162,254]. However, the rapid tissue wash-out of these ATP degradation products casts considerable doubt on this possibility [255]. Furthermore, the independence of allopurinol protective effects and alterations in tissue ATP content has been repeatedly demonstrated [242,256]. This is further strengthened by our findings in rabbits subjected to brief episodes of myocardial ischemia that I/R-induced alterations in myocardial antioxidant capacity were not
influenced by substantial depletion of tissue ATP levels. It, therefore, seems unlikely that the protection against I/R injury afforded by allopurinol treatment is directly related to processes involved in ATP homeostasis.

4.7 The Effects of Acute U74006F Treatment on I/R-Induced Alterations in Rabbit Myocardium

Although the marked manifestations of myocardial I/R injury appear during the post-ischemic reperfusion phase (see Fig. 9,10), acutely administered protective agents often must be present throughout the ischemic phase in order to be maximally effective [129,257]. In the present study, U74006F was found to abolish I/R-induced inactivation of mitochondrial ATPase when injected shortly before the onset of myocardial reperfusion (Table IV). Although some indication of cytoprotection was also seen when U74006F was administered prior to coronary artery ligation, this did not attain statistical significance. The decreased effectiveness of U74006F when given early in the experimental period could relate, at least in part, to its rapid rate of hepatic metabolism [258].

The beneficial effects of U74006F on mitochondrial integrity were not associated with any detectable increase in tissue ATP levels. Similar results were observed from previous studies in our laboratory involving membrane-active agents such as the verapamil derivative D-600, propranolol and halothane [216,259]. A recent report by Simidzhiev et.
al. [260] has shown that short-chain derivatives of α-tocopherol are able to stimulate mitochondrial ATPase activity and uncouple oxidative phosphorylation by membrane modifying actions that are unrelated to their antioxidant capacity. When taken together, these findings suggest that U74006F may have prevented I/R-induced decreases in mitochondrial ATPase activity by membrane perturbational effects rather than by virtue of its well documented radical scavenging properties.

The absence of detectable effects of U74006F on direct manifestations of myocardial oxidative injury, namely the depletion of tissue GSH and the increased susceptibility to in vitro oxidative challenge, was somewhat unexpected. The intravenous dose of U74006F used (3 mg/kg) was comparable to or greater than doses previously shown to afford significant protection in experimental models of concussive injury to the head [166] or spinal cord [167], subarachnoid hemorrhage and associated cerebral vasospasm [168,261] as well as in global cerebral ischemia [262]. The foregoing studies demonstrating beneficial effects of U74006F have been performed in several different types of animals, including rabbits [261], and using a variety of anesthetic agents, including pentobarbital [262]. It is therefore somewhat unlikely that the negative results reported here can be attributed to our choice of the pentobarbital-anesthetized rabbit as experimental model. Rather, our findings may be an indication that responsiveness to U74006F treatment is
critically dependent on the particular type of injury involved.

U74006F has been found most effective in reducing the extent of CNS injury induced by physical trauma in which ischemia is only a secondary consequence in association with vasospastic responses to injury. It has been suggested that trauma-induced hemorrhage in the CNS favours hemoglobin-catalysed production of reactive oxidants with resulting injury to vascular endothelial cells [263]. Given the intrinsic radical generating capability of these cells [264] and the relative deficiency of radical scavenging systems in the cerebrospinal fluid [265], necrosis would spread rapidly in an autocatalytic fashion. The fact that the probable initiating stimulus, namely radical attack on the endothelium, is extracellular in origin may account for the relatively high susceptibility of such processes to protection by U74006F administered immediately before or shortly after the onset of injury.

The situation with myocardial I/R damage differs from that described above in that both intracellular and extracellular sources of oxygen radical production are likely to be important in the initiation process [266]. Under these conditions, optimal protection by antioxidants might, therefore, require their repeated administration prior to the ischemic insult in order to achieve adequate intracellular levels of drug. This could also explain the need for a chronic treatment regimen rather than acute
administration in order to demonstrate protection against I/R injury by allopurinol (Fig. 11,12 & Table 2).

4.8 The Effects of Allopurinol Pretreatment on the Antioxidant Capacity of Erythrocytes and Functional Viability of Transplanted Lung Tissue

The protective actions of allopurinol in I/R injury have been documented in studies involving a variety of tissues [147-151,156,267]. The results obtained from the present study examining the effects of allopurinol pretreatment in a swine model of heart-lung transplantation indicate that allopurinol treatment produced a beneficial effect on the antioxidant capacity of erythrocytes, as reflected in the reduction in susceptibility of red cells to \textit{in vitro} peroxidative challenge (Fig. 17). These results and the finding that the extent of red cell protection in both donor and recipient animals correlated significantly with the functional viability of the transplanted lung (assessed by tissue water content) suggest that generalized alterations in tissue antioxidant capacity may be discernible from measurements of TBHP-induced formation of TBARS (including MDA) in red cells. Recent studies from our laboratory have shown that rats with chemically-induced diabetes exhibit increased susceptibility of red cell lipids to oxidative damage \textit{in vitre} and marked alterations in tissue antioxidant enzyme activities [268]. This abnormality in red cell antioxidant capacity based on
measurements of TBARS is also demonstrable in clinical diabetes to an extent that parallels the severity of secondary diabetic complications present [269].

It seems unlikely, based on a recent study by Zimmerman et al. [270], that the observed alterations in red cells induced by allopurinol treatment reflect changes in the antioxidant capacity of plasma. These investigators have shown that the antioxidant capacity of extracellular fluids (plasma and intestinal lymph) was unaltered in cats given allopurinol orally for two days at a dose of 50 mg/kg, the same as that used in the present study. The authors suggested that the powerful antioxidant properties of extracellular fluid attributable to the presence of numerous endogenous non-enzymatic scavengers may have precluded the demonstration of any further increase by allopurinol treatment.

If, however, alterations in red cell antioxidant capacity can be taken as a general indication of the situation in tissues, our findings concerning the time/dose dependence of allopurinol-induced protection may have an important bearing on the variability of reported results obtained with this agent in a variety of experimental models of I/R injury. In most investigations documenting protection by allopurinol, the drug was administered for varying periods of time prior to the ischemic episode [153-156, 255]. Treatment with allopurinol beginning 24 hours before the induction of myocardial ischemia in two studies
using dogs has yielded inconsistent results [132,271], while acute administration of allopurinol has usually failed to elicit myocardial protective effects [272-274]. The foregoing experimental results are in general accord with the time-course of allopurinol-induced protection of red cells observed in the present study, further strengthening the view that red cell susceptibility to in vitro oxidative challenge may provide a useful functional measure of generalized alterations in tissue antioxidant status produced by allopurinol and possibly other pharmacological agents. The inter-animal differences in response to allopurinol treatment might involve variability in the absorption of allopurinol following oral administration or in the metabolism of allopurinol which involves xanthine oxidase-mediated oxidation with resultant formation of superoxide radicals. Given that xanthine oxidase activity is also present in plasma [279], an increased oxy-radical production may occur in plasma following oral allopurinol administration with resulting alterations in red cell antioxidant status.

The high sensitivity of lung tissue to I/R-induced damage has greatly curtailed the use of heart-lung transplantation in the management of patients with end-stage pulmonary disease [275]. Several techniques for lung preservation during ischemia have been evaluated, including hypothermic storage, continuous perfusion and autoperfusion [191-193]. However, none of these has been entirely
successful in preventing vulnerable lung tissue from I/R injury following prolonged periods of ischemia.

In contrast to other organ systems, little work has been done to elucidate the mechanism of reperfusion injury in the lung following an episode of ischemia. Preliminary studies in our laboratory indicated that xanthine oxidase activity was undetectable in pig lungs (data not shown), suggesting that xanthine oxidase might not be an important source of radical production in ischemic lung tissue. While the role of xanthine oxidase in the development of tissue damage in pig lung is yet to be determined, it has been shown that xanthine oxidase inhibition by lodoximide can ameliorate I/R injury in isolated rat lungs [276].

The functional integrity of transplanted lungs as assessed in terms of lung water content as well as other indices of pulmonary function, such as arterial blood partial pressure of oxygen (pO₂), alveolar-arterial oxygen gradient (AaG), alveolar-arterial oxygen tension ratio (AaR) and pulmonary vascular resistance (PVR) (data not shown). The clinical usefulness of lung water measurements in the assessment of lung functional integrity has recently been commented upon by Staub [277]. The increase in lung water content, as observed in the present study, was often associated with an increased AaG, a decreased AaR as well as an increased PVR (data not shown). All these changes are suggestive of extensive capillary endothelial damage, possibly due to an increase in oxy-radical activity.
The observed parallelism between the preservation of pulmonary function and the protection of red cells against oxidative challenge suggests that allopurinol may exert beneficial effects by virtue of a generalized effect on tissue antioxidant capacity. This is consistent with our demonstration of allopurinol protection against the impairment in antioxidant capacity which accompanies myocardial I/R injury in open-chest anesthetized rabbits. On the other hand, antioxidant properties of erythrocytes have been shown to be beneficial in protecting against I/R- or oxidant-induced tissue injury [307,312]. Brown et al. [312] have demonstrated that reperfusion with human erythrocytes increased ventricular function and decreased myocardial hydrogen peroxide levels in isolated rat hearts subjected to a 20 min period of normothermic global ischemia. In contrast, reperfusion with erythrocytes that were deprived of catalase activity and/or GSH did not produce any protective effects. Thus, the enhancement of antioxidant capacity of erythrocytes by allopurinol pretreatment may, at least in part, be directly responsible for observed protection against I/R-induced damage in the myocardium and lung.
4.9 *Inhibitory Effects of Allopurinol and Oxypurinol on Transition Metal Ion-Catalysed Ascorbate Oxidation and Lipid Peroxidation*

A number of possible mechanisms other than xanthine oxidase inhibition by allopurinol have been implicated in its protection against I/R injury, including an increased efficiency of ATP salvage [162,163], facilitation of mitochondrial electron transfer [164] and direct inactivation of endogenously formed reactive species, such as hydroxyl radicals or myeloperoxidase-derived hypochlorous acid [165]. One mode of action not previously considered is the chelation of transition metal ions whose catalytic actions in oxy-radical mediated reactions have been shown to be important in the pathogenesis of oxidative damage in ischemic tissue [13,194]. The results of the present study demonstrating the inhibitory actions of allopurinol and its metabolite oxypurinol in the transition metal ion-catalysed oxidation of ascorbic acid and oxidation of erythrocyte membrane lipids suggest that the metal chelating actions of allopurinol and oxypurinol may be relevant to their protective actions against I/R injury.

Transition metal ions catalyse the non-enzymatic oxidation of ascorbate through the intermediacy of redox reactions in which ascorbate oxidation occurs in one-electron steps [225]. Since the mechanism of ascorbate oxidation likely involves formation of an ascorbate-transition metal ion complex preceding intramolecular electron transfer
transition metal chelators would be expected to interfere with this interaction, thereby decreasing the reaction rate. Allopurinol and oxypurinol, as well as the metal chelator EDTA are capable of suppressing the basal oxidation of ascorbate (Fig. 19a), presumably caused by transition metal ion contaminants present in the phosphates and double-distilled water. The stimulatory effects of exogenously added cupric ions on the oxidation of ascorbate were also inhibited by allopurinol in a concentration-dependent manner (Fig. 19b). The use of EDTA to minimize basal oxidation of ascorbate permitted measurement of the relatively modest increase in the rate of ascorbate oxidation induced by ferric ions at micromolar concentrations. Under the assay conditions employed, cupric ions were found to be two-fold more effective than ferric ions in catalysing ascorbate oxidation (Fig. 20). The cupric ion-catalysed reaction was also more susceptible to the inhibitory actions of allopurinol and oxypurinol than that catalysed by ferric ions. A similar trend was observed for uric acid, a compound closely related structurally to allopurinol and oxypurinol.

UV spectral analysis suggested the formation of an allopurinol-ascorbate-copper complex which is unstable in the presence of EDTA (Fig. 21, Table XIV). A similar complexation process has previously been implicated in the inhibition by uric acid of the cupric ion-catalysed oxidation of ascorbate, as reported by Lam et. al. [50].
The absence of similar spectral changes in the allopurinol-ascorbate-ferric chloride mixture presumably indicates that complex formation in this system is much less highly favoured. This is consistent with our observation that ferric chloride is less effective as a catalyst of ascorbate oxidation and its pro-oxidant action is less susceptible to allopurinol inhibition than that of cupric chloride. Although antioxidant enzymes, such as catalase and superoxide dismutase, have been shown to inhibit the cupric ion-catalysed oxidation of ascorbate, their protective effects are more likely attributable to protein binding of cupric ions rather than to scavenging of reactive oxygen-derived radicals [280]. In addition, neither the transition metal ion-catalysed oxidation of ascorbate nor the formation of the allopurinol-ascorbate-copper ion complex is affected by butylated hydroxytoluene (BHT), a lipophilic antioxidant whose radical scavenging activity is also detectable in some aqueous systems, such as the hydroxyl radical-mediated degradation of deoxyribose (data not shown). Moreover, allopurinol and oxypurinol also inhibit the cupric ion-TBHP-catalysed peroxidation of membrane lipids (Fig. 25), presumably by chelating cupric ions, thereby preventing their reaction with hydroperoxides which can generate reactive oxy-radicals [281]. The lack of inhibitory effect on lipid peroxidation induced by TBHP alone (i.e., in the absence of exogenously added transition metal ions) suggests that the actions of allopurinol or oxypurinol are not
mediated by direct free radical scavenging. This is further strengthened by the observation that increasing concentrations of allopurinol or oxypurinol did not cause a greater degree of inhibition of cupric ion-TBHP-induced lipid peroxidation (data not shown). All these seem to suggest that the inhibition by allopurinol of the transition metal ion-mediated processes is more likely attributable to its metal chelating properties rather than to an oxy-radical scavenging action, despite the fact that allopurinol has been shown to be capable of inactivating exogenously and endogenously generated oxy-radicals [157,165].

It is now well established that lipid peroxidation in a variety of systems requires the presence of iron in various catalytically active forms [222,282-285]. With regard to the mechanism of iron-catalysed lipid peroxidation, the nature of the oxidant species responsible for initiating the reaction is still unresolved [282,286,287]. Minotti and Aust [286,287] have proposed that a specific Fe$^{2+}$-O$_2$-Fe$^{3+}$ complex, or at least a 1:1 ratio of Fe$^{2+}$ to Fe$^{3+}$, acts as initiator of peroxidation in liposomal and microsomal systems. Other studies involving the peroxidation of rat brain synaptosomes [222] or rat liver microsomes [283] also support this hypothesis. On the other hand, Aruoma et al. [287] have recently presented evidence arguing against the essential requirement for a Fe$^{2+}$-Fe$^{3+}$ complex or a specific ratio of Fe$^{3+}$ to Fe$^{2+}$ in the initiation of lipid peroxidation. They have shown that the Fe$^{2+}$-dependent
peroxidation of liposomes made from ox-brain phospholipids was stimulated by Al$^{3+}$ and Pb$^{2+}$ in a non-additive manner and these effects were greater than those of Fe$^{3+}$. Nevertheless, the present study suggests the importance of attaining an optimal Fe$^{3+}$/Fe$^{2+}$ ratio in initiating the Fe$^{3+}$-induced lipid peroxidation. Our results indicate that the lag phase preceding the Fe$^{3+}$-stimulated oxidation of erythrocyte membrane lipids (Fig. 23) and the observed decrease in the formation of TBARS at high concentrations of Fe$^{3+}$ (Fig. 22) may both be manifestations of a suboptimal Fe$^{3+}$/Fe$^{2+}$ ratio. When only Fe$^{3+}$ is present in the reaction mixture, reductive processes leading to the attainment of a favourable Fe$^{3+}$/Fe$^{2+}$ ratio would be crucial in facilitating the initiation of lipid peroxidation reactions. In this regard, Minotti and Aust [282] have shown that the increase of Fe$^{2+}$ autoxidation produced by altering the citrate to Fe$^{2+}$ ratio could eliminate the lag phase of the citrate-Fe$^{2+}$-dependent peroxidation of microsomal phospholipids, presumably by facilitating the redox cycling of iron. In addition, the marked stimulatory effects of ascorbic acid and GSH on Fe$^{3+}$-induced lipid peroxidation, as described in the present study (Fig. 23), also agree with a recent finding suggesting a facilitating effect of iron reduction on the enhancement of Fe$^{3+}$-dependent peroxidation of liver microsomal lipids [288]. Superoxide anion radicals derived from GSH autoxidation [289] may lead to the generation of hydroxyl radicals through the iron-catalysed Haber-Weiss
reaction [290], resulting in a stimulatory effect on lipid peroxidation. Moreover, the increase in the maximal level of TBARS attained in ascorbate-stimulated peroxidation may indicate the enhancement of reactions in the propagating phase caused by ascorbate radicals generated from the interaction of Fe$^{3+}$ and ascorbic acid [225].

Allopurinol and oxypurinol, as well as other iron-chelators such as EDTA and uric acid, inhibit the ferric ion-induced oxidation of erythrocyte membrane lipids. Based on the aforementioned hypothesis that an optimal ratio of Fe$^{3+}$ to Fe$^{2+}$ is important for the initiation of iron-dependent lipid peroxidation [222,282], iron chelators may act by changing the redox potential of iron. A higher affinity of Fe$^{3+}$ for lipid regions of membranes than for allopurinol or oxypurinol might explain the weak inhibitory effects of allopurinol and oxypurinol on the Fe$^{3+}$-induced lipid peroxidation in erythrocyte membranes. However, the lipid-soluble antioxidant BHT strongly suppressed this lipid peroxidation. The finding that the inhibitory actions of allopurinol and oxypurinol in the Fe$^{3+}$-induced oxidation of erythrocyte membrane lipids were surmountable by increasing the concentration of Fe$^{3+}$ ions, similar to the results obtained with EDTA (Fig. 24), further suggests the involvement of metal chelation in the actions of allopurinol. Although the inhibitory effect of a low concentration of BHT can be partly overcome by increasing the concentration of Fe$^{3+}$ ions, this is probably due to the
complete depletion of exogenously added BHT as a result of the increasing production of oxidants [291], which eventually leads to a greater extent of lipid peroxidation. Moreover, the ability of BHT to reduce the extent of peroxidation induced by the Cu²⁺-TBHP mixture to a level lower than that produced by TBHP alone further suggests a free radical mode of action.

Under physiological conditions, most of the cellular iron is stored in an inactive (ferric) form in ferritin [94]. Superoxide radicals derived from activated neutrophils [308] or generated by xanthine oxidase [309] are able to release iron from ferritin which can catalyse the peroxidation of lipids [292]. These processes could be important in the mobilization of iron in ischemic tissues in which oxy-radical production is greatly enhanced. On the other hand, cellular copper is mostly incorporated into caeruloplasmin or amino acid complexes [310]. Large quantities of these copper complexes are released from liver as a component of the acute-phase response to a number of disease states, such as infections, arthritis and neoplasias [310]. It is therefore possible that this response also occurs during acute myocardial infarction. Although the copper ion-catalysed generation of hydroxyl radicals is only confined to the site of binding of the copper ions [311], the resulting damage to the binding protein may lead to the release of free copper ions. Thus, during the course of tissue ischemia and reperfusion, the availability of
catalytically active transition metal ions might be increased by a process of decompartmentalization, i.e., mobilization from cellular sites [292,293]. This, in turn, could lead to an enhancement of reactive oxygen radical generation and subsequent amplification of tissue damage.

The effective concentrations of allopurinol or oxypurinol shown in the present study to inhibit the transition metal ion-catalysed reactions are much higher than the plasma concentrations attained following allopurinol treatment in animals during the course of ischemia/reperfusion studies [270]. However, the fact that optimal protection by allopurinol against I/R injury is usually observed only following multiple drug dosing, as reported previously [155,156] and in the present study, may reflect the progressive attainment of tissue allopurinol or oxypurinol levels sufficient to inhibit the transition metal ion-catalysed generation of reactive oxidant species. Our data, therefore, support the hypothesis that the transition metal chelating actions of allopurinol and oxypurinol are relevant to their protective action against I/R injury.

4.10 The Effects of Allopurinol on Myoglobin-TBHP-Catalysed Uric Acid Oxidation and Lipid Peroxidation

The physiological function of myoglobin is generally believed to be concerned with the intracellular transport and storage of oxygen [197,294]. However, the existence of
a substantial intracellular oxygen gradient in myocytes argues against an essential role of myoglobin in the facilitation of oxygen diffusion to mitochondria [295]. Recently, it has been suggested that the redox cycling of myoglobin by ascorbate or GSH may serve as an electron "sink" for protecting against endogenously generated hydroperoxides in muscle [54,55]. Our results, which have demonstrated the strong inhibitory effects of ascorbic acid and GSH on the myoglobin-TBHP-catalysed oxidation of uric acid, are consistent with this hypothesis. The catalytic action of myoglobin in the oxidation of uric acid induced by hydroperoxides has long been known [220], but the physiological implications of this peroxidation reaction are still unclear. The inhibition by uric acid of the myoglobin-TBHP-induced lipid peroxidation, as described in the present study, may suggest the involvement of uric acid in the defense against oxidative damage in muscle. In this regard, a recent study by Becker et al. [296] has demonstrated the beneficial actions of uric acid in isolated guinea pig hearts perfused with uric acid-containing buffer, suggesting that uric acid can serve as a physiological radical scavenger and antioxidant, maintaining functional responsiveness of the coronary system and of the myocardium.

Hydroperoxides oxidize myoglobin, resulting in the production of both alkoxy radicals and ferryl heme oxidants, which are powerful initiators of lipid peroxidation reactions [202,229]. Our results have
demonstrated the stimulatory effect of a myoglobin-TBHP mixture on the peroxidation of erythrocyte membrane lipids (Fig. 27), which was strongly suppressed by the lipid-soluble antioxidant BHT (Fig. 28), presumably by virtue of its free radical chain terminating activity [291]. Allopurinol also slightly inhibited this myoglobin-TBHP-induced lipid peroxidation. The general finding that only those iron chelators with radical scavenging properties inhibit the hemoprotein-dependent lipid peroxidation [297] has suggested an essential role of free radical scavenging in the inhibition by allopurinol. The inhibition by allopurinol or oxypurinol of uric acid oxidation induced by myoglobin-derived oxidants, as shown in the present study (Fig. 26), would be consistent with this hypothesis. In this regard, both allopurinol and oxypurinol have been shown to possess hydroxyl radical scavenging properties in a system measuring the hydroxyl radical-induced degradation of deoxyribose [165]. On the other hand, allopurinol, by virtue of its transition metal chelating properties (shown in the foregoing studies), could possibly interfere with the interaction between heme-iron and hydroperoxide by binding the iron-containing heme prosthetic groups in myoglobin, thereby suppressing the generation of ferryl heme oxidants.

The important role of hydrogen peroxide in causing myocardial I/R injury has been suggested by various studies [124,298]. In this regard, the pathogenic actions of hydrogen peroxide are often attributed to its ability to
generate hydroxyl radicals through transition metal ion-catalysed reactions [84,224,284]. However, hydrogen peroxide produced in the ischemic myocardium would also react with myoglobin which is abundant in heart muscle [197]. During the course of myocardial ischemia and reperfusion, the increased production of hydrogen peroxide [124,298], which likely coincides with the depletion of tissue ascorbic acid and GSH [170,229], could result in the uncontrolled production of ferryl heme oxidants, subsequently leading to tissue damage. Ascorbic acid and its lipophilic derivative 2-octadecylascorbic acid, effective scavengers for myoglobin-derived oxidants [54,55], have been shown to protect against I/R injury in rat and dog hearts [127,131]. The results of the present study which demonstrated the inhibitory effects of allopurinol on myoglobin-hydroperoxide-induced uric acid oxidation and lipid peroxidation suggest that the allopurinol protection against I/R injury may, at least in part, be related to its inhibitory actions in hemoprotein-hydroperoxide oxidative reactions.

4.11 Summary and Conclusions

(1) A marked impairment in myocardial antioxidant capacity developed in association with the onset of irreversible I/R injury in rabbit hearts subjected to periods of
ischemia longer than 20 min followed by 60 min of reperfusion.

(2) Reperfusion of the myocardium after a 40 min period of ischemia resulted in a progressive decrease in myocardial antioxidant capacity. The protracted time-course of myocardial alterations dissociated them from the early burst of radical formation known to occur at the onset of post-ischemic reperfusion of the myocardium.

(3) When the time-dependent changes in functional indices of antioxidant status (TBHP-induced GSH depletion and formation of TBARS) of ischemic/reperfused myocardial tissues were analysed in relation to activities of antioxidant enzymes, evidence suggestive of functionally relevant impairment in Cu,Zn-SOD and GRD activities was found. These results and the demonstration of significant decreases in the activity of GSH-dependent antioxidant enzymes under acidotic conditions suggest that a transient impairment in the functioning of antioxidant enzymes may be involved in triggering irreversible myocardial I/R injury.

(4) Repetitive brief episodes of ischemia and reperfusion did not produce any discernible effects on myocardial antioxidant capacity despite the substantial decrease in tissue ATP levels. Moreover, ischemic
preconditioning produced by repetitive brief episodes of ischemia did not affect the severity of subsequently induced I/R injury. All these suggest that I/R-induced changes in myocardial antioxidant capacity and ATP levels can occur independently, although ATP depletion usually accompanies the development of irreversible I/R-induced injury.

(5) Isolated Langendorff-perfused rabbit hearts subjected to ischemia and reperfusion did not show any changes in myocardial antioxidant capacity, which contrasted with the marked impairment seen in blood-perfused intact hearts. When intact hearts were subjected to ischemia in vivo and then a subsequent reperfusion in vitro (i.e., Langendorff-perfusion), an impairment in myocardial antioxidant capacity became apparent. These suggest that blood elements may be a crucial factor involved in the development of I/R-induced oxidant injury.

(6) Chronic allopurinol pretreatment provided significant protection against I/R-induced alterations in myocardial antioxidant capacity, but not the decrease in tissue ATP levels. The chronic allopurinol regimen was found to enhance the activity of myocardial GRD, which may, at least in part, account for the protection against I/R injury.
Allopurinol and oxypurinol inhibited the transition metal ion-catalysed ascorbate oxidation and lipid peroxidation, likely as a consequence of their metal chelating actions. Similarly, myoglobin-TBHP-catalysed uric acid oxidation and lipid peroxidation were also suppressed by allopurinol. Taken together, the results suggest that allopurinol may favourably alter myocardial antioxidant capacity directly by virtue of its transition metal chelating properties and its antioxidant action in myoglobin-mediated oxidative processes. The finding that acute allopurinol or oxypurinol treatment did not protect against I/R injury suggests that the time-dependence of allopurinol-induced protection may reflect the gradual accumulation in tissues of allopurinol or its active metabolite oxypurinol to levels sufficient to exert antioxidant effects by transition metal chelation or other related antioxidant actions.

The acute administration of the 21-aminosteroid antioxidant U74006F under conditions comparable to those known to protect against trauma-induced damage in the central nervous system failed to reduce manifestations of oxidative injury in rabbit hearts subjected to ischemia and reperfusion. Although reactive free radicals have been implicated in both types of tissue damage, the observed difference in
susceptibility to protection by this steroidal antioxidant suggests that the molecular mechanisms involved are not identical. Given the evidence suggesting that a multiple treatment regimen and heavy metal chelation are important in determining the antioxidant actions of allopurinol, future experiments exploring the possible effects of chronic 21-aminosteroid pretreatment on myocardial susceptibility to I/R injury should probably utilize a compound such as U74500A which is more effective than U74006F as a chelator of iron.

(9) In the heart-lung transplantation study, erythrocytes from allopurinol-treated pigs showed a time/dose-dependent enhancement in antioxidant capacity as reflected in the decrease in MDA production following in vitro oxidative challenge. The extent of red cell protection in both donor and recipient animals correlated significantly with the functional viability of the transplanted lung tissue, as assessed by tissue water content. It is, therefore, suggested that the antioxidant status of red cells may provide a useful assessment of generalized alterations in antioxidant status produced by pharmacological interventions.

(10) Finally, the beneficial actions of allopurinol in various settings of I/R injury has suggested the clinical use of allopurinol in protection of tissues
against I/R damage, given its relatively low toxicity. The results obtained from the present investigation further support this proposal, and clinical studies are currently under way to evaluate the effects of chronic allopurinol pretreatment and the potential predictive value of red cell antioxidant capacity measurements in patients undergoing open-heart surgery.
5. REFERENCES


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