ROLE OF 15-F$_{2}$T-ISOPROSTANE IN THE PATHOGENESIS OF MYOCARDIAL ISCHEMIA-REPERFUSION INJURY: A NOVEL THERAPEUTIC APPROACH TO CARDIOPROTECTION WITH PROPOFOL

By

ZHENGYUAN XIA

M. B., Xian Ning Medical College, 1984
M. Sc., Hubei Medical University, 1991

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Pharmacology & Therapeutics
In Close Association with the Department of Anesthesiology

We accept this thesis as confirming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April 2004
© Zhengyuan Xia, 2004
ABSTRACT

Myocardial ischemia-reperfusion injury (IRI) is a major pathophysiologic factor contributing to post-operative cardiac dysfunction in patients undergoing coronary artery bypass surgery utilizing cardiopulmonary bypass. Reactive oxygen species (ROS)-mediated lipid peroxidation plays a critical role in mediating myocardial IRI. This thesis reports the results of four studies designed to investigate the role of 15-F_2-isoprostane, a reliable measure of lipid peroxidation that has bioactivity, in the pathogenesis of myocardial IRI and to explore the therapeutic potential of propofol.

In the first study we demonstrated for the first time that significant in vivo lipid peroxidation occurs early during myocardial ischemia and continues during reperfusion rather than primarily only during reperfusion in patients undergoing cardiac surgery utilizing cardiopulmonary bypass. The plasma decay patterns of 15-F_2-isoprostane during reperfusion parallel post-operative cardiac functional recovery.

In an in vitro study, a unique therapeutic regimen of propofol was developed to best utilize its antioxidant properties in order to attenuate ROS generation during myocardial ischemia and early reperfusion in isolated rat hearts. Propofol provides better cardiac protection when applied at clinically achievable high concentration before ischemia and during global myocardial ischemia and continued during early reperfusion, followed by a relatively lower concentration during the later phase of reperfusion. Of particular relevance is our identification, using the isolated perfused rat hearts, that 15-F_2-isoprostane is produced in situ during global myocardial ischemia. This finding provides evidence to support the use of antioxidant interventions during ischemia which would target the coronary endothelium and/or the cardiomyocytes.
A third study investigated whether or not aging could be a factor that adversely affects the cardiac protective effect of propofol on myocardial IRI. The results showed that propofol equally preserved myocardial endogenous antioxidant capacity in the young and middle-aged rat hearts and, more significantly, enhanced post-ischemic myocardial functional recovery in the middle-aged rat hearts relative to that in the young rat hearts. This finding provides evidence to support the notion that drug(s) with antioxidant properties (such as propofol) could be more effective in attenuating myocardial IRI in populations suffering from insufficient or decreased endogenous antioxidant capacity, such as the elderly. In our study, we identified a strong inverse correlation between myocardial 15-F₂-isoprostane levels and post-ischemic cardiac function in the isolated rat heart, suggesting 15-F₂-isoprostane itself could be a factor mediating myocardial IRI.

In the last study, we further explored whether 15-F₂-isoprostane can directly mediate myocardial IRI and if 15-F₂-isoprostane antagonism could be a potential adjunct therapy. We found that 15-F₂-isoprostane exacerbated myocardial IRI as evidenced by an increased myocardial infarct size, cellular damage and reduced post-ischemic myocardial function. 15-F₂-isoprostane antagonism abolished its deleterious effects. We also found evidence that 15-F₂-isoprostane may mediate myocardial IRI, at least in part, by increasing ET-1 production during later reperfusion.

It is hoped that the studies described in the thesis have enhanced knowledge concerning the role of 15-F₂-isoprostane in the pathogenesis of myocardial IRI, from its mechanism(s) of action to its clinical relevance. It is hoped that our findings can aid in the development of novel and effective therapeutic interventions against myocardial ischemia-reperfusion injury.
TABLE OF CONTENTS

Abstract...............................................................................................................................ii
TABLE OF CONTENTS........................................................................................................iv
List of Tables......................................................................................................................viii
List of Figures....................................................................................................................ix
Acknowledgements...........................................................................................................xii
Dedication...........................................................................................................................xiii

1. Chapter 1.........................................................................................................................1
   Literature Review.............................................................................................................1
   1.1. Ischemic Heart Disease — A Leading Cause Of Death In North America.............1
       1.1.1. Advantages And Limitations Of Current Therapeutic Interventions ..........1
       1.1.2. Sustained Significant Morbidity And Mortality........................................2
   1.2. Myocardial Ischemia-Reperfusion Injury (IRI).......................................................3
       1.2.1. Myocardial Ischemia..................................................................................3
       1.2.2. Myocardial Reperfusion: The Role Of Reactive Oxygen Species (ROS) In
             Mediating Myocardial IRI.............................................................................5
       1.2.3. ROS-Mediated Lipid Peroxidation: Available Measures And Their
             Limitations......................................................................................................7
   1.3. F2-Isoprostanes — A Reliable Index Of Endogenous Lipid Peroxidation.......8
       1.3.1. Mechanism Of F2-Isoprostane Formation..................................................8
       1.3.2. 15-F2t-Isoprostane — A Marker And Mediator Of Oxidant Injury............10
       1.3.3. Bioactivity Of 15-F2t-Isoprostane (15-F2t-IsoP).........................................12
       1.3.4. Methods Of Measurement Of 15-F2t-Isoprostane.....................................14
       1.3.5. 15-F2t-Isoprostane Metabolism..................................................................16
       1.3.6. Evidence For A Unique 15-F2t-Isoprostane Receptor.................................17
       1.3.7. 15-F2t-Isoprostane In Myocardial IRI........................................................18
   1.4. Antioxidant Interventions Against Myocardial IRI.............................................19
       1.4.1. Antioxidant Vitamins C And E..................................................................19
       1.4.2. Allopurinol.................................................................................................20
       1.4.3. N-Acetylcysteine.......................................................................................21
       1.4.4. Propofol....................................................................................................21
   1.5. Protective Effects And Limitations Of Ischemic Preconditioning On
       Myocardial IRI.......................................................................................................22
   1.6. Anesthesia And Myocardial IRI............................................................................23
       1.6.1. Inhalational Anesthesia.............................................................................24
       1.6.2. Intravenous Anesthesia.............................................................................25
### 3.4. Results

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.1</td>
<td>15-F_{2t}-Isop Generation During Ischemia-Reperfusion</td>
<td>65</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Tissue Antioxidant Capacity</td>
<td>65</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Contracture Development During Ischemia</td>
<td>70</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Functional Response To Ischemia –Reperfusion</td>
<td>70</td>
</tr>
<tr>
<td>3.4.5</td>
<td>Coronary Perfusion Pressure</td>
<td>72</td>
</tr>
<tr>
<td>3.4.6</td>
<td>Lipid Peroxidation And Post-Ischemic Myocardial Function</td>
<td>75</td>
</tr>
<tr>
<td>3.4.7</td>
<td>Pre-Ischemic Myocardial Depression And Post-Ischemic Myocardial Function</td>
<td>78</td>
</tr>
</tbody>
</table>

### 3.5. Discussion

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>78</td>
</tr>
</tbody>
</table>

### 3.6. Conclusion

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>82</td>
</tr>
</tbody>
</table>

### 4. CHAPTER 4

**Propofol Effects On Ischemic Tolerance Of Middle-Aged Rat Hearts: Effects Of 15-F_{2t}-Isop Formation And Tissue Antioxidant Capacity**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Preface</td>
<td>84</td>
</tr>
<tr>
<td>4.2</td>
<td>Introduction</td>
<td>84</td>
</tr>
<tr>
<td>4.3</td>
<td>Materials And Methods</td>
<td>86</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Heart Perfusion</td>
<td>86</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Experimental Protocol</td>
<td>87</td>
</tr>
<tr>
<td>4.3.3</td>
<td>15-F_{2t}-Isop Assays</td>
<td>88</td>
</tr>
<tr>
<td>4.3.4</td>
<td>Heart Tissue Antioxidant Capacity Determination</td>
<td>89</td>
</tr>
<tr>
<td>4.3.5</td>
<td>Data Analysis</td>
<td>90</td>
</tr>
<tr>
<td>4.4</td>
<td>Results</td>
<td>90</td>
</tr>
<tr>
<td>4.4.1</td>
<td>15-F_{2t}-Isop Generation During Ischemia-Reperfusion</td>
<td>90</td>
</tr>
<tr>
<td>4.4.2</td>
<td>Tissue Antioxidant Capacity</td>
<td>91</td>
</tr>
<tr>
<td>4.4.3</td>
<td>Effects Of Propofol On Contracture Development During Ischemia And Reperfusion</td>
<td>94</td>
</tr>
<tr>
<td>4.4.4</td>
<td>Coronary Perfusion Pressure</td>
<td>97</td>
</tr>
<tr>
<td>4.4.5</td>
<td>Left Ventricular Mechanics</td>
<td>97</td>
</tr>
<tr>
<td>4.4.6</td>
<td>Correlation Analysis</td>
<td>98</td>
</tr>
<tr>
<td>4.5</td>
<td>Discussion</td>
<td>103</td>
</tr>
<tr>
<td>4.6</td>
<td>Conclusion</td>
<td>109</td>
</tr>
</tbody>
</table>

### 5. CHAPTER 5

**Effects of 15-F_{2t}-isoP on myocardial IRI in isolated rat hearts: potential mechanism of 15-F_{2t}-isoP action**

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
</tr>
</tbody>
</table>
5.1. Preface ........................................................................................................... 110
5.2. Introduction ................................................................................................. 110
5.3. Materials and methods ................................................................................ 113
  5.3.1. Heart preparation .................................................................................. 113
  5.3.2. Experimental Protocol ....................................................................... 114
  5.3.3. Measurement of Endothelin-1 ............................................................. 115
  5.3.4. Measurement of CK-MB ..................................................................... 117
  5.3.5. 15-F$_2$-IsoP Assays ........................................................................... 117
  5.3.6. Myocardial Infarct Size Measurement .................................................. 118
  5.3.7. Statistical Analysis .............................................................................. 118
5.4. Results .......................................................................................................... 118
  5.4.1. Endothelin-1 Release and its Relation with 15-F$_2$-IsoP .................... 118
  5.4.2. 15-F$_2$-IsoP Generation During Ischemia-reperfusion ....................... 119
  5.4.3. CK-MB Release During Ischemia-reperfusion .................................... 122
  5.4.4. Contracture Development during Ischemia .......................................... 126
  5.4.5. Functional Response to Ischemia-reperfusion ..................................... 130
  5.4.6. Coronary Perfusion Pressure ............................................................... 130
  5.4.7. Myocardial Infarct Size ......................................................................
5.5. Discussion ..................................................................................................... 131
5.6 Conclusion ...................................................................................................... 135
6. CHAPTER 6 ...................................................................................................... 136
  General summary and conclusions ................................................................. 136
  6.1. Summary .................................................................................................... 136
  6.2. Conclusions ............................................................................................... 140
  6.3. Future directions for research .................................................................. 141
References .......................................................................................................... 144
Appendix I: ........................................................................................................... 192
  Propofol inhibition of TNF-alpha- induced vascular endothelial cell apoptosis:
  effects on Bcl-2 and Bax protein expression .................................................. 192
LIST OF TABLES

Table 2.1. Patients demographic and perioperative data .................................................44

Table 2.2. Cardioplegic perfusion data for cardiac surgery patients who do not need
inotrope or those who need ≥2 inotropes support post-operatively.........................45

Table 2.3. Plasma free 15-F_2-isoprostane assay precision analysis.................................46

Table 3.1. Changes of left ventricular developed pressure (LVDP), LV systolic
pressure (LVSP) and coronary perfusion pressure (CPP) (mm hg) during
myocardial ischemia and reperfusion.................................................................73

Table 4.1. Variations of coronary perfusion pressure (CPP) (mm Hg) of the
ischemic-reperfused rat hearts .................................................................................99
LIST OF FIGURES

Figure 1.1. 15-F_2t-isoprostane formation pathway.................................................................9

Figure 1.2. Molecular structures of 2,6-diisopropylphenol (propofol) and alphatocopherol (vitamin E)...................................................................................................................27

Figure 2.1. Changes of plasma free 15-F_2t-Isoprostane during ischemia-reperfusion in patients undergoing coronary artery bypass graft surgery utilizing cardiopulmonary bypass.................................................................48

Figure 2.2. Changes of plasma free 15-F_2t-Isoprostane during ischemia-reperfusion in patients who do not need inotrope or those who need ≥ 2 inotropes support post-operatively.................................................................49

Figure 2.3. Plasma free 15-F_2t-Isoprostane in the non-inotrope group decays exponentially after global myocardial ischemia-reperfusion.................................................................50

Figure 2.4. Correlation between post-operative cardiac index (CI) and changes of plasma free 15-F_2t-Isoprostane during early reperfusion.................................................................51

Figure 3.1. 15-F_2t-isoprostane release during ischemia and reperfusion...............................67

Figure 3.2. Formation of thiobarbituric acid reactive substances (TBARS), a measure of tissue antioxidant capacity, in heart tissues (represented as absorbance at 532 nm) in the presence of 1 mM t-butylhydroperoxide...........................................68

Figure 3.3-A. Effect of Propofol on left ventricular end-diastolic pressure (LVEDP), reflecting myocardial contracture (ventricular stiffness), during myocardial ischemia........................................................................................................69

Figure 3.3-B. Effect of Propofol on left ventricular end-diastolic pressure (LVEDP), during postischemic reperfusion........................................................................................................69

Figure 3.4. Relationship between 15-F_2t-IsoP generation upon reperfusion and coronary perfusion pressure (CPP) at 90 minutes of reperfusion........................................74

Figure 3.5. Correlation between the recovery of left ventricular developed pressure (LVDP) after 90 min of reperfusion and the formation of heart tissue thiobarbituric acid reactive substances (TBARS), a measure of tissue antioxidant capacity........................................................................................................76
Figure 3.6. Correlation between the changes of left ventricular developed pressure (LVDP) after reperfusion (from reperfusion 60 to 90 min) and the formation of heart tissue thiobarbituric acid reactive substances (TBARS) in the presence of 1 mM t-butylhydroperoxide.

Figure 4.1-A. Coronary effluent 15-F_2t-isoprostane release during reperfusion of the ischemic-reperfused young and middle-age rat hearts.

Figure 4.1-B. Coronary effluent 15-F_2t-isoprostane release during ischemia of the ischemic-reperfused young and middle-age rat hearts.

Figure 4.2. Thiobarbituric acid reactive substances (TBARS) formation (as reflected by absorbance at 532 nm) as a function of t-BHP concentration for heart tissues at reperfusion 90 min.

Figure 4.3. Left ventricular end diastolic pressure (LVEDP), reflecting myocardial contracture during ischemia of the ischemic-reperfused young and middle-age rat hearts.

Figure 4.4. Left ventricular end diastolic pressure (LVEDP), reflecting myocardial contracture during reperfusion following 40 min of global ischemia of the ischemic-reperfused young and middle-age rat hearts.

Figure 4.5. Post-ischemic Left ventricular developed pressure (LVDP).

Figure 4.6. Correlation between coronary effluent 15-F_2t-isoprostane release during ischemia and heart tissue antioxidant capacity. Heart tissue was sampled after 40 min of ischemia and 90 min of reperfusion.

Figure 4.7. Correlation between left ventricular developed pressure (LVDP) at 90 min of reperfusion (Re-90) and 15-F_2t-isoprostane release during the first 30 min of ischemia.

Figure 5.1-A. Coronary Effluent Endothelin-1 (ET-1) concentrations during myocardial ischemia-reperfusion.

Figure 5.1-B. Relationship between 15-F_2t-isoprostane (15-F_2t-isoP) and ET-1 concentration during the first 30 min of ischemia in the untreated control group.

Figure 5.2. Effect of SQ 29548 (SQ) on 15-F_2t-isoprostane (15-F_2t-isoP) release during myocardial ischemia-reperfusion.

Figure 5.3. Coronary effluent CK-MB concentration during myocardial ischemia-reperfusion.
Figure 5.4-A. Development of left ventricular end-diastolic pressure (LVEDP), reflecting myocardial contracture, during ischemia.......................124

Figure 5.4-B. Ischemic contracture onset time during myocardial ischemia........124

Figure 5.5. Variations of left ventricular end-diastolic pressure (LVEDP), reflecting myocardial stiffness, during post-ischemic reperfusion.................125

Figure 5.6. Recovery of left ventricular developed pressure (LVDP), reflecting effective myocardial contractility, during reperfusion......................128

Figure 5.7. Myocardial infarct size after 60 min of reperfusion following 40 min of ischemia.................................................................129

Figure A-1. Tumor necrosis factor (TNF) -alpha-induced apoptotic cell death in cultured human umbilical vein endothelial cells (HUVECs) measured by TUNEL staining...............................................................201

Figure A-2. The expression of anti-apoptotic Bcl-2 protein evaluated by immunoperoxidase technique.........................................................202

Figure A-3. The expression of pro-apoptotic Bax protein evaluated by immunoperoxidase technique.........................................................203

Figure A- 4-A. Changes of Bcl-2/Bax ratio of cultured HUVECs after TNF-alpha stimulation and propofol treatment...........................................204

Figure A- 4-B. Inverse correlation between endothelial cell apoptotic index (AI) and the ratio of bcl-2 over Bax proteins expression.........................204

Figure A-5. Nitric oxide (NO) concentration in the culture medium..............205

Figure A-6. Representative electron microscopy of endothelial cells (HUVECs) apoptotic conformational changes.......................................206

Figure A-7. H2O2 and TNF-alpha synergistically induced apoptotic cell death in cultured HUVECs...............................................................208

Figure A-8-A. Effects of H2O2 on TNF-mediated changes in Bcl-2 expression...210

Figure A-8-B. Effects of H2O2 on TNF-mediated changes in Bax (B) expression...210
ACKNOWLEDGEMENTS

I would like to express my gratitude to the many individuals who assisted me during the course of this study. In particular, I would like to acknowledge the Centre for Anesthesia & Analgesia, Department of Pharmacology & Therapeutics for the use of office and laboratory facilities and for the support of my study through Dr. Jean Templeton Hugill funding.

I would like to express my sincere gratitude to my supervisors Dr. David Ansley and Dr. David Godin for teaching me an incredible amount about both science and life. I am grateful for their never-ending support and encouragement throughout the course of my studies. Their friendship has made my years at U.B.C. most memorable. I would like to thank Dr. Ernie Puil and Dr. Karim Qayumi for giving me the opportunity to join their laboratory group meetings and for their teaching and support. I would also like to thank Dr. Michael Walker and Dr. Thomas Chang for allowing me to use their laboratory facilities and for their teaching.
DEDICATION

To my wife Lihui and daughter Weiyi

who continually provide inspiration.
CHAPTER 1

LITERATURE REVIEW

1.1. Ischemic heart disease — A Leading Cause Of Death In North America

Cardiac disease remains the leading cause of death for men and women in North America. Ischemic heart disease accounts for more than one in five cardiac-related deaths, annually.\(^1\) Consequently, much effort has been made with the aim of optimising conditions to effectively re-establish the blood perfusion (reperfusion) to the ischemic myocardium in order to salvage tissue at risk of irreversible damage.

1.1.1. Advantage And Limitations Of Current Therapeutic Interventions

The advent of treatments such as angioplasty, thrombolysis and coronary artery bypass grafting have improved the overall survival of individuals receiving treatment within the first few hours of a myocardial infarction. However, the mortality rate is increased within the first 24 hours following the onset of reperfusion compared to the situation if no reperfusion therapy is given.\(^2\) This may be attributable to the extension of subsequent myocardial damage and the “no-flow” phenomenon during reperfusion.\(^3; 4; 5\)

Intravenous thrombolytic therapy is the standard approach for managing patients with a cute myocardial infarction, based upon its widespread availability and ability to reduce patient mortality as demonstrated in randomised trials. Despite its proven efficacy,
thrombolytic therapy has limitations. Many patients are ineligible for treatment with thrombolytics. Of those given thrombolytic therapy, 10 to 15 percent have persistent occlusion or reocclusion of the infarct-related artery.

Recent studies have shown that angioplasty provides a short-term clinical advantage over thrombolysis which may not be sustained. Therefore, for patients with acute ST-segment elevation acute myocardial infarction (AMI) and contraindications to thrombolytics, coronary artery bypass grafting surgery is likely a better choice for therapeutic management. In fact, the rate of coronary artery bypass grafting (CABG) surgery has more than quadrupled in the last 20 years in Canada.

1.1.2. Sustained Significant Morbidity And Mortality

Despite advances in modern surgical and anesthetic techniques, the risks of morbidity and mortality associated with CABG surgery are significant since physicians encounter, with increasing frequency, patients who are elderly, suffering from increased disease severity, and concomitant medical illnesses.

Myocardial ischemia-reperfusion injury (IRI) is a major pathophysiologic factor in the high mortality rate from diseases of the cardiovascular system. Myocardial IRI contributes to early postoperative myocardial ischemia and dysfunction, two factors associated with increased morbidity, and prolonged intensive care unit (ICU) stay that have not been significantly reduced by current forms of therapy.
Myocardial IRI is a complex phenomenon, the mechanisms of which are incompletely understood. It is, however, well accepted that lipid peroxidation mediated by reactive oxygen species (ROS) plays a critical role in myocardial IRI.\textsuperscript{18-27}

1.2. Myocardial Ischemia-Reperfusion Injury (IRI)

1.2.1. Myocardial Ischemia

Myocardial ischemia and infarction are typically caused by a substantial reduction or complete interruption of regional blood supply following occlusion of its coronary artery. During CABG surgery utilizing cardiopulmonary bypass (CPB), the whole heart is subjected to ischemia (global myocardial ischemia) during the period of aortic cross-clamping. This deprives the heart of oxygen and metabolic nutrients essential for the maintenance of myocardial tissue integrity.

As with most living tissues, heart muscle harnesses and utilizes the energy in the form of adenosine triphosphate (ATP). Energy transfer within the cardiac myocyte is mediated through a series of interconnected cycles. The consumption of ATP: (1) provides the energy for the cross-bridge cycle of contractile elements; (2) decreases the proton gradient across the inner mitochondrial membrane (which drives ATP synthesis); (3) increases the oxidation of NADH; (4) increases flux through the Krebs cycle (the major source of reducing equivalents); (5) increases acetyl-CoA use; and finally (6) increases substrate consumption.
The major source of ATP in heart muscle is via phosphorylation of ADP coupled to respiratory chain activity. The heart’s content of ATP is normally about 20 μmol/g dry weight. At an oxygen consumption rate of about 80 μmol/min/g dry weight and a phosphorus/oxygen ratio of approximately 2.5, heart muscle replenishes about 33 μmol of ATP/g dry weight each minute. Therefore, ATP must be re-synthesized as quickly as it is broken down. Without continuous replenishment, the intracellular stores of ATP would be expected to be exhausted within <1 minute.

The deprivation of oxygen during myocardial ischemia interrupts mitochondrial oxidative phosphorylation, thereby compromising the supply of ATP. When cellular ATP is depleted during ischemia, the energy deficiency leads to the disruption of normal ion gradients (e.g. involving sodium, calcium, potassium and the hydrogen ion), resulting in intracellular edema and morphological alterations. Disruption of the plasma membrane integrity causes further changes in ionic gradients by a massive influx of sodium and efflux of potassium. The loss of the ion concentration gradients and the resultant cellular depolarization activate voltage-gated calcium channels, resulting in a massive uncontrolled calcium influx. The subsequent cytosolic calcium overload leads to the activation of intracellular Ca\(^{2+}\)-dependent proteases and phospholipases, resulting in extensive cardiomyocyte cellular damage, and ultimately irreversible necrosis.

Re-establishment of blood flow (i.e. reperfusion) to the ischemic myocardium is necessary in order to salvage dying cardiomyocytes.
1.2.2. Myocardial Reperfusion: The Role Of Reactive Oxygen Species (ROS) In Mediating Myocardial IRI

Reperfusion of the ischemic myocardium initiates two disparate types of cellular processes in the previously ischemic tissue as a result of oxygen re-introduction. Upon reperfusion, cells adjust to the reintroduction of molecular oxygen, and cellular repair begins. Paradoxically, however, in the meantime, a second mechanism triggers the immediate over-production of reactive oxygen species (ROS), which causes further cell injury, referred to as "ischemia-reperfusion injury" (IRI).

Reactive oxygen species are molecules or fragments of molecules containing unpaired electrons in their outermost orbits. Unpaired electrons tend to acquire an electron to form a pair; therefore, most ROS are highly chemically reactive and, as a result, short-lived. Important ROS in biological systems include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH), and peroxynitrite (ONOO$^-$), the product of nitric oxide (NO) combining with O$_2^-$. 33-38

The release of high levels of ROS associated with reperfusion can trigger lipid peroxidation of the unsaturated phospholipid components of cellular membranes, which is a crucial event determining the onset of irreversible cellular necrosis in the ischemic-reperfused tissue. 39; 40 Lipid peroxidation and the subsequent activation of phospholipases can initiate the formation and release of inflammatory mediators such as tumor necrosis factor-alpha, thromboxanes, leukotrienes and platelet activating factor, which can
adversely affect hemodynamic homeostasis and promote vascular endothelial cell and cardiomyocyte apoptotic cell death during reperfusion.

Previous studies have shown that during cardiac surgery utilizing cardiopulmonary bypass, ROS are generated during myocardial reperfusion, and these are important contributors to tissue injury. Moreover, a number of recent studies, including our own, have shown that profound systemic oxidative stress and lipid peroxidation occur before as well as during myocardial ischemia shortly after the onset of CPB in humans. This is likely due to the activation of leukocytes which subsequently release substantial amounts of cytotoxic ROS. Superoxide and NO, when present in equimolar concentrations, can combine to form ONOO⁻, a highly reactive and injurious free radical.

Myocardial antioxidant enzymes, including glutathione reductase, superoxide dismutase (SOD), and catalase, are activated in proportion to the degree of myocardial IRI. Host antioxidants can become depleted during CPB, presumably as a result of consumption by ROS. When ROS production exceeds host defense scavenging capacity, cellular injury results. An inverse correlation has been identified between preoperative total plasma antioxidant capacity and lipid peroxidation, the latter being an index of myocardial cellular injury. Furthermore, post-CPB coronary endothelial cell dysfunction appears to be partially mediated by ROS. This is of significance. A recent study has shown that apoptosis of endothelial cells precedes myocyte cell apoptosis in ischemia-reperfusion injury. This suggests that reperfusion induces the release of
soluble pro-apoptotic mediators (including ROS) from endothelial cells that promote myocyte apoptotic cell death.

1.2.3. ROS-Mediated Lipid Peroxidation: Available Measures And Their Limitations

The detection and measurement of lipid peroxidation has been most frequently used to support the involvement of the ROS reactions in pathophysiologic processes. Furthermore, these indices have been the basis for the development and use of antioxidant interventions to prevent oxidative injury.

Part of the difficulty in determining the role of oxidant stress in human disease has been the lack of a sensitive and specific indicator of oxidative damage. The thiobarbituric acid (TBA) assay that measures an aldehydic breakdown product of lipid hydroperoxides, namely malondialdehyde (MDA), has been used most frequently. However, TBA reacts with a variety of other biological compounds, such as prostaglandins, thromboxanes, carbohydrates, and sialic acids. Consequently, TBA and other commonly used assays, such as diene conjugation, are neither sufficiently specific nor sensitive enough to monitor small changes in free radical status and lipid peroxidation in vivo. Therefore, it has been recognized that one of the greatest needs in the field of oxygen-derived free radical research is the availability of a reliable non-invasive method to assess oxidative stress status in vivo in humans.
In 1990, Morrow and colleagues\textsuperscript{55} reported that a series of prostaglandin (PG) F\textsubscript{2}-like compounds are produced by the free radical-catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme. These compounds are termed F\textsubscript{2}-isoprostanes,\textsuperscript{56} and these provide a specific and unique measure of \textit{in vivo} lipid peroxidation (see below).

1.3. F\textsubscript{2}-Isoprostanes — A Reliable Index Of Endogenous Lipid Peroxidation

1.3.1. Mechanism Of F\textsubscript{2}-Isoprostane Formation

An interesting aspect related to the formation of F\textsubscript{2}-isoprostanes is that they are esterified phospholipids formed \textit{in situ} and they are subsequently released in the free form by phospholipases.\textsuperscript{56-58} Since only small amounts of arachidonic acid are present in the unesterified state and the vast majority of arachidonate is esterified to phospholipids\textsuperscript{58}, the level of F\textsubscript{2}-isoprostane formation could therefore serve as an ideal indicator of \textit{in vivo} lipid peroxidation.

Indeed, recently, a substantial body of evidence has been obtained indicating that the measurement of F\textsubscript{2}-isoprostanes, in urine or plasma, provides a reliable non-invasive approach to assessing lipid peroxidation status \textit{in vivo} and represents a major advance in our ability to assess oxidative stress in humans.\textsuperscript{56;59}
Figure 1.1. The isoprostane pathway. Free radical (i.e. reactive oxygen species) attack on arachidonic acid results in the formation of arachidonoyl radicals, which, following peroxidation, form 4 prostaglandin-H$_2$-like compounds that can then be fully reduced to form 4 prostaglandin F$_{2\alpha}$ regioisomers [those of the 15-series, 8-series, 12-series and 5-series], or rearrange to form prostaglandin E$_2$ and D$_2$ regioisomers. Each regioisomer comprises 8 diastereoisomers and so 64 different F$_2$-isoprostanes can be generated.
Depending on which of the labile hydrogen atoms of arachidonic acid is first abstracted by reactive oxygen species (ROS), three initial arachidonoyl radicals can be formed following ROS attack. These radicals form four prostaglandin-H$_2$-like compounds that can then be fully reduced to form 4 prostaglandin F$_{2a}$ regioisomers (i.e. 15-series, 5 series, 8-series and 12-series F$_2$-isoprostanes, Figure 1.1), or rearranged to form prostaglandin E$_2$ and D$_2$ regioisomers. Because each F$_2$-isoprostane regioisomer comprises 8 diastereoisomers, 64 different F$_2$-isoprostanes can be generated.

Most studies have focused on 15-F$_2t$-isoprostane (15-F$_2t$-IsoP), which is one of the most abundant F$_2$-isoprostanes produced in vivo, and one of the few isoprostanes commercially available.

1.3.2. 15-F$_2$-Isoprostane --- A Marker And Mediator Of Oxidant Injury

15-F$_2$-isoprostane (previously 8-epi-PGF$_{2a}$), has been the most extensively studied isomer of the F$_2$-isoprostane family. Much of the interest in this compound derives from the fact that it is not only one of the most abundant F$_2$-isoprostanes that is produced in vivo but it also possesses potent bioactivity. Measurement of 15-F$_2t$-IsoP has been shown to represent a sensitive and reliable marker of oxidative stress because: (1) it can be specifically and accurately measured in biological samples; (2) the level is increased in response to pro-oxidants, and (3) levels can be suppressed by dietary supplementation with antioxidants.
1.3.2.1. 15-F₂⁻Isoprostane As An Index Of Oxidant Stress

Reactive oxygen species-mediated non-enzymatic oxidation of arachidonic acid accounts for almost all of the formation of 15-F₂⁻isoprostane in vivo, although minute amounts of this compound may be produced as a by-product of the cyclooxygenase enzyme. Studies have shown that the physiological formation of 15-F₂⁻isoprostane in vivo is not affected by cyclooxygenase inhibition in humans, even when high doses of cyclooxygenase inhibitors are administrated. This indicates that the relative contribution of enzymatic generation of 15-F₂⁻isoprostane in vivo is inconsequential compared with the amounts formed via the ROS-mediated non-enzymatic pathway.

15-F₂⁻isoprostane is detectable in significant amounts in human plasma from normal volunteers at levels of 35 ± 6 pg/ml (n=12). Plasma levels of 15-F₂⁻isoprostane are known to increase during oxidant stress. It has been reported that there is a tendency for 15-F₂⁻isoprostane formation to increase with age in humans. This lends some support to the hypothesis that the normal aging process is associated with enhanced oxidant damage to important biological molecules over time.

Measurements of 15-F₂⁻isoprostane should potentially allow for exploration of the role of ROS in the pathophysiology of a wide range of human diseases. In particular, it could provide a valuable tool to define the clinical pharmacology of antioxidant agents. Previous clinical trials examining the effect of antioxidants to prevent or ameliorate some
of the pathology of diseases in which ROS have been implicated were hampered by insufficient information regarding what doses and combinations of antioxidants are maximally effective. Studies have shown that the formation of 15-F₂-isoprostane increases significantly in animals deficient in vitamin E ⁶³; ⁷⁰ and administration of antioxidants decreases the formation of 15-F₂-isoprostane in animal models of oxidant stress, and in humans with increased oxidative activity. ⁷¹; ⁷²; ⁷³ Of interest, vitamin E dose-dependently reduced plasma levels of 15-F₂-isoprostane in normal volunteers. ⁵⁶ This suggests that measurements of 15-F₂-isoprostane can be used to quantitatively define the effects of antioxidants to inhibit free radical processes in vivo in humans.

1.3.3. Bioactivity Of 15-F₂-Isoprostane

The discovery that 15-F₂-isoprostane can exert biological effects ⁶³; ⁷¹ reveals that this compound may not simply be a marker of lipid peroxidation, but may also participate as a mediator of oxidant injury.

In the early 1990's, studies showed that renal ischemia-reperfusion injury results in increased urinary excretion of 15-F₂-isoprostane by 300% over baseline levels. ⁷⁴ 15-F₂-isoprostane, when administrated to rats via intrarenal arterial infusion at low nanomolar concentrations, reduces glomerular filtration rate and renal blood flow by 40–50%. ⁷¹; ⁷⁴ However, systemic infusion of 15-F₂-isoprostane produced a fall in renal blood flow of ~50% without alterations in systemic blood pressure, suggesting a selective effect of 15-F₂-isoprostane on renal vasculature. The primary action of 15-F₂-isoprostane
in the glomerulus is constriction of the afferent renal arteriole, leading to a drop in glomerular capillary pressure.\textsuperscript{74,75}

More recently, experimental studies have shown that 15-F\textsubscript{2t}-isoprostane can exert effects on the heart.\textsuperscript{76} Concentration-dependent coronary vasoconstriction with parallel decreases in left ventricular developed pressure (LVDP) were seen in the isolated perfused guinea pig heart at a dose range of $10^{-8} - 10^{-5}$ M 15-F\textsubscript{2t}-isoprostane.\textsuperscript{76} Interestingly, in the isolated perfused rat heart, 15-F\textsubscript{2t}-isoprostane did not affect coronary flow at concentrations up to $3 \times 10^{-6}$ M.\textsuperscript{77} However, 15-F\textsubscript{2t}-isoprostane exerted a vasoconstriction at concentrations as low as 10 nM when rat hearts were either subjected to a short period (30 min) of low flow ischemia and subsequent re-establishment of normal perfusion, or when the hearts were subjected to varying periods of oxidant stress.\textsuperscript{77} Furthermore, 15-F\textsubscript{2t}-isoprostane-mediated vasoconstriction increases with the duration of oxidant stress in the isolated perfused rat heart.\textsuperscript{77} This supports the notion that 15-F\textsubscript{2t}-isoprostane can function as a mediator that can exacerbate oxidant injury.

Increasing evidence supports the possibility that 15-F\textsubscript{2t}-isoprostane could be a mediator of oxidant injury in humans. Elevated levels of 15-F\textsubscript{2t}-isoprostane have been demonstrated in the pericardial fluid of patients suffering from congestive heart failure, the degree of elevation correlating with the functional severity of disease.\textsuperscript{78} 15-F\textsubscript{2t}-isoprostane was found to accumulate in coronary arteries from patients with coronary artery disease and it may, therefore, be involved in the process of atherogenesis\textsuperscript{79} and possibly in the pathology of ischemic heart syndromes.
1.3.4. Methods Of Measurement Of 15-F_2t-Isoprostane

1.3.4.1. Gas chromatography (GC)-mass spectrometry (MS)

Gas chromatography-mass spectrometry (GC-MS) has been widely used for the analysis of the 15-F_2t-isoprostane (15-F_2t-IsoP). Compared to the classical procedure for the isolation and quantitative determination of plasma prostanoids, which involves chromatography on a C_{18} and Si cartridge followed by thin-layer chromatography (TLC) prior to final determination, the procedure for GC-MS measurement of 15-F_2t-IsoP has been improved. The improvements include the following: (1) recovery of prostaglandin(PG)-F_2-like compounds during the C_{18} chromatography step is improved; (2) the Si cartridge and the TLC steps are replaced with an aminopropyl cartridge, further improving the recovery of PGF2-like compounds; and (3) gas chromatographic separation of PGF2-like compounds is improved.

However, despite the improvements mentioned above, the overall recovery of PGF_2-like compounds from GC-MS procedures is not complete (~75%). The assay is still time consuming. The availability of the instrument and the capacity to analyse large amounts of clinical material has significantly limited the application of this method.
1.3.4.2. Radioimmunoassay And Enzyme Immunoassay

Immunoassay has always been an important tool for the measurement of low levels of endogenous prostaglandins or their exogenous analogues. Enzyme immunoassay (EIA) is more readily available. However, the accuracy of EIA for the analysis of 15-F₂-t-IsoP has been questioned, due to the potential for cross-reactivity of the antibody with other isoprostane isomers.

Recently, an enzyme immunoassay and a radioimmunoassay (RIA) for measuring urinary concentrations of 15-F₂-t-IsoP have been developed by raising antibodies against this compound. The antisera had high titers (>1/300,000) and provided highly sensitive assays (IC₅₀, 8 and 24 pg/ml, for EIA and RIA, respectively), and cross-reactivity with other isoprostane isomers was negligible. The intra-assay precision (<10%) is acceptable. Measurements of urinary 15-F₂-t-IsoP by immunoassay were validated using different antisera and by comparison with GC-MS. The results obtained by EIA or RIA were highly correlated with those obtained from GC-MS.

It is important to note that the measurements of F₂-isoprostanes by EIA or RIA and GC-MS are not equivalent. GC-MS estimates F₂-isoprostane concentration from a peak encompassing a number of F₂-isoprostanes isomers (15-F₂-t-IsoP being one of the major F₂-isoprostanes isomers detected), while EIA or RIA methods measure a specific isoprostane (i.e., 15-F₂-t-IsoP in most studies). Therefore, direct comparison of F₂-
isoprostane levels derived from GC-MS and levels derived from EIA or RIA may be inappropriate.

The work described in this thesis utilizes the EIA method to measure 15-F_2t-IsoP in studies designed to specifically address the role of 15-F_2t-IsoP in the pathogenesis of myocardial IRI.

1.3.5. 15-F_2t-Isoprostane Metabolism

In general, knowledge about the metabolic fate of 15-F_2t-isoprostane is very limited. The t_{1/2} of the clearance of 15-F_2t-isoprostane from the circulation in the rat is \(~16\) min. \(^{57}\) It has been postulated that, analogous to the metabolism of other prostanoids, the lung is the major site of metabolic clearance of F_2-isoprostanes from the circulation. \(^{56}\) This postulation is supported by the finding that the creation of a porta-caval shunt and the ligation of the hepatic artery in rats, completely eliminating clearance of 15-F_2t-isoprostane by the liver, but only prolonged the t_{1/2} of the clearance of 15-F_2t-isoprostane from the circulation by an incremental amount of 5 minutes – i.e., from 16 to 21 min. \(^{57}\) In rabbits, plasma 15-F_2t-isoprostane concentration was maximum at 1.5 min after the intravenous administration of the substance. \(^{81}\) The level decreased rapidly thereafter. The plasma distribution phase half-life (α-phase) of 15-F_2t-isoprostane was found to be about 1 min and the terminal elimination phase half-life (β-phase) was 4 min. \(^{81}\)
Experimental studies have shown that 15-F$_2$-isoprostane is readily transported by a prostaglandin transporter (PGT), which is expressed at high levels in various rat tissues, most notably the lung. This indicates that PGT probably represents the predominant route by which certain prostanoids, including F$_2$-isoprostanes, are transported across plasma membranes.

The urinary concentration of 15-F$_2$-isoprostane reaches its height at 20 min following the intravenous administration of the substance in rabbits. The total excretion of radioactivity in the urine was about 80% at 4 hours after the administration of tritium-labelled 15-F$_2$-isoprostane. This suggests that, at least in the rabbit, urinary elimination is an important route for 15-F$_2$-isoprostane clearance. The major urinary metabolite of 15-F$_2$-isoprostane in the rabbit is $\alpha$-tetranor-15-keto-13,14-dihydro-15-F$_2$-isoprostane and about 7% 15-F$_2$-isoprostane is found unconverted in the urine. In humans, the major urinary metabolite of 15-F$_2$-isoprostane is 2,3-dinor-5,6-dihydro-15-F$_2$-isoprostane, which represented 29% of the radiolabeled 15-F$_2$-isoprostane.

1.3.6. Evidence For A Unique 15-F$_2$-Isoprostane Receptor

It has been previously determined that the renal vasoconstricting actions of 15-F$_2$-isoprostane are completely prevented or reversed by SQ 29548, a thromboxane A$_2$ (TxA$_2$) receptor antagonist. This suggests that 15-F$_2$-isoprostane may exert its vasoconstricting effect via activation of the TxA$_2$ receptor.
Fukunaga and colleagues, using cultured rat aortic smooth muscle cells, found specific binding sites for \[^{3}H\]SQ 29548 and for \[^{125}I\] BOP, a TxA\(_{2}\) agonist. They found that both ligands were displaced from these binding sites by 15-F\(_{2}\)-isoprostane, but with significantly lesser potency than labelled SQ 29548 or I-BOP. In contrast, 15-F\(_{2}\)-isoprostane stimulated inositol 1,4,5-trisphosphate production and DNA synthesis in these cells with significantly greater potency than any of the TxA\(_{2}\) agonists tested, effects only partially inhibited by SQ 29548. In human TxA\(_{2}\) receptor cDNA-transfected cells, competition by 15-F\(_{2}\)-isoprostane for specific \[^{3}H\]SQ 29548 binding was negligible. These findings suggest the existence of distinct F\(_{2}\)-isoprostane binding sites, i.e., F\(_{2}\)-isoprostane receptors, although these receptors bear homology to the TxA\(_{2}\) receptor. The existence of a unique receptor for F\(_{2}\)-isoprostane was further evidenced by radiolabeled binding studies.

1.3.7. 15-F\(_{2}\)-Isoprostane In Myocardial IRI

Using GC-MS as a measure of 15-F\(_{2}\)-isoprostane, it was first documented clinically in 1997 that urinary levels of 15-F\(_{2}\)-isoprostane increased during myocardial reperfusion in patients undergoing elective coronary artery bypass graft (CABG) surgery using cardiopulmonary bypass (n=5). However, due to the rapid clearance from the circulation of 15-F\(_{2}\)-isoprostane and its relatively delayed appearance in the urine, urinary levels of 15-F\(_{2}\)-isoprostane might not accurately reflect the acute changes of circulatory 15-F\(_{2}\)-isoprostane levels during myocardial IRI. Furthermore, the clinical
relevance of the role of 15-F_2t-isoprostane in myocardial IRI could not be addressed due to the limited number of patients studied.

Most recent studies \(^{43; 66}\) have shown that plasma levels of 15-F_2t-isoprostane significantly increased within 3 min and continued for 50 min during CPB, or until 30 min after aorta de-clamping. Also, coronary endothelial and myocardial tissue 15-F_2t-isoprostane levels are increased during CPB in patients. \(^{19}\) Furthermore, the decay patterns of plasma 15-F_2t-isoprostane are predictive the recovery of post-operative cardiac function. \(^{43}\) These studies suggest that 15-F_2t-isoprostane may play an important role in the pathogenesis of myocardial IRI.

1.4. Antioxidant Interventions Against Myocardial IRI

Theoretically, ROS scavengers, such as enzymatic scavengers, antioxidants, and iron chelators, may be useful therapeutic adjuncts to control or attenuate ROS-mediated myocardial IRI, as demonstrated by numerous experimental studies. \(^{88-90}\) However, enzymatic scavengers or antioxidants used experimentally are not all clinically applicable.

1.4.1. Antioxidant Vitamins C And E

Clinically, high-dose vitamin C (ascorbic acid) has been demonstrated to effectively scavenge ROS, decreasing cell membrane lipid peroxidation, \(^{41; 91}\) indices of
myocardial injury, improving pulmonary endothelial function\(^{92}\) and post-operative hemodynamics.\(^{91}\) Controversy exists regarding whether endogenous vitamin E (alpha-tocopherol) is depleted during CPB surgery.\(^{50, 93, 94}\) Previous studies have shown that vitamin E supplementation reduces plasma concentrations of hydrogen peroxide\(^{95}\) and decreases cell membrane lipid peroxidation following CPB.\(^{41}\) It has been demonstrated that preoperative supplementation with a combination of vitamin E, vitamin C and allopurinol in patients undergoing CABG\(^{96}\) reduced cardiovascular dysfunction and decreased the incidence of perioperative myocardial infarction.

Most recent clinical trials using a combination of vitamin E and C supplements in CABG surgery revealed no detectable attenuation in peri-operative myocardial injury.\(^{97}\) Of interest, long-term vitamin E supplementation failed to reduce lipid peroxidation in individuals at cardiovascular risk.\(^{98}\)

In summary, despite the general acceptance of ROS-mediated lipid peroxidation as an important contributor to myocardial IRI, clinically available antioxidant interventions have yielded no convincing clinical benefit.

1.4.2. Allopurinol

Allopurinol is an inhibitor of the enzyme xanthine oxidase, a pivotal generator of ROS during reperfusion injury.\(^ {99, 100}\) Studies have suggested that in the heart, xanthine oxidase is a major source of free radical formation.\(^ {101}\) Allopurinol has been shown to decrease myocardial formation of cytotoxic ROS,\(^ {51, 102}\) to lower markers of myocardial
cellular injury, and to improve post-operative myocardial functional recovery following CPB. However, other studies have demonstrated no improvement in either myocardial function or myocardial cellular injury with allopurinol, casting doubt on its therapeutic potential, although theoretically it should be a good choice for an antioxidant intervention. In some cases, differences in the nature of the allopurinol treatment regimen may explain these discrepancies. However, the lack of myocardial xanthine oxidase activity in some species, notably the human heart, unlike the rat heart, may account for some of the discrepancies regarding the effects of allopurinol on myocardial IRI. However, allopurinol has been shown to attenuate myocardial IRI of the rabbit heart that lacks xanthine oxidase. This would suggest that mechanisms other than inhibition of xanthine oxidase can contribute to the protective effects of allopurinol against myocardial IRI.

1.4.3. N-acetylcysteine

N-acetylcysteine has been used as a radical scavenger to prevent liver damage associated with acetaminophen overdose. Recently, N-acetylcysteine has come into use during cardiac surgery. High-dose N-acetylcysteine, before or during bypass surgery, appears to act as a free radical scavenger and reduce the neutrophil oxidative burst response. However, the therapeutic potential of N-acetylcysteine in attenuating myocardial IRI has yet to be proved.

1.4.4. Propofol
Propofol is an intravenous anesthetic that has antioxidant properties and is now commonly used for anesthesia to patients undergoing CABG surgery. It may provide a promising antioxidant therapy for myocardial IRI in patients undergoing cardiac surgery utilizing CPB (see section 1.7 of this Chapter, as well as Chapters 3 and 4 for a detailed discussion).

1.5. Protective Effects And Limitations Of Ischemic Preconditioning On Myocardial IRI

It has been shown that repeated brief coronary occlusions increase myocardial resistance towards prolonged episodes of ischemia. This phenomenon, which renders the heart more tolerant to ischemia with subsequent limitation of infarct size, has been termed “ischemic preconditioning”. It has been described in a variety of species, including humans. Preconditioning may also protect the heart against postischemic dysfunction and ventricular arrhythmias. Although the beneficial effects seem to be transient, they re-appear at 24 hours, representing a "second window of protection." Ischemic preconditioning has been considered one of the most powerful mechanisms of myocardial protection so far identified.

Ischemic preconditioning, however, has several disadvantages that may limit its clinical application. Firstly, brief periods of ischemia could induce numerous untoward changes in the myocardium, including some that may persist for days. One of these is referred to as the "stunned myocardium", which represents "prolonged postischemic
contractile dysfunction of myocardium salvaged by reperfusion. The mechanism of stunning is believed to involve generation of oxygen radicals as well as alterations in calcium homeostasis. Stunning has been observed in several clinical scenarios, including after percutaneous transluminal coronary angioplasty, unstable angina, stress-induced ischemia, after thrombolysis, and after cardiopulmonary bypass. It is likely that classical ischemic preconditioning might not be sufficient to attenuate the stunning of surviving myocardium that renders the ventricle akinetic during early reperfusion.

Secondly, a recent study has shown that pathological conditions such as diabetes mellitus prevent ischemic preconditioning in patients with a first acute anterior wall myocardial infarction. Similarly, oral sulfonylurea hypoglycemic agents prevent ischemic preconditioning in human myocardium, and may cause excess cardiovascular mortality in these patients. Consequently, the application of ischemic preconditioning to diabetic patients, a high-risk population, is limited.

In addition, in patients with chronic coronary stenosis, their myocardium may have already been in a naturally ischemic preconditioned status. It is, therefore, questionable whether further ischemic preconditioning prior to cardiopulmonary bypass surgery in these patients would be beneficial or even detrimental.

1.6. Anesthesia And Myocardial IRI

The contribution of anesthesia to the course of myocardial IRI has not been adequately addressed. Controversies exist about the potential risks or benefits of
anesthetics on myocardial IRI in the clinical setting. It is generally agreed that volatile anesthetics (such as isoflurane and sevoflurane) and intravenous anesthetics (e.g., propofol) reduce the myocardial damage caused by ischemia and reperfusion.\textsuperscript{121} The proposed mechanisms for protection by anesthetic agents may include an anesthetic preconditioning (APC) effect by volatile anesthetics\textsuperscript{122, 123} and an antioxidant effect by propofol.\textsuperscript{124-126}

1.6.1. Inhalational Anesthesia

Laboratory studies have shown that volatile anesthetics such as sevoflurane and isoflurane can reduce post-ischemic myocardial infarct size.\textsuperscript{127} Volatile anesthetics could exert myocardial protective effects through an ischemic preconditioning-like mechanism (i.e. anesthetic preconditioning). It should be noted, however, that volatile anesthetic-mediated anesthetic preconditioning (APC) primarily requires the generation of ROS in advance (i.e., before ischemia) as a trigger of myocardial preconditioning.\textsuperscript{127, 128} Theoretically, the additional generation of ROS in a population with preexisting high degrees of oxidative stress, for example, chronic heart failure and diabetes, may stimulate increased mitochondrial permeability transition, releasing large amounts of ROS (involving radical-induced radical release).\textsuperscript{129} This could overwhelm endogenous antioxidant defenses, resulting in extensive lipid peroxidation and cellular destruction.\textsuperscript{129}

Indeed, experimental studies\textsuperscript{130, 131} have shown that myocardial IRI can be accentuated by the interaction of inhalational anesthetics and the xanthine oxidase system, a major source of free radical formation.\textsuperscript{101} This is an important issue, given the
fact that during CPB surgery patients are already under oxidative stress resulting from dramatically increased ROS originating from the ischemic-reperfused heart and systemic circulation. This systemic oxidative stress is likely to give rise to secondary myocardial damage. In addition, isoflurane-induced preconditioning may be attenuated or prevented by diabetes or acute hyperglycemia, a pathological condition that is associated with increased post-operative cardiac dysfunction. Of interest, one experimental study has shown that N-acetylcysteine can restore isoflurane-induced preconditioning against myocardial infarction during hyperglycemia in rats (on a time limited basis, ie. less than 40 min of ischemia). It is yet to be proven if this could be clinically applicable. Hence, the potential clinical utility of APC may be limited and alternative approaches to APC are required. Sevoflurane, a new inhalational anesthetic that is in clinical use, decreased superoxide during ischemia and reperfusion in the isolated guinea pig heart and attenuated post-ischemic myocardial dysfunction. This is promising. Further studies are needed to confirm the cardioprotective effects of sevoflurane in clinical settings. Taken together, these studies imply a preference for the use of intravenous anesthetic agents in settings where oxidative stress may already be elevated.

1.6.2. Intravenous Anesthesia

The intravenous anesthetic propofol may have potential benefit over isoflurane anesthesia in facilitating post-operative myocardial functional recovery. However, propofol, when administrated to achieve a plasma concentration between 2 to 4 μg/ml,
did not demonstrate greater effects than volatile anesthetic sevoflurane or desflurane in attenuating post-operative myocardial cellular damage using myocardial troponin I as a specific indicator. The dosage and the timing of propofol application may need to be adjusted in order to optimize cardiac protection.

1.7. Propofol: An Anesthetic and Antioxidant

Propofol is a short-acting intravenous anesthetic, which is highly lipid-soluble. It is widely used in both ambulatory and hospitalized patients and is increasingly used for cardiac anesthesia and for perioperative sedation. Propofol permits both efficient control of anesthetic depth as well as rapid and controllable recovery. Propofol’s antioxidant activity may have contributed to its protective effects in attenuating myocardial IRI in animal models.

1.7.1. Structure

Propofol, 2,6-diisopropylphenol (molecular weight 178.27), is chemically similar to phenol-based free radical scavengers such as the endogenous antioxidant vitamin E (alpha-tocopherol) (Figure 1.2). All these compounds carry a hydroxyl substituent on the phenyl ring, which is known to confer free radical scavenging properties.
Figure 1.2. Molecular structures of 2,6-diisopropylphenol (propofol) and alpha-tocopherol (vitamin E).
1.7.2. Basic Pharmacokinetics

Propofol anesthesia offers several advantages over other intravenous anesthetics, notably rapid onset and emergence, rapid return to spontaneous ventilation and reduction in time to tracheal extubation. Administration of propofol, 2-2.5 mg/kg, intravenously over 15 seconds or less, produces unconsciousness within about 30 seconds. Awakening is very rapid and complete, with minimal residual central nervous system effects - this is likely the most important advantage over other drugs used for anesthesia induction, especially for ambulatory patients.

After intravenous administration of propofol, distribution occurs with a half-life ($t_{1/2\alpha}$) of 2-8 minutes. The elimination half-life ($t_{1/2\beta}$) of propofol is approximately 30-60 minutes. The drug is rapidly metabolized in the liver by conjugation to glucuronide and sulfate derivatives which are excreted in the urine. Less than 1% of the administered dose of drug is excreted unchanged in the urine.

The blood clearance of propofol is greater than hepatic blood flow, suggesting that tissue uptake and metabolism contribute to the removal of this drug from the blood. This property of propofol is useful in patients with impaired ability to metabolize other anesthetics.
Despite the rapid clearance of propofol by metabolism, there is no evidence of impaired elimination in patients with moderate cirrhosis or renal dysfunction. Patients older than 60 years of age exhibit a reduced plasma clearance of propofol. Therefore, there may be a modest cumulative effect in elderly patients receiving continuous intravenous infusions of propofol.

1.7.3. Effects Of Cardiopulmonary Bypass On Propofol Pharmacokinetics

Propofol is a weak organic acid that is bound extensively (97-98%) to plasma albumin, with a free fraction in the plasma of about 2-3%. It is likely the unbound, rather than the total plasma, concentration that is related to the anesthetic action of propofol. It has been reported that hemodilution during cardiopulmonary bypass (CPB) is associated with decreases in the concentration of plasma proteins, a decrease in total plasma propofol concentration disproportional to the decreases in the concentration of plasma proteins, and an increase in the fraction of unbound propofol. As a result, the unbound concentration of propofol may remain relatively constant during CPB.

The decrease in total propofol concentration with the initiation of CPB is probably mainly a result of the hemodiluting effect of the priming solution in the extra-corporeal circuit. Binding of propofol to the extracorporeal circuit may be an additional factor in decreasing plasma concentration of propofol in patients undergoing CPB, since the decrease of propofol concentration is up to 50% more than that predicted by hemodilution alone.
During the later phase of CPB, total propofol concentration increases to pre-bypass values. This increase in propofol concentration may result from decreases in volume of distribution during hypothermia.\textsuperscript{155,158}

The increase in the fraction of unbound propofol during CPB may be a consequence of heparin administration. The administration of heparin during CPB tends to increase the free fraction of propofol in plasma and these changes were reversed after protamine.\textsuperscript{155} Heparin activates lipoprotein lipase, resulting in the hydrolysis of plasma triglycerides into non-esterified fatty acids.\textsuperscript{159,160} Non-esterified fatty acids could then competitively inhibit the binding of various drugs to the plasma proteins and may contribute to the increase in the unbound fraction of the drug\textsuperscript{161} as noted above.

1.7.4. Propofol Antioxidant Activity

Tissue damage caused by the activity of reactive oxygen species (ROS) contributes to myocardial IRI.\textsuperscript{162,163-166} As a consequence of the action of ROS, lipid peroxidation can occur. Under physiological conditions, the polyunsaturated fatty acids in the cell membrane are protected against lipid peroxidation by endogenous antioxidants including the lipid soluble chain-breaking antioxidants such as vitamin E (\(\alpha\)-tocopherol).\textsuperscript{167,168} The hydroxyl (OH) group of \(\alpha\)-tocopherol donates the hydrogen atom. \(\alpha\)-Tocopherol terminates the chain reaction of lipid peroxidation by scavenging lipid
peroxyl radicals (LOO') by a process of hydrogen donation. In this reaction, α-tocopherol itself becomes a phenoxyl radical which is much less reactive than LOO' (reaction 1).  

\[ \alpha\text{-tocopherol-OH} + \text{LOO}' \rightarrow \alpha\text{-tocopherol-O'} + \text{LOOH} \quad (\text{reaction 1}) \]

Propofol (2,6-diisopropylphenol) also contains a phenolic OH-group (figure 1.2) and it has been reported to act as an antioxidant via the similar mechanism to α-tocopherol according to spin resonance spectroscopy, 141, 169 one of the most specific methods for characterizing radical species. Propofol reacts with peroxynitrite (ONOO-), a reactive radical formed from the reaction of superoxide anion with nitric oxide, 170, 171 to form a phenoxyl radical as demonstrated by electron spin resonance. 172 This might be a very important aspect of protocol’s antioxidant capacity, since peroxynitrite is more potent than superoxide anion or nitric oxide in mediating myocardial ischemia-reperfusion injury. 47, 163-165, 173-175

Propofol has also been shown to directly and dose-dependently scavenge ROS generated either by stimulated human leucocytes or in cell-free systems, 176 as examined using luminal chemiluminescence, a sensitive assay for monitoring free radicals and reactive oxygen metabolites produced by enzymes, cell or organ systems. 177, 178

An in vitro study has demonstrated that propofol, when applied at a concentration of 8 µM, can completely suppress lipid peroxidation in isolated liver mitochondria from
rats. Propofol's capability to inhibit mitochondrial lipid peroxidation may prove to be an important mechanism of its protective effect against myocardial IRI. A recent study has shown that the capacity of mitochondria to produce both ROS and lipid peroxidation increases upon reperfusion in rat hearts subjected to ischemia and reperfusion.

1.7.5. Propofol Effects On IRI In Isolated Hearts

Propofol, at 25 and 50 μM, attenuated both mechanical dysfunction and metabolic damage induced by exogenous hydrogen peroxide in isolated rat hearts. Hydrogen peroxide significantly decreased left ventricular developed pressure (i.e., it produced mechanical dysfunction) and decreased tissue concentrations of ATP and creatine phosphate and also increased tissue lipid peroxidation. Propofol (25 μM and 50 μM) completely suppressed hydrogen peroxide-induced tissue lipid peroxidation and significantly attenuated mechanical and metabolic alterations induced by hydrogen peroxide.

Propofol significantly improved functional and metabolic recovery in ischemic-reperfused isolated rat hearts at 25 μM, 50 μM or 100 μM when the isolated hearts were subjected to 15 or 25 min of global ischemia followed by 20 to 30 min of reperfusion. It is unknown whether propofol could exert cardioprotective effects if the duration of global ischemia is prolonged.
However, when applied at 3 or 10 μM, propofol had no significant effect on myocardial function before or after global ischemia, nor did it suppress free radical formation in isolated rat hearts during reperfusion as measured with high performance liquid chromatography. These concentrations of propofol (3 or 10 μM) are likely lower than the concentration needed to attenuate coronary artery endothelial damage induced by inflammatory cytokines during myocardial ischemia and reperfusion. Recent studies have shown that apoptosis of coronary artery endothelial cells precedes and affects myocyte cell apoptosis in ischemia-reperfusion injury in the rat heart. Our recent study has demonstrated that propofol suppresses cytokine-induced vascular endothelial cell apoptosis at concentrations ≥ 12.5 μM (see Appendix I).

1.7.6. Propofol Anesthesia In Cardiopulmonary Bypass Surgery

Propofol anesthesia has the advantage of rapid and complete awakening, resulting in early extubation after surgery and early discharge from the intensive care unit. It has been used widely since the early 1990's to produce total intravenous anesthesia in cardiac surgeries using CPB.

Hypotension is considered to be a major “adverse” effect of propofol anesthesia. This is a result of a decrease in peripheral vascular resistance. However, propofol did not significantly depress myocardial contractility as compared to the inhalational anesthetics isoflurane or enflurane during cardiac surgery.
Propofol significantly attenuated, but did not completely prevent, myocardial lipid peroxidation during CABG surgery using CPB when it was infused continuously at 3-6 mg/kg/hr.\textsuperscript{191} This dosage of propofol achieves plasma concentrations of up to about 5µg/ml (28 µM) based on a previous study in our laboratory.\textsuperscript{125} Interestingly, enhancing red blood cell antioxidant capacity during CPB with propofol, an intravenous anesthetic with antioxidant properties,\textsuperscript{140,141} was related to improved post-operative cardiac functional recovery.\textsuperscript{125}

Recent studies have shown that 15-F\textsubscript{2t}-isoprostane is a vasoconstrictor in human saphenous veins and internal mammary arteries.\textsuperscript{192,193} Taken together with the fact that level of 15-F\textsubscript{2t}-isoprostane is increased during coronary reperfusion clinically,\textsuperscript{87,194} one may postulate that 15-F\textsubscript{2t}-isoprostane produced at sites of free radical generation may play an important role in internal mammary artery and/or coronary spasm in situations of oxidant stress such as coronary bypass surgery, contributing to post-ischemic reperfusion injury. It can be hypothesized that 15-F\textsubscript{2t}-isoprostane may not only serve as a specific marker of oxidative myocardial damage, but also play a causative role in exacerbating myocardial IRI. One can further hypothesize that interventions aimed to reducing the formation, or antagonizing the action, of 15-F\textsubscript{2t}-isoprostane during myocardial ischemia-reperfusion should attenuate post-ischemic myocardial dysfunction.

The overall aims in this thesis were: 1) to investigate the role of 15-F\textsubscript{2t}-isoprostane in myocardial ischemia-reperfusion injury during cardiac surgery, and, 2) to
develop a clinically applicable therapeutic regimen for propofol in order to effectively target ROS-mediated myocardial IRI. Furthermore, the potential mechanism(s) whereby 15-F_{2\alpha}-isoprostane may exacerbate post-ischemic myocardial dysfunction will be explored.
CHAPTER 2

THE RELATIONSHIP BETWEEN PLASMA 15-F_{2T}-ISOPROSTANE
CONCENTRATION AND EARLY POSTOPERATIVE CARDIAC FUNCTION
FOLLOWING CARDIAC SURGERY

2.1. Preface

This investigation was supported by internal department development funding to Dr. D.M. Ansley from the Department of Anesthesia, University of British Columbia. A manuscript reporting the studies in this Chapter has been published in *The Journal Thoracic and Cardiovascular Surgery* 2003;126:1222-3 (Ref.43), co-authored with D.M. Ansley and B.S. Dhaliwal. B.S. Dhaliwal contributed to plasma sample collection and clinical hemodynamic data analysis.

2.2. Introduction

Warm heart surgery has changed the nature of cardiac surgery, and is commonly employed today.195 Although the overall mortality rate has declined, significant morbidity is still be associated with the procedure. The reported risk of early postoperative myocardial dysfunction is 6-25%.196,197 Low cardiac output syndrome is associated with increased operative mortality, myocardial infarction rate, prolonged intensive care unit and hospital lengths of stay.196 This problem reflects inadequate intraoperative myocardial protection and/or incomplete revascularization.
Ischemia-reperfusion injury (IRI) is a major pathophysiologic factor. This is a complex phenomenon, whose mechanisms are incompletely understood. The generation of oxygen derived free radicals initiates a series of events, which culminate in loss of cellular integrity and function.

Although well documented in animal studies using different methods, the role of oxidant stress in humans, particularly myocardial IRI, remains unclear. Part of the difficulty has been the lack of a sensitive and specific indicator of oxidative damage. The measurement of F$_2$-Isoprostanes appears to be both a specific and sensitive index of oxidative stress status \textit{in vivo}.\textsuperscript{56; 198; 199} Of special interest, 15-F$_2$-isoprostane (15-F$_2$-isoP) exerts biologic activity on the heart. Concentration dependent coronary vasoconstriction, with parallel decreases in cardiac function have been documented in experimental models.\textsuperscript{76; 200}

Evidence documenting the clinical relevance of oxidant stress in cardiac surgery is lacking. 15-F$_2$-isoP formation has been implicated in PTCA-associated complications such as coronary vasospasm,\textsuperscript{201} but investigation of a role in myocardial stunning is required. The relationship between 15-F$_2$-isoP formation and postoperative cardiac function, if one exists, is unknown.

Our overall goal was to establish the role of 15-F$_2$-isoP in the pathogenesis of myocardial IRI, in an effort to develop a novel strategy to improve outcomes following
cardiac surgery. We hypothesized that the intraoperative level of plasma 15-F_{2\text{-isoP}} is a determinant of postoperative cardiac function. We tested this hypothesis in the setting of warm heart surgery.

2.3. Materials and methods

2.3.1. Patient data and surgical technique

Following institutional ethics board approval and written informed consent, 30 patients scheduled for CABG (n=24) or combined valve replacement/CABG (n=6) procedures were enrolled in this study. Patients with a history of preoperative use of Vitamin C or E, acute or evolving myocardial infarction, preoperative hemodynamic instability, hepato-renal dysfunction, and age > 80 years, were excluded.

Cardiac catheterization was performed in all patients between 1 and 12 weeks preoperatively to determine the extent of coronary artery disease and left ventricular function. Left ventricular ejection fraction was measured by contrast ventriculography.

Patients continued their cardiac medications up to the time of surgery. Perioperative monitoring included five lead ECG for ST-segment analysis (Marquette® Solar 8000) of leads I,II,III,V_s, aVR, aVL,aVF. Standard criteria were utilized to diagnose perioperative myocardial ischemia. Invasive hemodynamic monitoring included arterial, central venous and/or pulmonary artery catheterization for determination of mean arterial, central pressures, and cardiac output.
After median sternotomy and systemic heparinization, aortic and venous cannulae were inserted. Patients received epsilon aminocaproic acid (AMICAR®) 10 grams in two divided doses prior to, and at the initiation of, cardiopulmonary bypass. Full cardiopulmonary bypass was established at a patient temperature of 34-37°C (esophageal) with a non-pulsatile flow rate of 2 l/min/m² and use of a membrane oxygenator. The heart was arrested by antegrade infusion of intermittent warm high potassium blood cardioplegia (8:1 with Normosol R; potassium 20 meq/liter; mean, hematocrit approximately 80 g/L) via an aortic root catheter at 300 ml/min and continuous cross clamping of the aorta in all patients. In 9 patients, additional intermittent cold (10°C) low blood potassium cardioplegia was subsequently applied. Intermittent warm low blood potassium cardioplegia was applied in 21 patients. Low potassium cardioplegia was used throughout the procedure, unless electrical activity dictated the need to use the high potassium solution. Distal anastomoses were initially performed, then cardioplegia was delivered down the graft as each was completed. The last anastomosis in each patient was internal mammary to anterior descending coronary artery. Proximal anastomoses were performed during continuous aortic side clamping.

2.3.2. Data and samples collection

All patient data were collected prospectively in our data base. Cardiac output was determined in triplicate, by the thermodilution technique prebypass, 30, 60, 120, 240 and 360 min following surgery. Cardiac index was derived from mean cardiac output.
Central venous blood sampling was conducted at baseline, 30 min global ischemia, and at 10, 30, and 120 min reperfusion. Blood was draw into Vacutainer® tubes containing EDTA, temporarily stored on ice (during operation room to laboratory transportation) and immediately centrifuged at 0°C to separate the plasma. The plasma was snap frozen with liquid nitrogen and stored at -70 °C until analysis.

Low cardiac output syndrome was defined by cardiac index < 2.2 L.min.m\(^2\) despite optimization of preload, afterload and heart rate. The hemodynamic support required to achieve a cardiac index greater than or equal to 2.2 l/min/m\(^2\) was recorded. Inotropic support was defined as any use of dopamine > 4 µg/kg/min, epinephrine > 0.04 µg/kg/min, or milrinone 0.25-1.0 µg/kg/min, alone or in combination, for greater than 30 min duration during the first six postoperative hours.

2.3.3. 15-F\(_{21}\)-IsoP enzyme immunoassay

Unlabeled 15-F\(_{21}\)-IsoP (8-iso-PGF\(_{2\alpha}\)) standard and the enzyme immunoassay (EIA) kit were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The EIA kit was tested for assay accuracy prior to direct assay for plasma free 15-F\(_{21}\)-IsoP concentration. The EIA procedures used to measure free plasma F\(_2\)-IsoP were according to the methods provided by the manufacturer with a minor modification. In brief, plasma samples were removed from -70 °C storage and thawed on ice. For the assay, 50 µl standards and samples were first added in duplicate to the 96-well plate provided in the kit, followed by addition of isoprostane acetylcholinesterase tracer and antibody. The prepared plates were then incubated for 18 hours at room temperature. On the next day,
the plates were washed 5 times with wash buffer, followed by addition of Ellman's reagent. After optimal development by using an orbital shaker, the plates were read at 405 nm, and the values of the unknowns were expressed as picograms per milliliter plasma. The plasma samples were re-analyzed in duplicate or triplicate after being diluted with phosphate-buffered saline (PBS) whenever the first direct assay values of free plasma 15-F_{2t}-IsoP were above 250 pg/mL or inconsistent results occurred.

2.3.4. Data analysis

Patient data were analysed as a whole (n=30) and by subgroups according to inotrope requirements for postoperative hemodynamic stabilization: Group I (no inotropes, n=7); Group II (≥ 2 inotropes, n=6). All data are presented as mean ± SEM. Student t test was used to compare pre-anesthesia and during CPB patient data. Isoprostane data were compared by ANOVA with Bonferroni's corrections (GraphPad Prism; NCSS). One-way repeated measure ANOVA and Tukey's Multiple Comparison test was applied for within group comparison. Correlations were evaluated by the Pearson test. The differences were considered significant at P < 0.05.

2.4. Results

2.4.1. Patient profile and perioperative data
The patient demographic and perioperative data for thirty patients is presented in Table 2.1. Four patients were monitored with central venous pressure only. Cardiac index data was complete in 26/30 patients. No perioperative myocardial ischemia was detected in any patient. Differences in maintenance cardioplegia techniques were not associated with differences in outcome variables.

Seven patients separated easily from CPB and remained stable postoperatively (group I). Six patients needed two or more inotropes for hemodynamic stabilization (group II). Patients in the two subgroups were demographically similar in terms of age (60.3 ± 2.3 vs 69.0±3.4 yr), gender (male/female, 5/2 vs 4/2), preoperative left ventricular ejection fraction (LVEF) (58.2±3.0 vs 55.7±6.3%), incidence of recent MI (one patient in group I vs three patients in group II) and dyslipidemia (five patients in group I vs four in group II).

There was no significant difference between group I and group II in duration of ACC, CPB, mean systemic oxygenation during surgery, hemoglobin content of cardioplegia, time between doses of cardioplegia, or systemic oxygen tension during CPB (Table 2.2).

2.4.2. Evaluation of the assay

To assess the linearity and accuracy of the assay, EIA buffer spiked with 15-F2t-IsoP standard was assayed using serial dilutions. The intra-assay mean values (triplicate
assays) were 98%, 90%, 103%, 103% of the expected values of 7.8, 15.6, 62.5, 125 pg/mL, respectively. Values were 144% and 118% for the expected values of 3.9 and 250 pg/mL. The best assay linearity relationship exists between 7.8 to 125 pg/mL (R2 = 0.9998).

Three plasma samples of known concentrations of 15-F$_{2t}$-IsoP (obtained after preliminary direct EIA assay in duplicate) were assayed six times on one sample plate, or assayed in 12 separate assays to assess intra-assay and inter-assay precision. The intra- and inter-assay coefficient of variation was less than 9% or less than 14.7% when plasma free 15-F$_{2t}$-IsoP was in the range of about 14 or 40 to 58 pg/mL, respectively (Table 2.3).
Table 2.1. Demographic and perioperative data

[mean ± SEM]

<table>
<thead>
<tr>
<th></th>
<th>(N = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>67.7 ± 1.6</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>22/8</td>
</tr>
<tr>
<td>Body surface area (m²)</td>
<td>1.97 ± 0.04</td>
</tr>
<tr>
<td>NYHA Class</td>
<td>II-IV</td>
</tr>
<tr>
<td>Pre-operative LVEF (%)</td>
<td>56.3 ± 2.2</td>
</tr>
<tr>
<td>Duration of ACC (min)</td>
<td>100.9± 8.5</td>
</tr>
<tr>
<td>Duration of CPB (min)</td>
<td>132.1± 10.6</td>
</tr>
<tr>
<td>Volume of cardioplegia (ml)</td>
<td>5874 ± 785</td>
</tr>
</tbody>
</table>

ACC = Aorta Cross-clamping
CPB = Cardiopulmonary Bypass
LVEF = left ventricular ejection fraction
Table 2.2. Perfusion data for the non-inotropes and ≥ 2 inotropes group [mean ± SEM]

<table>
<thead>
<tr>
<th></th>
<th>Non-inotrope (N=7)</th>
<th>≥2 inotropes (N=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plegia (blood:crystalloid)</td>
<td>7.6±0.4</td>
<td>8±0.0</td>
</tr>
<tr>
<td>Ratio (x : 1)</td>
<td>0.24±0.01</td>
<td>0.23±0.01</td>
</tr>
<tr>
<td>Intra-op Hct</td>
<td>6.91±0.31</td>
<td>6.82±0.37</td>
</tr>
<tr>
<td>Plegia Hb (g/dL)</td>
<td>198.1±9.1</td>
<td>176.8±7.3</td>
</tr>
<tr>
<td>Time interval for plegia (min)</td>
<td>20.9±2.3</td>
<td>19.5±2.8</td>
</tr>
<tr>
<td>Duration of ACC(min)</td>
<td>112.7±31.3</td>
<td>108.5±16.1</td>
</tr>
<tr>
<td>Duration of CPB(min)</td>
<td>141.0±36.6</td>
<td>150.8±25.0</td>
</tr>
</tbody>
</table>

ACC = Aorta Cross-clamping; CPB = Cardiopulmonary Bypass. P > 0.05 between groups for all parameters.
Table 2.3. Plasma free 15-F2t-Isoprostane assay precision

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean (pg/mL)</th>
<th>SD</th>
<th>CV(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>6</td>
<td>13.81</td>
<td>0.97</td>
<td>7.0</td>
</tr>
<tr>
<td>Sample 2</td>
<td>6</td>
<td>42.35</td>
<td>5.71</td>
<td>13.48</td>
</tr>
<tr>
<td>Sample 3</td>
<td>6</td>
<td>58.40</td>
<td>7.75</td>
<td>13.27</td>
</tr>
<tr>
<td><strong>Inter-assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>12</td>
<td>13.54</td>
<td>1.21</td>
<td>8.96</td>
</tr>
<tr>
<td>Sample 2</td>
<td>12</td>
<td>40.11</td>
<td>5.15</td>
<td>12.85</td>
</tr>
<tr>
<td>Sample 3</td>
<td>12</td>
<td>55.00</td>
<td>8.06</td>
<td>14.65</td>
</tr>
</tbody>
</table>

N: number of measures; SD: standard deviation;
CV: coefficient of variation
2.4.3. Plasma free 15-F$_2$-isoprostane concentration

The lowest detected plasma free 15-F$_2$-isoP concentration was 12 pg/mL at baseline in one patient. Eleven out of a total of 150 plasma samples were re-analyzed at different dilutions due to free 15-F$_2$-isoprostane values exceeding 250 pg/mL.

For the group as a whole (n = 30), plasma 15-F$_2$-isoP increased significantly during ischemia, and remained significantly elevated after 10 min of reperfusion (P<0.001 vs baseline, Figure 2.1). 15-F$_2$-isoP did not differ from baseline at reperfusion 30 min and onwards (P>0.05 vs baseline). There was no relation between plasma 15-F$_2$-isoP and duration of ACC or CPB.

Figure 2.2 depicts the different patterns of plasma 15-F$_2$-isoP formation in group I and group II. Plasma 15-F$_2$-isoP in group I underwent exponential decay (t$_{1/2}$ = 71.4 min, Figure 2.3) after ischemia, and did not differ significantly from baseline at reperfusion 10 min and onwards (P>0.05 vs baseline, Figure 2.2). In contrast, plasma 15-F$_2$-isoP remained elevated during reperfusion in group II, with levels unchanged in 2 patients and increased in 4 patients between 10-30 min reperfusion. In 6/7 patients requiring no inotropes, the isoprostane concentration decreased primarily between 10-30 min reperfusion.
Figure 2.1. Changes of plasma free 15-F$_2$-Isoprostane during ischemia-reperfusion. Values were mean ± SEM. N=30. Rep-10, Rep-30 and Rep-120 represent 10, 30, and 120 min of reperfusion. * P < 0.001 vs baseline. # P < 0.01 vs ischemia.
Figure 2.2. Changes of plasma free 15-F₂tı-Isoprostane during ischemia-reperfusion in non-inotrope and ≥ 2 inotropes groups. Values were mean ± SEM. Light bar indicates non-inotrope group (N = 7), solid bar indicates ≥ 2 inotropes group (N = 6). * P < 0.05, ** P < 0.001 vs baseline. # P < 0.01 vs ischemia. Difference between groups was not significant (P > 0.05).
Figure 2.3. Plasma free 15-F2t-Isoprostane in the non-inotrope group decays exponentially after global myocardial ischemia-reperfusion. Values were the mean of seven (N = 7). Isoprostane decay half-life (t1/2) 71.4 minutes (Goodness of fit of the decay curve: $R^2 = 0.97$. GraphPad Prism program). Average duration of ischemia =122 min.
Figure 2.4. Correlation between post-operative cardiac index (CI) and changes of plasma free 15-F₂t-Isoprostane during early reperfusion. CI was significantly negatively correlated with percentage increase of 8-F₂α-isoprostane from reperfusion (rep) 10 to rep 30 min (r = -0.73, 95%CI: -0.87 to -0.47, P < 0.0001). N = 26. CI data not available in 4 patients.
2.4.4. 15-F$_2$-isoprostane and postoperative cardiac index

A significant negative correlation was observed between postoperative cardiac index and percentage change in plasma 15-F$_2$-isoP concentration from 10 to 30 min reperfusion for n=26 patients (Figure 2.4, $r = 0.8361$; 95% CI: -0.9496 to -0.5258, $P = 0.0004$). Postoperative cardiac index did not correlate with baseline 15-F$_2$-isoP, nor the 15-F$_2$-isoP concentration during ischemia ($P > 0.1$).

2.5. Discussion

The aim of our study was to determine the relationship between plasma 15-F$_2$-isoP generation and early postoperative cardiac depression following warm heart surgery. The principal findings include: 1) 15-F$_2$-isoP generation occurs despite application of warm blood cardioplegia; 2) the pattern of intraoperative 15-F$_2$-isoP generation varies between hemodynamically stable and unstable patients; 3) an inverse relationship exists between early postoperative cardiac function and the percentage change in 15-F$_2$-isoP during early reperfusion.

The use of warm heart techniques in CABG surgery is associated with superior postoperative cardiac performance, reduced incidence of low cardiac output syndrome and increased tolerance to prolonged duration of aortic crossclamping, compared to
hypothermic protection and CPB. This clinical practice is common and has changed the outcome of cardiac surgery.

Low cardiac output syndrome is recognized as a high risk scenario complicating postoperative recovery of patients undergoing cardiac surgery. The approach to myocardial protection during warm heart surgery is important. The incidence of low output syndrome increases beyond 8-10% when time between doses of cardioplegia exceeds 13 min, and/or hemoglobin content of cardioplegia is less than 8 g/dl. The mechanism(s) of cardiac dysfunction has yet to be elucidated. The systemic inflammatory response to cardiopulmonary bypass has been previously implicated. The direct cardio-depressant effects of pro-inflammatory cytokines, nitric oxide, or the vasoconstrictor substance endothelin-1 (ET-1) are associated with postoperative hemodynamic instability. Oxidant stress influences the release of these factors.

In an effort to find a sensitive and specific in vivo marker of oxidant stress, recent attention has focused on the measurement of prostaglandin isomers known as isoprostanes. Isoprostanes are chemically stable end products of lipid peroxidation formed in vivo, that circulate in plasma, exert biologic activity (coronary vasoconstriction, platelet activation and adhesion) and are excreted in the urine.

Using GCMS, Delanty and Reilly et al. reported an increase in urinary 8-epi-prostaglandin F2α (15-F2α-isoP) during cardiac surgery or angioplasty. They used sampling intervals different from our study. They did not measure 15-F2α-isoP levels
during the ischemic interval. Their results are limited because of the small patient sample size and the lack of documented clinical sequelae in response to 15-F_2t-isop formation.

Iuliano et al provided first evidence of cardiac 15-F_2t-isop formation. They measured increased levels of F_2-isoprostane in the coronary sinus blood of 12 patients during percutaneous transluminal coronary angioplasty. They suggested a potential role for isoprostane in PTCA-associated complications like vasospasm and myocardial stunning.

Clermont et al. recently described the systemic production of free radicals during hypothermic CPB in eleven patients with normal preoperative ejection fraction. The pattern and amount of free radical activity measured in peripheral and in coronary sinus blood during the ischemia and early reperfusion (25 min) was characterized by ESR spin trapping. The amount of oxidant stress was correlated to the duration of CPB and was associated with a decrease in plasma antioxidant status despite adequate systemic antioxidant reserve. They concluded that systemic oxidant stress during CPB participates in myocardial damage. The clinical effect of oxidant stress on cardiac function was not evaluated.

Our study characterizes isoprostane formation predominantly during global ischemia, subject to variation in exponential decay (metabolism/excretion) during the course of surgery. Our findings demonstrate that 15-F_2t-IsoP formation is clinically significant, correlating inversely with early postoperative myocardial function when it
remains elevated following surgery. We observed an average time of 19 min between doses of cardioplegia containing slightly less than 80g/L hemoglobin. This may explain the decline in postoperative cardiac index observed in 20% of our patients. Since these factors were similar between subgroups, our findings suggest that the recovery of postoperative cardiac function may be related, at least in part, to isoprostane formation and its elimination. The half-life of elimination of 15-F_{2\alpha}-IsoP is relevant, reflecting the duration of early postoperative myocardial depression previously characterized by Breisblatt et al.\textsuperscript{16} We feel it is significant that cardiac depression occurred without any evidence of perioperative myocardial infarction. Whether isoprostane acts directly or is involved in another mechanism of cardiac depression, is beyond the scope of this study but is the subject of current investigation.

GC-Mass spectrometry is considered the "gold standard" for 15-F_{2\alpha}-IsoP analysis, but is expensive and technically difficult.\textsuperscript{209} The availability of the instrument and the need to analyse large amounts of clinical material has limited its clinical application. Enzyme immunoassay (EIA) is more readily available, but the use of EIA to measure isoprostane is controversial. The accuracy of EIA has been questioned, due to the potential for cross-reactivity of antibodies in immunoassays, differential metabolism of different isomers, and differential loss during sample preparation.

The use of EIA for 15-F_{2\alpha}-IsoP has recently been validated by GC-Mass spectrometry and the results from both methods were significantly correlated.\textsuperscript{80} The measurement of free 15-F_{2\alpha}-IsoP has been performed and validated via
radioimmunoassay of unextracted plasma. We used the same rabbit 15-F2t-IsoP antibody to directly measure 15-F2t-IsoP in unextracted plasma. This approach to EIA yielded reliable and acceptable results. It is important to note however, we found that the EIA result is most reliable within a narrow range (7.8 to 250 pg/mL).

Plasma free F2t-IsoP is about one third of the total F2-Isoprostanes esterified to lipoproteins in humans. The baseline free plasma 15-F2t-IsoP levels we measured are comparable to the results measured in healthy adults by Mori and colleagues who used capillary gas chromatography/electron capture negative ionization mass spectrometry (GC-ECNI-MS), and Iuliano and colleagues during PTCA. Our findings are in keeping with 15-F2t-IsoP being the most abundant isomer in plasma. Direct comparison of clinical results measured with EIA and GCMS is inappropriate, as they do not measure exactly the same compounds and the presence of co-migrating substances in GC/MS analysis may produce incorrect results. Therefore, our data is meaningful in terms of the patterns we identified.

We relied on central venous samples for our assay, which makes it difficult to localize the site of origin for 15-F2t-IsoP formation. The levels we measured are similar to those measured in coronary sinus blood by Iuliano et al, suggesting cardiac formation. In a separate pilot study mimicking conditions of CABG in a non-working rat heart model, we found the ischemic myocardium to be a source of 15-F2t-IsoP formation. While this does not exclude systemic formation of 15-F2t-IsoP, we suspect
that myocardium is a major source, at least during reperfusion. Cardiac endothelium and coronary plaque are likely sites of origin.\textsuperscript{78, 79}

15-F\textsubscript{2t}-IsoP formation occurred in a manner unrelated to the duration of CPB. Alterations in its formation, metabolism and elimination during CPB, by as yet unknown mechanisms, may explain these effects. F2 isoprostanes are metabolized by the liver to a dinor metabolite, or cleared by the kidney. While we excluded patients with pre-existent hepato-renal dysfunction, we can not exclude that a prolonged low flow or cardiac output state could reduce the clearance of 15-F\textsubscript{2t}-Isoprostane and lead to accumulation in plasma.

2.6. Conclusion

In summary, we studied perioperative 15-F\textsubscript{2t}-IsoP formation in patients undergoing CABG surgery. To the best of our knowledge, we provide first evidence that 15-F\textsubscript{2t}-IsoP formation and metabolism may be a factor in postoperative recovery of cardiac function. Although elevated primarily during ischemia, the pattern of 15-F\textsubscript{2t}-IsoP degradation during reperfusion appears clinically relevant. Besides being a marker of oxidant stress, 15-F\textsubscript{2t}-IsoP may be a prognostic indicator of postoperative cardiac dysfunction. Based on our results, persistent intraoperative elevation of 15-F\textsubscript{2t}-IsoP during reperfusion is associated with increased need for hemodynamic stabilization following CABG surgery in humans. This occurs independent of the timing and hemoglobin content of cardioplegic protection, two factors in low cardiac output syndrome. A role for 15-F\textsubscript{2t}-IsoP in the pathogenesis of myocardial IRI is suggested.
Postoperative myocardial ischemia was not evident. The mechanism of cardiac depression has yet to be determined. Determination of the mechanism(s) will be important in the development of new therapies that prevent or treat postoperative cardiac depression.
CHAPTER 3
DOSE-DEPENDENT PROTECTION OF PROPOFOL IN MYOCARDIAL IRI IN RATS: EFFECTS ON 15-F2T-ISOP FORMATION

3.1. Preface

A manuscript reporting studies described in this Chapter has been published in Canadian Journal of Physiology and Pharmacology 2003;81:14-21 (Ref. 213) and is co-authored with D.V. Godin, T. K. Chang and D. M. Ansley. T. K. Chang provided technical assistance with the assay for 15-F2T-isoprostane.

3.2. Introduction

Oxidant stress has been implicated in myocardial ischemia/reperfusion injury (IRI) and reactive oxygen species (ROS), such as the superoxide anion and hydroxyl radicals, whose formation increase during reperfusion, have been implicated.\textsuperscript{214-217} The development of an effective antioxidant therapy for the treatment of myocardial IRI is of interest. However, study of the effects of “traditional” antioxidants vitamin E and C supplementation has yielded no beneficial effects in humans with major cardiovascular risk factors, \textsuperscript{98} nor reduced myocardial injury after cardiac surgery. \textsuperscript{97}
Previous work from our laboratory has demonstrated that the intravenous anesthetic propofol enhances red cell and tissue antioxidant capacity both in vitro and in vivo. It is unknown, however, if enhancing myocardial antioxidant capacity with propofol protects against the oxidant stress associated with ischemia/reperfusion damage. A recent study reported that propofol failed to reduce ROS formation during reperfusion in the isolated reperfused rat heart. The concentration of propofol used, however, may not have been sufficient to enhance myocardial antioxidant capacity.

The purpose of the present study was to determine the effect of propofol on 15-F₂-IsoP generation and functional recovery during myocardial ischemia-reperfusion in an isolated rat heart model and to explore a novel approach to antioxidant enhancement.

3.3. Materials and methods

3.3.1. Heart preparation

The study was approved by the Committee of Animal Care of the University of British Columbia. Animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (250~300g) were anesthetized with pentobarbital (70mg/kg intraperitoneally) and heparinized with sodium heparin (1000 IU/kg, intraperitoneally). After median thoracotomy, hearts were quickly excised and immersed in ice-cold Krebs-Henseleit (KH) solution to stop contractions. Hearts were gently squeezed to remove residual blood to prevent clot formation. Hearts were retrogradely perfused via the aorta in a non-
working "Langendorff" preparation at a constant flow rate of 10ml/min using a peristaltic pump. The perfusion fluid (pH 7.4; temperature, 37°C) was KH solution that contained: 120mM NaCl; 20 mM NaHCO₃; 4.63 mM KCl; 1.2 mM MgCl₂; 1.25 mM CaCl₂; 1.17 mM KH₂PO₄; 8 mM glucose. The perfusate was bubbled with a mixture of 95% O₂ and 5% CO₂. The perfusate solution and the bath temperature were maintained at 37°C using a thermostatically controlled water circulating system. Coronary perfusion pressure (CPP) was measured via a side arm of the perfusion cannula connected to a pressure transducer (Statham p23 ID, Gould Electronics, Cleveland). A latex water-filled balloon fixed to a pressure transducer was inserted through the mitral valve into the left ventricle for the determination of left ventricular (LV) developed pressure (LVDP), which was calculated by subtracting end-diastolic pressure (LVEDP) from LV peak systolic pressure (LVSP). Rates of maximum LV pressure development and relaxation (positive dp/dt and negative dp/dt, respectively) were calculated with a differentiator. LVEDP was adjusted to approximately 5 mmHg before the start of the experiment by adjusting the volume in the intraventricular balloon. Hearts were perfused within 30 to 40 seconds after excision. Exclusion criteria included heart preparation times longer than 60 seconds and/or LVSP lower than 70 mmHg after 10 min of equilibration.

3.3.2. Experimental Protocol

All hearts were initially equilibrated for 10 min (BS10), then they were randomly allocated one of the three experimental groups (n=6 each): ischemia-reperfusion untreated control group (control), low-concentration propofol group (lo-P) and high-concentration propofol group (hi-P). A sham perfused group (Sham, n=6) was included
to test if coronary effluent 15-F$_2$-IsoP levels change over time during the period of observation for 15-F$_2$-IsoP. After BS10, propofol was applied for 10 min at 5 µg/ml (lo-P) or 12 µg/ml (hi-P) before inducing global ischemia for 40 min by stopping perfusion flow. Control hearts were equilibrated for another 10 min before inducing global ischemia for 40 min. During ischemia, saline (control) or lo-P or hi-P in saline was perfused through the aorta at 60 µl/min using a mini-pump. KH was perfused during 90 min of reperfusion in the control group. Either lo-P or hi-P in KH was perfused for the first 15 min of reperfusion. To minimize the direct negative inotropic effects of propofol on myocytes, lo-P in KH was applied during reperfusion for 75 min in both groups. The perfusion flow rate (10 ml/min) was based on the result of a pilot work which showed that hearts sham-perfused without ischemia beat well and remained hemodynamically stable for 150 min (the duration of the experiment) in our experimental set-up. It has been previously shown that up to 300 nM 15-F$_2$-IsoP had no effect on coronary flow in sham-perfused isolated rat hearts and therefore we did not include this experiment in our study. Hearts were electrically paced at a rate of 300 beats/min, prior to and following, but not during the ischemic period when hearts ceased to beat spontaneously.

Baseline effluent perfusate was sampled at BS10. Effluent samples during ischemia were collected during the first 30 min of ischemia (I-30). Also, effluent was sampled at 0.5 (Re-0.5), 5 (Re-5), 10 (Re-10) and 30 (Re-30) min of reperfusion in control and propofol-treated groups. Coronary effluent was sampled at the corresponding time points in the sham group. The effluent samples were stored at -70 °C until analysis
for free 15-F$_2$-IsoP. LV function was continuously monitored using a polygraph. At the end of the 90 min reperfusion period, hearts were immediately removed from the cannula, frozen with liquid nitrogen and stored at -70 °C. Hearts were assayed for tissue thiobarbituric acid-reactive substances (TBARS) following *in vitro* exposure to the peroxidizing agent t-butylhydroperoxide (t-BHP) within 48 hours of storage.

3.3.3. Heart tissue antioxidant capacity determination

Myocardial tissue antioxidant capacity was determined by exposure of tissue homogenates to the peroxidizing agent t-BHP. The oxidation of tissues by t-BHP results in the formation of numerous lipid byproducts, which form a chromogen when incubated with thiobarbituric acid (TBA) and are therefore collectively termed TBARS. Lower tissue antioxidant capacity will result in a greater amount of TBARS formation in the presence of t-BHP. The level of TBARS in the sample is estimated from the absorbance at 532 nm. Heart tissue samples (300 mg) were thawed and homogenized on ice in 3 ml Tris-EDTA buffer using a Polytron homogenizer for 30 s at 25% power. The resulting homogenates were used for *in vitro* forced peroxidation using t-BHP and subsequent determination of TBARS, as previously described.$^{217}$ In brief, 400 μL of tissue homogenate was combined with 400 μL t-BHP (in 0.9% saline/2 mM sodium azide to produce final concentrations of t-BHP ranging from 0.5 to 10mM). These suspensions were incubated for 30 min at 37 °C, and then 400 μL of cold 28% (w/v) TCA-0.1 M sodium arsenite was added. The mixture was centrifuged at 12,000g for 5 min at 4 °C, and 800 μL of supernatant was removed and added to 400 μL of thiobarbituric acid
(0.5% in 25 mM NaOH). The samples were boiled for 15 min, and the absorbance at 532 nm was measured spectrophotometrically.

3.3.4. 15-F₂t-IsoP Assays

Enzyme-linked immunoassay (EIA) was used to measure free 15-F₂t-IsoP levels according to the methods provided by the manufacturer (Cayman Chemical, Ann Arbor). EIA provides a sensitive measure for 15-F₂t-IsoP with a limit of quantification as low as 3.9 pg/ml. In brief, effluent samples were removed from -70 °C storage and thawed on ice. Fifty μL standards and samples were added in duplicate to the 96-well plate provided in the kit, followed by addition of 15-F₂t-IsoP acetylcholinesterase tracer and antibody. The prepared plates were then incubated overnight at room temperature. On the next day, the plates were washed 5 times with wash buffer, followed by addition of Ellman's reagent. After optimal development, the plates were read at 405 nm, and the values of the unknowns were expressed as picograms 15-F₂t-IsoP per milliliter effluent. The samples were coded and the investigator responsible for 15-F₂t-IsoP assays was blinded until the completion of the assay.

3.3.5. Statistical analysis

All data are presented as mean ± SEM. 15-F₂t-IsoP and hemodynamic data were compared by two-way ANOVA with Bonferroni's correction (GraphPad Prism).
correlation between 15-F_{2t}-IsoP concentrations and myocardial function was evaluated by the Pearson test. P<0.05 was considered statistically significant.

3.4. Results

3.4.1. 15-F_{2t}-IsoP generation during ischemia-reperfusion

As shown in Figure 3.1, baseline (BS10) 15-F_{2t}-IsoP values did not differ among groups. 15-F_{2t}-IsoP levels increased during ischemia (P < 0.01 vs BS10) and remained elevated at Re-0.5 (P < 0.05 vs BS10) in all the three experimental groups. Propofol reduced effluent 15-F_{2t}-IsoP release during ischemia and the early phase of reperfusion. During ischemia, 15-F_{2t}-IsoP levels were higher in the control than in the lo-P (P< 0.01 vs control) and hi-P groups (P<0.05 vs control) groups. At Re-0.5, effluent 15-F_{2t}-IsoP concentrations in the control group (22.5 ± 2.5 pg/ml) were also significantly higher than the corresponding values in the lo-P (14.6 ± 1.8 pg/ml, P < 0.05) and hi-P (12.0 ± 2.3 pg/ml, P < 0.05) groups. Levels of 15-F_{2t}-IsoP decreased rapidly after reperfusion in the ischemic/reperfused groups and these were not statistically differ from baseline values at Re-5 (P > 0.05). 15-F_{2t}-IsoP did not change over time during the observation period in the sham group.

3.4.2. Tissue antioxidant capacity
Heart tissue TBARS formation following *in vitro* peroxide challenge (as reflected by absorbance at 532 nm) was significantly higher in the control group than in the propofol treatment groups (Figure 3.2) at 1mM \( t \)-BHP, a concentration that provides a sensitive measure of rat tissue TBARS.\(^{217}\) The final \( t \)-BHP concentration of 1 mM was considered critical as it is a compromise between concentrations sufficiently high to produce adequate levels of TBARS but sufficiently low to avoid non-specific bleaching of the color produced by the TBA reaction. Tissue antioxidant capacity was found to be lower in the control group. Tissue TBARS formation in hi-P was lower than that in lo-P (\( P < 0.05 \)).
**Figure 3.1.** 15-F₂-isoprostane (15-F₂-IsoP) release during ischemia and reperfusion. BS10 indicates 10 minutes after equilibration; Ische indicates ischemia, samples were collected during the first 30 minutes of ischemia; Re-0.5, Re-5, Re-10 and Re-30 indicate 0.5, 5, 10 and minutes after reperfusion respectively. *P<0.05 or P<0.01 vs control; †P<0.05 or P<0.01 vs BS10.
Figure 3.2. Formation of thiobarbituric acid reactive substances (TBARS), a measure of tissue antioxidant capacity, in heart tissue (represented as absorbance at 532 nm) in the presence of 1 mM t-butylhydroperoxide. Hearts were assayed for *in vitro* TBARS formation after 90 min of reperfusion following 40 min of ischemia. * P < 0.001 vs control; † P < 0.05 vs Hi-P group.
**Figure 3.3.** Effect of Propofol on left ventricular end-diastolic pressure (LVEDP), reflecting myocardial contracture (ventricular stiffness), during ischemia (A) and reperfusion (B). Values were mean ± SEM, n=6 for all groups. *P<0.05, or P<0.01 vs control. †P<0.05 Lo-P vs Hi-P.
3.4.3. Contracture development during ischemia

Increases in LVEDP were indicative of contracture (ventricular stiffness) of isolated hearts during ischemia ("ischemic contracture"). The LVEDP increased progressively during ischemia in the control group (Figure 3.3A). Propofol at the high (12 μg/ml) but not at the low (5 μg/ml) concentration, reduced LVEDP (P < 0.05, hi-P vs control at 35 and 40 min ischemia). At 35 min of ischemia, the magnitude of LVEDP in the hi-P group was also less than in the lo-P group (7.3 ± 3.3 vs 26.5±7.1 mm Hg, P < 0.05). In both lo-P and hi-P groups, the latency to the onset of contracture was significantly increased (21.2 ± 2.1 and 24.5 ± 2.3 min in lo-P and hi-P, P < 0.05 vs 15.0 ± 1.1 min in control).

3.4.4. Functional response to ischemia -reperfusion

One of the main effects of propofol on functional recovery during reperfusion was a difference in LVEDP (an indicator of reperfusion-induced increase in "ventricular stiffness"). During reperfusion, LVEDP in the control group increased over time and peaked at 65.7 ± 10.4 mmHg at 90 min of reperfusion (Re-90) (Figure 3.3B). The low concentration of propofol attenuated the increase of LVEDP at 60 min of reperfusion (Re-60) and Re-90 (P < 0.05 vs control). The magnitude of LVEDP was less in the hi-P than in the lo-P group after Re-30 (P < 0.05, hi-P vs lo-P). In fact, the high concentration of propofol completely prevented the increase in ventricular stiffness during the experiment.
The LVDP in the untreated control group recovered to a maximum of 78.2 ± 7.9 % of its baseline value at Re-30 (Table 3.1) and decreased progressively thereafter. At Re-90, LVDP in the control group was lower than its baseline value (P < 0.05, Table 3.1). The low concentration propofol prevented the progressive decrease of LVDP seen in the control group after Re-30. Application of hi-P for 10 min prior to ischemia and during the first 15 min of reperfusion was associated with a significant decrease in LVDP and LVSP prior to ischemia and at Re-10. However, LVDP rapidly recovered to baseline values after Re-30 in the hi-P group (Table 3.1), and was about 25% higher than that in the lo-P group at Re-30. The rapid recovery of LVDP observed in hi-P coincided with the reduction in propofol concentration from 12 µg/ml to 5 µg/ml after 15 min of reperfusion. The LVDP, as a percentage of baseline values at Re-90, was significantly higher in the hi-P (P < 0.01) and lo-P groups (P < 0.05) than in the controls, despite the fact that propofol at 5 µg/ml may have inhibited myocardial contractility. Propofol (5 µg/ml) inhibition of myocardial contractility was evidenced by the significant decrease of LVDP from baseline to pre-ischemia in the lo-P group (Table 3.1).

Since one of the major goals of this study was to optimize the long-term protective effects of propofol, administration of propofol was not discontinued during reperfusion. This mimicked the clinical study of propofol used as the principal anesthetic and also possibly to increase myocardial antioxidant status during cardiopulmonary bypass surgery, and then continued at reduced dosage for post-operative sedation.\textsuperscript{125} This approach however, makes it inappropriate to compare post-ischemic LVDP values in the propofol treatment groups directly with that in the control group because of the potential confounding negative inotropic effect of propofol. The within-group percentage changes
of LVDP from Re-60 to Re-90 (the later phase of reperfusion in this study) best reflected the post-ischemic myocardial function preservation at different experimental conditions and served as a meaningful index for comparison between groups. The percentage decrease of LVDP from Re-60 to Re-90 in hi-P (-3.0±2.6%) was less than that in either control (-19.1±6.6%, P < 0.05) or in lo-P (-11.4±2.5%, P < 0.05). The value for lo-P did not differ statistically from that of the control group (P > 0.05).

After 90 min of reperfusion, LV relaxation (-dp/dt) in the control group was 44.2±4.7% of baseline, whereas LV development (+dp/dt) was 65.7±10.4% of its baseline (BS10) value. Recovery as a percentage of the baseline (BS10) value for the rates of LV -dp/dt paralleled the responses observed with LVDP in the hi-P, lo-P and control groups. Hi-P, but not lo-P, also resulted in significantly better recovery of LV +dp/dt (P < 0.05) at Re-90 compared to the control group.

3.4.5. Coronary perfusion pressure

Coronary perfusion pressure (CPP) increased significantly after 60 min of reperfusion in the control and lo-P groups (P < 0.05 vs BS10, Table 3.1). The low concentration of propofol did not significantly reduce CPP during reperfusion (P > 0.05 vs control). However, the high concentration of propofol reduced CPP by 20% before ischemia (Table 3.1) and prevented the significant increase of CPP after reperfusion seen in all the other groups. The CPP value at Re-90 in the hi-P group was lower than that in the control (P < 0.05).
Table 3.1. Left Ventricular Developed Pressure (LVDP), LV Systolic Pressure (LVSP) and Coronary Perfusion Pressure (CPP) (mm Hg)

<table>
<thead>
<tr>
<th></th>
<th>BS10</th>
<th>BS20</th>
<th>Re-10</th>
<th>Re-30</th>
<th>Re-60</th>
<th>Re-90</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVDP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>86.6 ± 5.2</td>
<td>95.6 ± 4.8</td>
<td>49.0 ± 15.0*</td>
<td>67.3 ± 6.2</td>
<td>57.5 ± 6.7</td>
<td>47.1 ± 8.4*</td>
</tr>
<tr>
<td>%BS10</td>
<td>110.9 ± 3.9</td>
<td>57.3 ± 17.2</td>
<td>78.2 ± 7.9</td>
<td>66.7 ± 7.7</td>
<td>54.3 ± 8.0</td>
<td></td>
</tr>
<tr>
<td>Lo-P</td>
<td>80.0 ± 4.1</td>
<td>61.3 ± 1.9*</td>
<td>41.7 ± 11.7*</td>
<td>55.5 ± 12.0</td>
<td>69.0 ± 7.7</td>
<td>60.3 ± 4.0</td>
</tr>
<tr>
<td>%BS10</td>
<td>77.1 ± 2.0*</td>
<td>52.6 ± 14.7</td>
<td>73.9 ± 11.5</td>
<td>86.3 ± 8.5</td>
<td>75.8 ± 4.3*</td>
<td></td>
</tr>
<tr>
<td>Hi-P</td>
<td>78.2 ± 5.1</td>
<td>42.3 ± 7.1**</td>
<td>32.5 ± 6.1*</td>
<td>76.5 ± 7.4</td>
<td>67.5 ± 7.0</td>
<td>66.0 ± 8.3</td>
</tr>
<tr>
<td>% BS10</td>
<td>53.2 ± 7.2*</td>
<td>41.5 ± 7.4</td>
<td>97.6 ± 6.4</td>
<td>85.9 ± 4.9*</td>
<td>63.8 ± 6.5*</td>
<td></td>
</tr>
<tr>
<td>LVSP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>91.7 ± 5.3</td>
<td>100.5 ± 4.8</td>
<td>85.2 ± 6.5</td>
<td>106.8 ± 3.6</td>
<td>116.5 ± 5.8*</td>
<td>112.8 ± 5.0</td>
</tr>
<tr>
<td>Lo-P</td>
<td>84.8 ± 4.0</td>
<td>65.7 ± 2.0**</td>
<td>59.2 ± 5.7**</td>
<td>77.0 ± 8.8*</td>
<td>92.8 ± 5.7*</td>
<td>87.5 ± 5.7*</td>
</tr>
<tr>
<td>Hi-P</td>
<td>83.0 ± 5.1</td>
<td>46.8 ± 6.9**</td>
<td>38.7 ± 6.2**</td>
<td>82.8 ± 7.1*</td>
<td>73.5 ± 7.2*</td>
<td>73.2 ± 9.2*</td>
</tr>
<tr>
<td>CPP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>57.2 ± 6.6</td>
<td>58.3 ± 6.9</td>
<td>60.0 ± 6.8</td>
<td>79.7 ± 11.2</td>
<td>95.7 ± 17.0*</td>
<td>103.8 ± 14.7*</td>
</tr>
<tr>
<td>Lo-P</td>
<td>52.7 ± 1.6</td>
<td>48.5 ± 2.6</td>
<td>50.5 ± 1.4</td>
<td>64.2 ± 4.9</td>
<td>79.2 ± 11.2*</td>
<td>86.7 ± 10.6*</td>
</tr>
<tr>
<td>Hi-P</td>
<td>50.8 ± 1.54</td>
<td>40.5 ± 2.5*</td>
<td>45.0 ± 4.4</td>
<td>52.5 ± 3.9</td>
<td>54.8 ± 5.1</td>
<td>62.0 ± 6.9*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n=6 for each group). BS10=baseline, BS20=pre-ischemia; Re-10, Re-30, Re-60 and Re-90 refer to 10, 30, 60 and 90 min after reperfusion. There is no significant difference in BS10 among groups for LVDP, LVSP and CPP. *P<0.05 or P<0.01, vs control; †P<0.05 or P<0.01 vs baseline.
Figure 3.4. Relationship between 15-F_2t-IsoP generation upon reperfusion and coronary perfusion pressure (CPP) at 90 minutes of reperfusion (n=18). CPP was positively correlated (r=0.74, 95%CI: 0.4180 to 0.8972, P=0.0004) with 15-F_2t-IsoP generation upon reperfusion.
3.4.6. Lipid peroxidation and post-ischemic myocardial function

Figure 3.4 depicts a strong positive correlation between effluent 15-F$_2$-isoP levels at Re-0.5 and CPP at Re-90 (r = 0.74, P = 0.0004). A weak, but significant negative correlation was obtained between LVDP recovery at Re-90 as a percentage of baseline and effluent 15-F$_2$-isoP levels at Re-0.5 (r = -0.585, P = 0.011). In general, effluent 15-F$_2$-isoP levels at Re-0.5 correlated with tissue TBARS formation at Re-90 (n=18, r=0.66, P<0.003), but this association resulted primarily from a good correlation between 15-F$_2$-isoP release and tissue TBARS formation in the propofol treatment groups (n=12, r =0.74, P=0.005). On the contrary, two control hearts that released the least 15-F$_2$-isoP at Re-0.5 in the group ended up with highest tissue TBARS formation (indicating lowest tissue antioxidant capacity preservation). Taking into consideration the immediate and rapid increase in CPP upon reperfusion in these two hearts, the lower release of 15-F$_2$-isoP at Re-0.5 most likely resulted from the sequestration of 15-F$_2$-isoP in the inadequately (or non-) reperfused tissue.

A negative correlation was obtained between heart tissue TBARS formation and LVDP recovery at Re-90 as a percentage of baseline (r = -0.71, Figure 3.5), and especially between tissue TBARS formation and changes of LVDP from Re-60 to Re-90 (r = -0.72, P =0.0007, Figure 3.6), which best represented post-ischemic myocardial function preservation in this model.
Figure 3.5. Recovery of left ventricular developed pressure (LVDP) after 90 min of reperfusion inversely correlated with the formation of heart tissue thiobarbituric acid reactive substances (TBARS), a measure of tissue antioxidant capacity ($r = -0.71$, 95% CI: -0.8837 to -0.3629, $P = 0.001$). Heart tissue was assayed for in vitro TBARS formation after 90 min of reperfusion following 40 min of ischemia, in the presence of 1mM t-butylhydroperoxide.
Figure 3.6. Changes of left ventricular developed pressure (LVDP) after reperfusion (from reperfusion 60 to 90 min) inversely correlated with the formation of heart tissue thiobarbituric acid reactive substances (TBARS) in the presence of 1 mM t-butyldihydroperoxide (r = -0.72, 95% CI: -0.8898 to -0.3672, P = 0.0007). It indicated a positive correlation between post-ischemic preservation of myocardial function and tissue antioxidant capacity.
3.4.7. Pre-ischemic myocardial depression and post-ischemic myocardial function

Administration of hi-P (12 µg/mL) and lo-P (5 µg/mL) for 10 min before ischemia decreased myocardial contraction as evidenced by the significantly lower pre-ischemic LVDP values in the propofol treatment groups than in the control (Table 3.1). Hi-P decreased LVDP (-46.8±7.2% reduction from baseline) more than lo-P (-22.9±2.0%, P < 0.05). This contrasts with a slight increase of pre-ischemic LVDP in the control group relative to its baseline value (P > 0.1). In general, a weak negative correlation was obtained between the percentage change of LVDP from baseline to pre-ischemia and the post-ischemic percentage recovery of LDVP at Re-90 (n=18, r=-0.52, P =0.03), the later phase of reperfusion. However, this relationship primarily existed in the lo-P and control groups (n=12, r = -0.61, P =0.04), but not in the two propofol treatment groups (r =0.13, P=0.6).

3.5. Discussion

To our knowledge, this is the first study to use 15-F_{2\text{-isoP}} as an index of the effects of propofol on lipid peroxidation in an isolated rat heart model of ischemia-reperfusion. In this study, we found: 1) lipid peroxidation occurs during both global myocardial ischemia and reperfusion; 2) propofol (at 5 µg/ml and 12 µg/ml) significantly inhibits lipid peroxidation during myocardial IRI; 3) post-ischemic cardiac functional preservation positively correlated with heart tissue antioxidant capacity and inversely correlated with the extent of lipid peroxidation during reperfusion; 4) the protective effect
of propofol on post-ischemic preservation of cardiac function and heart tissue antioxidant capacity are concentration-dependent, being greater at 12 μg/mL than at 5 μg/mL in this model.

Baseline 15-F₂t-isoP values in this model were within the limits of quantification of the assay. The baseline generation of 15-F₂t-isoP in our model likely represents release from normal myocardial tissue and not from ischemic insult during heart isolation, because of careful organ harvesting and reperfusion within 40 seconds after excision. 15-F₂t-isoP is detectable in the rat heart and plasma under control conditions. Since large quantities of isoprostanes can be generated ex vivo, we wanted to ensure that the measured 15-F₂t-isoP was generated by the heart during ischemia, and not produced during collection or storage. We found that the values did not change when effluent was stored overnight at room temperature (data not shown). This is a similar finding to that of Morrow and Robert, who found that urinary 15-F₂t-isoP levels did not increase when urine was incubated at 37 °C for 5 days. It is unlikely that 15-F₂t-isoP would be generated in substantial amounts in samples containing very small amounts of lipid, ex vivo.

In our experimental model, a small volume of physiological saline was infused during ischemia in order to quantitatively assess the extent of lipid peroxidation during global ischemia and validate a novel antioxidant therapy regimen involving propofol. We found that this delayed the onset of ischemic contracture as compared to the observation of Ko et al and of our own pilot work, where the onset of ischemic contracture was about 5 min when saline infusion during ischemia was not incorporated (n = 3; data not
shown). The precise mechanism for the delay of ischemic contracture onset by infusing saline during ischemia is uncertain. It might be attributable, at least in part, to the wash-out of 15-F_{2t}-isoP during ischemia by the infused saline. 15-F_{2t}-isoP is a potent vasoconstrictor. Pretreatment with vasoconstrictors such as norepinephrine or the calcium channel agonist BAY K 8644 has been shown to accelerate and exacerbate ischemic contracture in isolated rat hearts. In a pilot study we infused 15-F_{2t}-isoP (100 nM) during global myocardial ischemia. This increased the magnitude of ischemic contracture and shortened the time to peak contracture (data not shown). In addition, propofol, when administrated at a concentration of 5.3 μg/mL (30 μM) for 10 min immediately before global ischemia, did not slow the onset of ischemic contracture, nor did it reduce the magnitude of ischemic contracture. Propofol at 5 μg/mL, when applied both before and during ischemia, significantly slowed the onset of ischemic contracture in our study, and this was accompanied by a significant decrease in 15-F_{2t}-isoP release during ischemia as compared with the control group. These data implicate oxidative stress and the generation of 15-F_{2t}-isoP as potential contributors to myocardial ischemic contracture.

We found that ROS-mediated lipid peroxidation (as indicated by release of 15-F_{2t}-isoP) occurred mainly during ischemia and in the early phase of reperfusion in this model (Figure 3.1). In our experimental design, we decreased the propofol concentration from 12 μg/mL (hi-P) to 5 μg/mL (lo-P) after 15 min of reperfusion in order to avoid or reduce the possible direct negative inotropic effects of propofol on myocytes. The fact that heart tissue TBARS formation in the hi-P group was significantly lower than that in the lo-P group (Figure 3.2) after the completion of the experiment further indicated that
tissue antioxidant status was compromised mainly during ischemia and early reperfusion. This is because the major difference between these two groups is that propofol was applied at higher concentration during ischemia and the first 15 min of reperfusion. It validated the necessity for reducing propofol concentration during the late phase of reperfusion when antioxidant therapy is the major consideration to the use of high dose propofol. In fact, continuous application of hi-P (12 μg/mL) beyond the early phase of reperfusion compromised or prevented myocardial function recovery, based on our pilot work. This may have potential clinical significance.

Forty minutes of ischemia induced a profound ischemic insult, as evidenced by the magnitude of the ischemic contracture (Figure 3.3A). Myocardial ischemia and the subsequent reperfusion were accompanied by significant myocardial lipid peroxidation as evidenced by the significant increase in coronary effluent 15-F_{2\alpha}-isoP concentration during ischemia and the early phase of reperfusion. It is noteworthy that coronary effluent 15-F_{2\alpha}-isoP levels at Re-0.5 were negatively correlated with myocardial functional recovery after reperfusion 90 min. Tissue TBARS formation negatively correlated with post-ischemic myocardial function recovery (Figure 3.5) and cardiac function preservation after prolonged reperfusion (Figure 3.6). This provides indirect evidence that the decrease of endogenous tissue antioxidant capacity and the subsequent increase of lipid peroxidation during myocardial ischemia and reperfusion are major mediators of myocardial IRI.

Applying hi-P during ischemia and the early phase of reperfusion provided more long-lasting myocardial protection than lo-P. This is evidenced by the significantly lower
percentage decrease of LVDP from Re-60 to Re-90 in the hi-P than in the lo-P group. Interestingly, in our study, hi-P (12 µg/mL), but not lo-P (5 µg/mL), completely prevented the increase of LVEDP during reperfusion (Figure 3.3B) which was apparently not completely achieved by Mathur et al.\textsuperscript{223} They applied propofol at 6.2 µg/mL (35 µM) plus HOE 642 (a sodium ion-hydrogen ion exchange inhibitor) for 15 min before global ischemia and throughout 60 min of reperfusion in isolated rat hearts and achieved greater post-ischemic myocardial functional recovery than using propofol or HOE 642 alone. Our findings indicate that oxidant stress might be a major and/or initial contributor to myocardial IRI, whereas other mechanisms such as ion imbalances may be secondary mediators of myocardial IRI. Our study indicated that hi-P enhanced heart tissue antioxidant capacity to a greater extent than lo-P (Figure 3.2).

It should be noted that antioxidant vitamins (vitamin E and C) are not able to prevent ROS production from other sources in whole blood despite their ability to reduce ROS release from polymorphonucleated cells.\textsuperscript{224} Vitamin E and C impaired vascular function in the experimental diabetic rat.\textsuperscript{225} In contrast, propofol effectively preserved heart tissue antioxidant capacity and myocardial function. Our present study suggests that when applied in adequate amount, antioxidant therapy with propofol could potentially reduce myocardial IRI during cardiac surgery in high-risk patients, such as those with diabetes.

3.2. Conclusion
Our results support the hypothesis that propofol facilitates myocardial functional recovery following ischemia and reperfusion primarily by preventing lipid peroxidation. This does not exclude the possible role of other actions of propofol, including preservation of high energy phosphates\textsuperscript{223} and facilitation of metabolic recovery\textsuperscript{226} as intermediate mechanisms of cardio-protection. These, however, are not likely the major mechanism of protection, since the degree of pre-ischemic myocardial depression did not correlate with post-ischemic myocardial function recovery in the two propofol treatment groups. In terms of clinical implications: clinically achievable high concentrations of propofol (12 \mu g/mL),\textsuperscript{125} administrated primarily during myocardial ischemia and the early phase of reperfusion, can provide greater cardiac protection than when applied at a lower concentration. Antioxidant therapy should be focused on the period of ischemia and the earliest stages of reperfusion. The concern regarding cardiovascular depression with the use of high-dose propofol would likely be minimized if it were administered primarily during the period of cardiopulmonary bypass support.
CHAPTER 4

PROPOFOL EFFECTS ON ISCHEMIC TOLERANCE OF MIDDLE-AGED RAT HEARTS: EFFECTS OF 15-F_{2\alpha}-ISOP FORMATION AND TISSUE ANTIOXIDANT CAPACITY

4.1. Preface

This investigation was supported, in part, by funding from the Centre for Anesthesia and Analgesia, Dept. of Pharmacology & Therapeutics, The University of British Columbia. A manuscript reporting studies described in this Chapter has been published in *Cardiovascular Research 2003;59:113-21* (ref.227) and is co-authored with D. V. Godin and D. M. Ansley.

4.2. Introduction

Aging is known to be associated with biochemical and functional changes in the heart. Animal studies have shown an age-related decrease in recovery of cardiac function with post-ischemic reperfusion. Clinical studies have shown that age is one of the best predictors for operative mortality in patients undergoing cardiac surgery using cardiopulmonary bypass (CPB). However, the post-operative outcomes in terms of long-term survival and freedom from angina were excellent in senescent as compared with middle-aged patients. Thus, it is important to determine if the susceptibility of the myocardium to ischemia-reperfusion injury (IRI) varies with age, and to identify treatments that will effectively protect less tolerant myocardium.
Recent studies have shown that middle-aged rat hearts became more vulnerable to ischemic insult and that rat coronary arteries became more sensitive to the vasoconstrictor endothelin-1 before and after ischemia-reperfusion, particularly during the period of maturation from youth to adulthood. Oxidative stress may occur during myocardial ischemia-reperfusion and contribute to IRI secondary to lipid peroxidation of cell membranes. It has been reported that reactive oxygen species (ROS) and products of oxidation increase with age, accompanied by a reduction in tissue antioxidant capacity.

Based on these observations, we postulate that antioxidant intervention could increase ischemic tolerance and enhance postischemic myocardial functional recovery of the middle-aged rat hearts. Our previous work has demonstrated that the intravenous anesthetic propofol enhances red cell and tissue antioxidant capacity both in vitro and in vivo. We have recently found that enhancing myocardial antioxidant capacity with propofol protects against the oxidant stress associated with ischemia-reperfusion damage in young rat hearts. We hypothesize that propofol may provide effective protection against myocardial IRI of the more vulnerable middle-aged rat hearts and that this protection is related to the reduction of lipid peroxidation during ischemia-reperfusion. The hypothesis was tested in an isolated rat heart model, using 15-F_{2\alpha}-isoprostane (15-F_{2\alpha}-isoP, previous name 8-epi-PGF_{2\alpha}), a specific and reliable index of lipid peroxidation, as a measure of oxidative injury.
4.3. Materials and methods

4.3.1. Heart perfusion

The study was approved by the Committee of Animal Care of the University of British Columbia. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-25, 1996). Young (Y, 10 weeks, weighing 250-300 g) or middle-aged (M, 20 weeks, weighing 550-620 g) Male Sprague-Dawley rats were anesthetized with pentobarbital (70mg/kg, intraperitoneally) and heparinized with sodium heparin (1000 IU/kg, intraperitoneally). After thoracotomy, hearts were quickly excised and immersed in ice-cold Krebs-Henseleit (KH) solution to stop contractions. Hearts were gently squeezed to remove residual blood and thereby to prevent clot formation. Hearts were retrogradely perfused via the aorta as non-working "Langendorff" preparations at a constant flow rate using a peristaltic pump. The perfusion flow rate was 10 ml/min in young and 15 ml/min in middle-aged rat hearts. The choice of different perfusion flow rates was based on our pilot study results showing: 1) these flow rates yielded a comparable initial coronary perfusion pressure of about 50 mmHg in the hearts; 2) hearts beat well and remain hemodynamically stable for a duration of 150 min (the duration of our study) when sham-perfused without ischemia and reperfusion (n=3 each for young and middle-aged rat hearts). The flow rate for young rat hearts was the same as that used by Mathur et al., whereas the flow rate for the middle-aged rat hearts was comparable to that achieved by Goodwin et al. The perfusion fluid (pH 7.4; temperature, 37°C) was KH solution that contained: 120mM NaCl; 20mM NaHCO3; 4.63 mM KCl; 1.2mM
MgCl\(_2\); 1.25 mM CaCl\(_2\); 1.17 mM KH\(_2\)PO\(_4\) and 8 mM glucose. The perfusate was bubbled with a mixture of 95% \(O_2\) and 5% \(CO_2\). The perfusate solution and the bath temperature were maintained at 37°C using a thermostatically controlled water circulating system. During the experiment, the heart was in a chamber with circulating water in its jacket thermostatically controlled at 37°C. The chamber was properly sealed during the experiment, and the inside chamber environmental temperature was continuously monitored with a thermometer and maintained between 36.9 °C to 37.1 °C during ischemia. Coronary perfusion pressure (CPP) was measured via a side arm of the perfusion cannula connected to a pressure transducer (Statham p23 ID, Gould Electronics, Cleveland). A latex water-filled balloon fixed to a pressure transducer was inserted through the mitral valve into the left ventricle for the determination of left ventricular (LV) developed pressure (LVDP), which was calculated by subtracting end-diastolic pressure (LVEDP) from LV peak systolic pressure (LVSP). LVEDP was adjusted to approximately 5 mmHg before the start of the experiment by adjusting the volume in the intraventricular balloon. Hearts were perfused within 30 to 40 seconds after excision. Exclusion criteria included heart preparation times longer than 60 seconds and/or LVSP lower than 70 mmHg after 10 min equilibration.

4.3.2. Experimental Protocol

All hearts were initially equilibrated for 10 min (baseline, BS), and they were then randomly divided into one of four groups (n=6 each): ischemia-reperfusion untreated control groups of young (C-Y group) and middle-aged (C-M group) rat hearts, propofol treatment groups of young (P-Y group) and middle-aged (P-M group) rat hearts. Previous
studies in our laboratory have shown that the carrier vehicle for propofol was devoid of antioxidant activity, and therefore it was deemed unnecessary to include vehicle controls into an experimental protocol.\textsuperscript{126}

After the initial equilibration, propofol was applied for 10 min at 12 μg/ml (67 μM) in the P-Y and P-M groups prior to inducing global ischemia by stopping the perfusion flow for 40 min. Control (non-propofol-treated) hearts were equilibrated for another 10 min prior to inducing global ischemia for 40 min. During ischemia, saline (controls) or propofol (P-Y and P-M) in saline (12 μg/ml) was perfused through the aorta at 60 μl/min using a mini-pump. KH was perfused during the 90 min of reperfusion in the control groups. Propofol 12 μg/ml in KH was perfused for the first 15 min of reperfusion, followed by propofol 5 μg/ml in KH for 75 min in both propofol treatment groups. This reduction in propofol concentration during reperfusion was effected to avoid or reduce the possible direct negative effects of propofol on myocytes.\textsuperscript{219} Hearts were electrically paced at a rate of 300 beats/min, prior to and following, but not during the ischemic period. LV function was continuously monitored using a polygraph. At the end of the 90 min of reperfusion period, hearts were immediately removed from the cannula, precooled in liquid nitrogen and stored at -70 °C. Hearts were assayed for tissue thiobarbituric acid reactive substances (TBARS) formation within 48 hours of storage.

4.3.3. 15-F$_2$-isoP Assays

Effluent perfusate was sampled at baseline (BS), during the first 30 min of ischemia (I-30) and at 0.5 (Re-0.5), 5 (Re-5) and 30 (Re-30) min of reperfusion in control
and propofol-treated groups. Effluent perfusate samples at baseline and after reperfusion were collected over a 10-second period, while the samples during ischemia were collected over a period of 30 min. The effluent samples were stored at -70 °C until analysis for free 15-\(F_2\)-isoP.

Enzyme-linked immunoassay (EIA) was used to measure free 15-\(F_2\)-isoP levels as mentioned before (Chapter 3.3.4).

4.3.4. **Heart tissue antioxidant capacity determination**

Tissue antioxidant capacity was determined by exposure of tissue homogenates to the peroxidizing agent t-butylhydroperoxide (t-BHP). The oxidation of tissues by t-BHP results in the formation of numerous lipid byproducts, which are collectively termed TBARS. The levels of TBARS in the sample were estimated from the absorbance at 532 nm (18). Heart tissue samples (300 mg) were thawed and homogenized on ice in 3 ml Tris-EDTA buffer using a Polytron (PT-10, Brinkman Instruments, Canada) homogenizer for 30 s at 25% power. The resulting homogenates were used for *in vitro* forced peroxidation and subsequent determination of TBARS as previously described.\(^{240}\) In brief, 400 \(\mu\)L of tissue homogenate were combined with 400 \(\mu\)L t-BHP (in 0.9% saline/2 mM sodium azide to produce t-BHP concentrations ranging from 0.5 to 10mM). These suspensions were incubated for 30 min at 37 °C, then 400 \(\mu\)L of cold 28% (w/v) TCA-0.1 M sodium arsenite was added. The mixture was centrifuged at 12,000g for 5 min at 4 °C, and 800 \(\mu\)L of supernatant was removed and added to 400 \(\mu\)L of thiobarbituric acid
90

(0.5% in 25 mM NaOH). The samples were boiled for 15 min, and the absorbance at 532 nm was measured spectrophotometrically.

4.3.5. Data Analysis

All data are presented as mean ± SEM. Effluent 15-F_{2t}-isoP concentration and hemodynamic data were compared by two-way ANOVA with Bonferroni's correction (GraphPad Prism). One-way repeated measures ANOVA and Tukey's Multiple Comparison test were applied for within-group comparison. The correlation relationships were evaluated by the Pearson test. P < 0.05 (two-tailed) was considered significant.

4.4. Results

4.4.1. 15-F_{2t}-isoP generation during ischemia-reperfusion

Baseline 15-F_{2t}-isoP values were low (5.0 ± 0.5, 6.1 ± 1.0, 4.5 ± 0.4 and 4.0 ± 0.1 pg/ml in C-Y, P-Y, C-M and P-M groups, respectively) and did not differ among the various experimental groups (P>0.1). Since perfusion flow rates were different between young and middle-aged rat hearts, 15-F_{2t}-isoP production at baseline and during reperfusion was converted according to the flow rates and presented as the amount of 15-F_{2t}-isoP produced per minute (pg/min) in the effluent. 15-F_{2t}-isoP production during ischemia was, however, presented as pg/mL.

As shown in Figure 4.1, 15-F_{2t}-isoP levels increased significantly at Re-0.5 (P<0.05 or P<0.01 vs baseline) in all experimental groups. It decreased to close to
baseline levels in P-Y and P-M groups (P>0.05 vs baseline) at Re-5 and in C-Y and C-M groups at Re-30 (Figure 4.1A). During early reperfusion, 15-F₂-isOP levels fell more rapidly in the C-Y than in the C-M group. At Re-5 min, a significant decrease of 15-F₂-isOP from Re-0.5 was seen in the C-Y, but not in the C-M group. Propofol significantly reduced 15-F₂-isOP production at 0.5 min of reperfusion in young (P<0.05, P-Y vs C-Y) and at 5 min of reperfusion in the middle-aged rat hearts (P<0.05, P-M vs C-M). 15-F₂-isOP was produced in substantial amounts during ischemia in the C-Y and C-M groups (Figure 4.1B). Propofol significantly reduced 15-F₂-isOP production during ischemia in both young and middle-aged rat hearts.

4.4.2. Tissue antioxidant capacity

As shown in Figure 4.2, tissue TBARS formation following in vitro peroxide challenge (as reflected by absorbance at 532 nm) was significantly higher in control groups than in propofol treatment groups at t-BHP concentrations higher than 0.5 mM (P<0.01 or P<0.001 P-Y vs C-Y or P-M vs C-M). At a t-BHP concentration of 2 mM, tissue TBARS formation in C-M was found to be significantly higher than that in the C-Y group (P = 0.03, TBARS absorbance = 0.519 ± 0.019 in C-M vs 0.429 ± 0.028 in C-Y). This indicated lower tissue antioxidant preservation in the C-M group than in the C-Y group. Nevertheless, propofol treatment reduced TBARS absorbance at 2 mM t-BHP to the same level in the P-M (0.214 ± 0.017) as in the P-Y (0.222 ± 0.014) group, suggesting that propofol could more effectively preserve tissue antioxidant capacity during ischemia-reperfusion in middle-aged rat hearts than in young rat hearts.
Figure 4.1. Coronary effluent 15-F_{2\alpha}-isoprostane release during ischemia (B) and reperfusion (A). Effluent was sampled at baseline, 0.5 (Re-0.5), 5 (Re-5) and 30 (Re-30) min of reperfusion for a duration of 10 second. Effluent during ischemia was collected for the first 30 min. Values are mean ± SEM. *P < 0.05 or P < 0.01 vs baseline; †P < 0.05 P-Y vs C-Y or P-M vs C-M group; ‡P<0.05 Re-5 or Re-30 vs Re-0.5 min; P > 0.05 P-M vs P-Y or C-M vs C-Y.
Figure 4.2. Thiobarbituric acid reactive substances (TBARS) formation (as reflected by absorbance at 532 nm) as a function of t-BHP concentration for heart tissues at reperfusion 90 min. Heart tissue were sampled after 40 min of ischemia and 90 min of reperfusion. Values are mean ± SEM. *P < 0.01 P-Y vs C-Y or P-M vs C-M group; #P < 0.05 C-M vs C-Y; P > 0.05 P-M vs P-Y.
4.4.3. Effects of propofol on contracture development during ischemia and reperfusion

LVEDP increased progressively during ischemia in the two control groups (Figure 4.3). Maximum ischemic contracture (reflected as peak LVEDP) in the C-M group (38.3±3.8 mmHg) was not significantly different from that in the C-Y (37.8±3.0 mmHg) group (Table 1). There was no significant difference in the onset time of ischemic contracture and time to peak LVEDP between C-Y and C-M groups. LVEDP increased more rapidly in the C-M group after 25 min of ischemia. LVEDP at ischemia 30 min (and onwards) was significantly higher than that at ischemia 25 min (P = 0.03) in the C-M group (Figure 4.3). The gradual increase in LVEDP in the C-Y group only became significant after 40 min of ischemia. Propofol significantly reduced LVEDP in young rat hearts after 35 min of ischemia, and completely abolished ischemic contracture in middle-aged rat hearts during the 40 min period of ischemia (Figure 4.3).

Reperfusion was associated with a generalized elevation in LVEDP in both C-Y and C-M groups (Figure 4.4) when compared to the LVEDP of approximately 5 mmHg before ischemia. The LVEDP further increased over time after 30 min of reperfusion in the C-Y and C-M groups. The LVEDP at 5 min of reperfusion was 7.8 ± 1.2 mmHg in P-Y and 5.8 ± 0.5 mmHg in the P-M group and was significantly lower than the corresponding values in C-Y and C-M (Figure 4.4). LVEDP did not increase over time during the 90 min period of reperfusion in either the P-Y or the P-M group.
Figure 4.3. Left ventricular end diastolic pressure (LVEDP), reflecting myocardial contracture during ischemia. Values are mean ± SEM. *P < 0.05 vs C-Y group; †P < 0.01 vs C-Y and C-M groups. #P < 0.05 vs ischemia 25 min of the same group. Propofol (67 μM) completely abolished ischemic contracture (i.e., LVEDP = 0) in all six hearts in the P-M group.
Figure 4.4. Left ventricular end diastolic pressure (LVEDP), reflecting myocardial contracture during reperfusion following 40 min of global ischemia. Values are mean ± SEM. *P < 0.05 or P < 0.01 vs C-Y or C-M groups. P > 0.05 C-Y vs C-M or P-Y vs P-M groups.
4.4.4. Coronary Perfusion Pressure

As shown in Table 4.1, initial CPP values were comparable in C-Y, P-Y, C-M and P-M groups. CPP values increased gradually after reperfusion in the C-Y and C-M groups and were significantly higher than baseline after 30 min of reperfusion in the C-M group and after 60 min of reperfusion in the C-Y group. Administration of propofol for 10 min before ischemia significantly reduced CPP in young but not in the middle-aged rat hearts. Propofol prevented the increase of CPP after reperfusion that was seen in the control groups. The CPP was significantly lower in P-Y and P-M than in C-Y and C-M, respectively, after 90 min of reperfusion.

4.4.5. Left ventricular mechanics

Figure 4.5 depicts the pre- and post-ischemic values of LVDP, a measure of myocardial contractile function. Baseline LVDP values did not differ among the 4 experimental groups. Propofol administration for 10 min before ischemia reduced LVDP by 43.2% in P-Y and 36.5% in P-M groups. The pre-ischemia values of LVDP in P-Y and P-M were significantly lower than the corresponding values at baseline (P<0.01) prior to inducing ischemia. The LVDP in the C-Y and C-M groups recovered to 82.0 ± 8.6 % and 87.2 ± 9.0% of the corresponding baseline values, respectively, at the 30 min (Re-30) reperfusion time-point and decreased progressively thereafter. At 90 min of reperfusion (Re-90), LVDP in the C-Y and C-M groups was significantly lower than their baseline values (P<0.05). Propofol treatment resulted in better recovery of LVDP in middle-aged than in young rat hearts following reperfusion. LVDP in the P-M group was
significantly higher than that in the P-Y group after 60 min (Re-60) and 90 min of reperfusion. At Re-90, LVDP in the P-M was significantly higher than that in the C-M group (P<0.05).

Since one of the major goals of this study was to optimize the long-term protective effects of propofol, administration of propofol was not discontinued during reperfusion. For a better comparison among groups, the percentage change of LVDP from Re-60 to Re-90 was calculated. The percentage decrease of LVDP from Re-60 to Re-90 in the C-M group (-27.8±1.8%) was significantly higher than that in the C-Y group (-17.9±3.9%) (P<0.05), both being significantly higher than that in the corresponding propofol treatment groups. The percentage decrease of LVDP from Re-60 to Re-90 did not differ between P-Y (-3.6±2.7%) and P-M (-6.7±2.9%) groups.

4.4.6. Correlation Analysis

Figure 4.6 depicts the relationship between heart tissue antioxidant capacity (as reflected in peroxide-induced TBARS generation) at the end of 90 min of reperfusion and coronary effluent 15-F_{2t}-isoP production during ischemia. Tissue TBARS was significantly positively correlated with effluent 15-F_{2t}-isoP production during ischemia (R = 0.68, P = 0.0003), indicating an inverse correlation between tissue antioxidant capacity preservation and 15-F_{2t}-isoP production during ischemia. Tissue TBARS levels did not correlate with 15-F_{2t}-isoP generation during reperfusion.
Table 4.1. Coronary perfusion pressure (CPP) (mm Hg)

<table>
<thead>
<tr>
<th></th>
<th>BS</th>
<th>Pre-isch</th>
<th>Re-10</th>
<th>Re-30</th>
<th>Re-60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re-90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-Y group</td>
<td>53.0 ± 4.8</td>
<td>55.0 ± 5.2</td>
<td>59.2 ± 5.5</td>
<td>79.7 ± 9.5</td>
<td>95.5 ± 15.1**</td>
</tr>
<tr>
<td>P-Y group</td>
<td>50.8 ± 1.5</td>
<td>40.8 ± 2.7*</td>
<td>46.2 ± 4.9</td>
<td>53.0 ± 4.2*</td>
<td>53.0 ± 3.8*</td>
</tr>
<tr>
<td>C-M group</td>
<td>54.2 ± 3.3</td>
<td>56.5 ± 3.6</td>
<td>60.2 ± 2.2</td>
<td>71.3 ± 3.9*</td>
<td>84.2 ± 7.8**</td>
</tr>
<tr>
<td>P-M group</td>
<td>53.5 ± 2.3</td>
<td>48.0 ± 2.5</td>
<td>48.5 ± 3.0*</td>
<td>58.8 ± 5.4</td>
<td>66.7 ± 8.4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n=6 for each group). BS = baseline, Pre-isch = pre-ischemia; Re-10, Re-30, Re-60 and Re-90 refer to 10, 30, 60 and 90 min after reperfusion. CPP did not differ among groups at baseline. *P<0.05, **P<0.01, vs BS; †P<0.05 P-Y vs C-Y or P-M vs C-M group.
Figure 4.5. Left ventricular developed pressure (LVDP). BS and Pre-isch indicate baseline and pre-ischemia respectively. Re-10, Re-30, Re-60 and Re-90 indicate 10, 30, 60 and 90 min of reperfusion following 40 min of global myocardial ischemia. Values are mean ± SEM. * P < 0.05 or P < 0.01 vs BS; †P < 0.05 or P < 0.01 P-Y vs C-Y group or P-M vs C-M group. # P < 0.05 P-M vs P-Y group. P > 0.05 C-M vs C-Y group.
Figure 4.6. Correlation between coronary effluent 15-F₂-isoprostane release during ischemia and heart tissue antioxidant capacity. Heart tissue were sampled after 40 min of ischemia and 90 min of reperfusion. Tissue TBARS formation in the presence of the peroxidizing agent t-BHP (2 mM) was significantly positively correlated with effluent isoprostane generation during ischemia ($R = 0.68$, 95% CI: 0.3798 to 0.8498, $P = 0.0003$), indicating an inverse correlation between tissue antioxidant capacity preservation and isoprostane generation during ischemia.
Figure 4.7. Correlation between left ventricular developed pressure (LVDP) at 90 min of reperfusion (Re-90) and 15-F_{2\alpha}-isoprostane release during the first 30 min of ischemia. 15-F_{2\alpha}-isoP generation during ischemia was significantly negatively correlated with LVDP at Re-90 (R = -0.65, 95% CI: -0.8324 to -0.3277, P = 0.0007, A) and the percentage recovery of LVDP at Re-90 to baseline (R = -0.65, 95% CI: -0.8338 to -0.3318, P = 0.0006, B).
At 90 min of reperfusion, LVDP, as well as its percentage recovery to baseline values, was highly negatively correlated with heart 15-F_2t-isoP generation during ischemia (P < 0.001, Figure 4.7). A weak but significant correlation existed between 15-F_2t-isoP generation during ischemia and peak ischemic contracture (P = 0.04) and between 15-F_2t-isoP production at reperfusion 5 min and coronary perfusion pressure at reperfusion 90 min (P = 0.04).

Changes of LVDP from Re-60 to Re-90 were inversely correlated with changes of CPP from Re-60 to Re-90 in ischemic-reperfused middle-aged rat hearts (R= -0.68, P=0.015, n=12). Such a relationship did not exist in the young rat hearts (P=0.8). This extends the finding of Goodwin et al^{236} that middle-aged (5-month old) rat hearts became more sensitive to vasoconstriction induced by endothlin-1, whose release was increased in isolated ischemic-reperfused rat hearts.^{241}

4.5. Discussion

Previous laboratory studies investigating the effect of propofol on myocardial IRI have focused primarily on young animal hearts.^{142; 143; 213; 218; 223; 226} To our knowledge, this is the first study to compare the cardioprotective effects of propofol on isolated hearts from young and middle-aged rats and to use 15-F_2t-isoP as an index of lipid peroxidation to explore the mechanism of the protection. This approach is important to understanding the effects of age, which could identify patient populations that could benefit most from antioxidant intervention during clinical settings of myocardial IRI.
The principal findings of this study include the following. 1) lipid peroxidation occurs during global ischemia and reperfusion in both young and middle-aged rat hearts; 2) myocardial tissue antioxidant capacity preservation following IRI is highly negatively correlated with the extent of lipid peroxidation that occurs during prolonged global ischemia rather than during reperfusion; 3) 15-F$_2$-isoP generation during ischemia is highly negatively correlated with post-ischemic myocardial functional recovery; 4) propofol applied before and during ischemia as well as during early reperfusion significantly reduced 15-F$_2$-isoP generation during ischemia and abolished ischemic contracture in middle-aged rat hearts.

In attempts to clarify the contribution of ROS to myocardial IRI, experiments using a wide array of antioxidants have yielded conflicting results. Certain pathophysiological mechanisms may predominate depending on the conditions of the experiment, such as the duration of ischemia, the timing of the antioxidant intervention as well as the nature and dosage of the antioxidant used. If significant cellular necrosis has occurred during the preceding ischemic event, antioxidant interventions during reperfusion may have little effect. Cardiac mitochondria, critical to the energy status and function of the heart, are a source of ROS during ischemia and reperfusion, and exhibit increased rates of ROS production with age. Based on previous studies from our laboratory, we postulate that the cardioprotective effects of propofol on IRI are mostly attributable to its antioxidant properties.
15-F$_{2t}$-isoP is a chemically stable end product of lipid peroxidation, which is detectable in rat heart and plasma under control conditions. EIA provides a sensitive measure for 15-F$_{2t}$-isoP in biological specimens. Baseline 15-F$_{2t}$-isoP values in our model of myocardial IRI were within the limits of detection of the assay. The baseline generation of 15-F$_{2t}$-isoP in our model likely represents release from normal myocardial tissue rather than an ischemic insult during heart isolation, because of careful organ harvesting and reperfusion within 40 seconds after excision. Since large quantities of isoprostanes can be generated \textit{ex vivo}, it was important to ensure that the measured 15-F$_{2t}$-isoP was generated by the heart during ischemia, and not produced during collection or storage. We found that the values did not change when effluent was stored overnight at room temperature (data not shown). This is similar to a report by Morrow et al, who found that urinary F$_2$-isoprostane levels did not increase when urine was incubated at 37 °C for 5 days.

Forty minutes of ischemia induced a profound ischemic insult, as evidenced by the magnitude of the ischemic contracture in the two control groups. It is noteworthy that the magnitude of the ischemic contracture in the C-M group significantly increased every five minutes when the duration of ischemia exceeded 25 min, which was not the case in the C-Y group. This indicates that middle-aged rat hearts are more vulnerable to ischemic insult after prolonged ischemia. Interestingly, propofol abolished ischemic contracture in middle-aged but not in young rat hearts. The precise mechanism underlying this effect is uncertain. It seems reasonable to postulate, however, that antioxidant therapy would be more effective in hearts with decreased endogenous antioxidant capacity. This will
attenuate or prevent subsequent cellular damage resulting from ROS-mediated membrane lipid peroxidation. On the other hand, propofol may inhibit rat cardiomyocyte calcium channels at a concentration as low as 6 µM. This inhibition increases in a concentration-dependent fashion when propofol concentration exceeds 50 µM. This property of propofol could have contributed, in part, to the reduction or abolition of ischemic contracture in the propofol treatment groups. It has been shown that contracture development in rat cardiomyocyte is potentiated by a rise in intracellular calcium concentration. In addition, propofol may attenuate contracture development by enhancing energy preservation during myocardial ischemia-reperfusion. Energy depletion may play an important role in the acceleration of contracture development once the contracture has been initiated in the presence or absence of a rise in intracellular calcium. The strong negative correlation between 15-F$_2$-isoP generation during ischemia and post-ischemic myocardial function recovery highlights the role of ROS in mediating cellular injury early during ischemia. Susceptibility of heart tissue to ex vivo TBARS formation in the presence of the peroxidizing agent t-BHP provided a functional measure of tissue antioxidant capacity. The significant correlation between tissue TBARS formation and 15-F$_2$-isoP generation during ischemia but not during reperfusion indicates that the reduction in tissue antioxidant capacity occurred primarily during the ischemic phase in this experimental model. This would suggest that antioxidant interventions aimed at protecting against myocardial IRI are likely to be most effective if undertaken immediately prior to the ischemic insult.

The propofol concentration (67 µM or 12µg/ml) used in the present study is high, but still clinically achievable, based on our previous study. A recent study from our
laboratory has shown that the cardiac protective effect of propofol was concentration-dependent, being greater at 12 μg/ml, when used primarily before and during ischemia as well as during the early phase of reperfusion, than at 5 μg/ml in this experimental model. Therefore, only the highest concentration of propofol was used in the current study before and during ischemia and in the early phase of reperfusion. Propofol significantly reduced 15-F_2t-isop generation during ischemia and reperfusion in both young and middle-aged rat hearts. It is noteworthy that, during early reperfusion, 15-F_2t-isop levels decreased more rapidly in the C-Y than in the C-M group (Figure 4.1A). This is coincident with a more profound percentage decrease of LVDP from Re-60 to Re-90 in the C-M group. This is of clinical significance. We have recently shown that the percentage changes of plasma free 15-F_2t-isop concentration during the early phase of myocardial reperfusion inversely correlates with post-operative cardiac index.

A recently published study has shown that propofol may offer myocardial protection by inhibiting the mitochondrial permeability transition (MPT), another major cause of reperfusion injury, at concentrations low as 2 to 4 μg/mL (11 to 22 μM) in ischemic-reperfused rat hearts. Inhibition of the MPT by propofol, when applied at low concentrations, is likely attributable to the well-known membrane stabilizing action of lipophilic anesthetic molecules and the antioxidant properties of propofol. Propofol 2 μg/m did not inhibit MPT when applied directly to isolated de-energised mitochondria. Indeed, accumulating evidence strongly suggests oxidative stress as the link between excessive mitochondrial calcium overload and MPT (for a review, see ref.252). It has been proposed that MPT is not a consequence of the opening of a pre-formed pore, but the consequence of oxidative damage to pre-existing membrane
proteins. Propofol, however, may directly inhibit rat heart MPT at concentrations ≥ 50 μM. This is of importance. Clinically, propofol failed to offer appreciable protection against myocardial IRI when administrated using a target-controlled infusion system with propofol plasma concentrations between 2 to 4 μg/mL (concentration provided by author in response to letter-to-editor by Ansley and Xia). Interestingly, propofol, when used at a high concentration of 67 μM before and during ischemia as well as during the early phase of reperfusion, enhanced myocardial function recovery in middle-aged rat hearts 90 min after reperfusion compared to untreated control and young rat hearts. Clinically, the application of high-concentrations of propofol (average 11 μg/mL) before and during myocardial ischemia in a patient population 35 years of age or older resulting in better myocardial function recovery 12 to 24 hours post-operatively comparing to application of propofol at lower concentrations. Improved cardiac functional recovery by propofol 90 min after reperfusion in the middle-aged rat hearts and 12-24 hours after cardiac surgery in patients is similar in effect to the “first window” and “second window” of protection phenomena observed after ischemic or pharmacological preconditioning.

The proposed “preconditioned state” in the heart induced by propofol, when applied at high concentrations as aforementioned, is unlikely to be mediated by the activation of mitochondrial K_{ATP} (mK_{ATP}) channels, a mechanism by which volatile anesthetics are claimed to mimic cardiac preconditioning. Propofol, at concentrations between 10 to 200 μM, did not affect mK_{ATP} channel activity in cultured myocytes isolated from male Sprague-Dawley rat hearts. We propose that propofol, primarily at high concentrations, may “precondition” the heart via mechanism(s) downstream of mK_{ATP} channel activation. Indeed, accumulating evidence supports mK_{ATP} as a trigger
and/or a mediator rather than an end-effector in myocardial preconditioning and protein kinase C (PKC) is likely one of the kinases downstream from mK<sub>ATP</sub> that may be involved (for a review, see ref. 254). Propofol stimulated purified rat brain PKC activity <i>in vitro</i>,<sup>257</sup> and attenuated isoproterenol-stimulated increases in intracellular calcium via activation of PKC activity in rat cardiomyocytes.<sup>258</sup> Therefore, activation of PKC could be a potential signal pathway through which propofol may "precondition" the heart. Also, it is important to note that inhibition of the MPT in early reperfusion could represent a distal effector mechanism of myocardial "preconditioning" with mK<sub>ATP</sub> activation acting as a trigger or an intermediate step.<sup>259</sup> Taken together, propofol cardiac protection might involve the activation of PKC activity before ischemia, and the inhibition of the MPT, directly<sup>253</sup> or indirectly through reducing oxidative stress,<sup>252; 259</sup> during reperfusion.

4.6. Conclusion

In summary, our present study suggests adequate antioxidant therapy with propofol as a potentially useful means to reduce myocardial IRI under clinical conditions (e.g. coronary revascularization and heart transplantation), especially in the more vulnerable population of the middle-aged patients.<sup>234</sup> Propofol may mediate cardiac protection through a variety of mechanisms or signal pathways, with inhibition of ROS-mediated lipid peroxidation likely being one of the major mechanisms of protection.
1. CHAPTER 5: Effects of 15-F$_2$-isoP on myocardial IRI in isolated rat hearts: potential mechanism of 15-F$_2$-isoP action

5.1. Preface

A manuscript entitled "15-F$_2$-isoprostane exacerbates myocardial ischemia-reperfusion injury of isolated rat hearts: effects on endothelin-1 generation" reporting results of studies presented in this Chapter has been submitted for publication and is co-authored with D. M. Ansley, K. H. Kuo, D. V. Godin, M. J. Walker and M. Tao. K. H. Kuo provided technical assistance with the myocardial infarct size measurements. M. J. Walker provided valuable suggestions and M. Tao provided assistance in collecting and concentrating the coronary effluent samples. We thank Drs E. Puil and T. K. Chang for kindly allowing us to use their laboratory facilities.

5.2. Introduction

Myocardial ischemia-reperfusion injury (IRI) and its sequelae, cardiac depression and arrhythmogenesis, have been shown experimentally to result, at least in part, from the disruptive action of reactive oxygen species (ROS) on membrane lipids and intracellular proteins required for cellular integrity and function. The release of high levels of ROS during ischemia-reperfusion can overwhelm endogenous antioxidant defenses, a crucial event determining the onset of irreversible cellular necrosis secondary to extensive lipid peroxidation.
A recent advance in free radical biology has been the discovery of isoprostanes, stable in vivo end products of arachidonic acid peroxidation.\textsuperscript{63} Of the variety of isoprostanes detected,\textsuperscript{56, 62} 15-F\textsubscript{2r}-isoprostane (15-F\textsubscript{2r}-IsoP)\textsuperscript{61} has been found to be a specific, reliable marker of oxidative stress. This has facilitated investigation of the role of ROS in a variety of disease states, most notably cardiovascular disease. Of interest, 15-F\textsubscript{2r}-IsoP possesses potent biological activity, including vasoconstriction and platelet activation under pathophysiological conditions.\textsuperscript{71} 15-F\textsubscript{2r}-IsoP has no effect on coronary flow in the absence of ischemia in the isolated rat heart (up to a concentration of 256 nM), but significantly reduces coronary flow in the hypoxic or post-ischemic reperfused rat heart (at 30 nM).\textsuperscript{77}

Clinically we identified an inverse correlation between the speed of decay of plasma 15-F\textsubscript{2r}-IsoP concentrations during early phase of reperfusion and post-operative cardiac functional recovery in patients undergoing coronary artery bypass surgery utilizing cardiopulmonary bypass (CPB).\textsuperscript{43} We also have found that 15-F\textsubscript{2r}-IsoP generation during myocardial ischemia and reperfusion in an isolated rat heart model is inhibited by treatment with 2,6 diisopropylphenol (propofol), an anesthetic with powerful antioxidant properties. Propofol administration is associated with improved post-ischemic cardiac functional recovery\textsuperscript{227} These studies have prompted us to postulate that 15-F\textsubscript{2r}-IsoP, in addition to being a marker of oxidative stress, may also play a major role in mediating myocardial IRI.
The characteristics of 15-F_{2t}-IsoP production and its effects under conditions of ischemia and reperfusion are quite similar to those of endothelin-1 (ET-1). Endothelin-1 is one of the most potent vasoconstrictors known, and it has been postulated to contribute to post-ischemic myocardial dysfunction \(^{263,264}\). ET-1 release has been shown to increase during and after myocardial ischemia \(^{265,266}\) and its vasoconstrictor effect appears to be increased (potentiated) during post-ischemic reperfusion in isolated hearts. \(^{267,268}\)

We recently determined that antioxidant therapy during cardiac surgery utilizing CPB significantly reduces plasma malondialdehyde (MDA) levels as well as ET-1 concentrations, and this is associated with improved post-operative cardiac function \(^{269}\). It is plausible that antioxidant therapy reduced ET-1 release during myocardial ischemia-reperfusion by reducing 15-F{\textsubscript{2t}}-IsoP production.

We hypothesize that 15-F_{2t}-IsoP can exacerbate myocardial ischemia-reperfusion injury and that the mechanism of 15-F_{2t}-IsoP action may involve the release and/or enhancing the production of ET-1 during cardiac ischemia and reperfusion. Our hypothesis was tested in an isolated rat heart model, using SQ 29548, a thromboxane A\textsubscript{2} receptor (TXA\textsubscript{2}) antagonist used to abolish the vasoconstrictive actions of 15-F_{2t}-IsoP. \(^{200}\)
5.3. Materials and methods

5.3.1. Heart preparation

This study was approved by the Committee of Animal Care of the University of British Columbia. Animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (280~320g) were anesthetized with pentobarbital (70mg/kg intraperitoneally) and heparinized with sodium heparin (1000 IU/kg, intraperitoneally). After median thoracotomy, hearts were quickly excised and immersed in ice-cold Krebs-Henseleit (KH) solution to stop contractions. Hearts were gently squeezed to remove residual blood to prevent clot formation. Hearts were retrogradely perfused via the aorta in a non-working "Langendorff" preparation at a constant flow rate of 10ml/min using a peristaltic pump. The perfusion fluid (pH 7.4; temperature, 37°C) was KH solution that contained (in mM): NaCl 118; NaHCO$_3$ 24; KCl 4.63; MgCl$_2$ 1.2; CaCl$_2$ 1.25; KH$_2$ PO$_4$ 1.17; glucose 11. The perfusate was bubbled with a mixture of 95% O$_2$ and 5% CO$_2$. The perfusate solution and the bath temperature were maintained at 37°C using a thermostatically controlled water circulating system. Coronary perfusion pressure (CPP) was measured via a side arm of the perfusion cannula connected to a pressure transducer (Statham p23 ID, Gould Electronics, Cleveland). A latex water-filled balloon fixed to a pressure transducer was inserted through the mitral valve into the left ventricle for the determination of left ventricular (LV) developed pressure (LVDP), which was calculated by subtracting end-diastolic pressure (LVEDP) from LV peak systolic pressure (LVSP). LVEDP was adjusted to approximately 5 mmHg before the start of the experiment by
adjusting the volume in the intraventricular balloon. Exclusion criteria included heart
preparation times longer than 60 seconds and/or LVSP lower than 65 mmHg after 10 min
of equilibration.

5.3.2. Experimental Protocol

All hearts were initially equilibrated for 10 min (BS10). They then were
randomly assigned to a sham group or one of the four experimental groups (n=7 per
group): ischemia-reperfusion untreated control (control), 15-F_2t-IsoP (IsoP), 15-F_2t-IsoP
plus SQ 29548 (IsoP-SQ) and SQ 29548 (SQ) alone groups. After BS10, 15-F_2t-IsoP 100
nM (IsoP), SQ 29548 1μM (SQ) or 15-F_2t-IsoP 100 nM plus SQ 29548 1μM (IsoP+SQ)
were applied for 10 min, respectively in the corresponding groups, before global ischemia
(40 min) was induced by stopping perfusion flow. Control hearts underwent an additional
10 min period of equilibration before global ischemia was induced. During ischemia,
saline (control), 15-F_2t-IsoP 100 nM (IsoP), SQ 29548 1μM (SQ) or 15-F_2t-IsoP 100 nM
plus SQ 29548 1μM (IsoP+SQ) in saline was perfused through the aorta at 60 μl/min
using a mini-pump. KH was perfused during 60 min of reperfusion in the control group.
Either 15-F_2t-IsoP, SQ 29548 or 15-F_2t-IsoP plus SQ 29548 in KH was perfused for the
first 15 min of reperfusion.

The perfusion flow rate (10 ml/min) was based on the result of a pilot study which
showed that hearts sham-perfused without ischemia beat well and remain
hemodynamically stable for 120 min (the duration of the experiment) in our experimental
set-up. The reagent concentrations utilized were chosen based on findings in the
literature, demonstrating that: 1) 15-F$_2$-IsoP 100 nM had no effect on coronary flow in sham-perfused rat hearts but significantly reduced coronary flow in ischemic-reperfused rat hearts$^{200}$; 2) SQ 29548, a thromboxane A2 (TXA2) receptor antagonist$^{85}$, abolished 15-F$_2$-IsoP (56 nM)-induced reduction in coronary flow in ischemic-reperfused rat hearts at a concentration of 0.1 μM$^{200}$; and 3) SQ 29548, at 1 μM, abolished 15-F$_2$-IsoP (>300 nM)-induced reduction in coronary flow in isolated perfused guinea pig heart$^{76}$.

Baseline effluent perfusate was sampled at BS10. Effluent samples during ischemia were collected during the first 30 min of ischemia (isch). Also, effluent was sampled at 1 (Re-1), 5 (Re-5), 30 (Re-30) and 60 (Re-60) min of reperfusion in the four experimental groups or at the corresponding time points in the sham group. Aliquots of the effluent samples were immediately stored at -70 °C until analysis for cardiac specific creatine kinase (CK-MB) in all study groups and for 15-F$_2$-IsoP in the sham, the control and the SQ groups. Another portion of the effluent sample was initially concentrated (see below) and then stored at -70 °C for the analysis of ET-1 concentration. At the end of the 60 min of reperfusion, 37°C 1% 2,3,5-triphenyltetrazolium in buffer (0.1 M phosphate buffer adjusted to pH 7.4) was pumped into the heart at 1 ml/gm/min for 15 min until the epicardial surface became deep red. The hearts were then stored in 10% formaldehyde for later analysis of myocardial infarct size.

5.3.3. Measurement of Endothelin-1

Measurement of ET-1 concentrations in the coronary effluent was performed using a commercially available human ET-1 enzyme immunometric assay kit (human
ET-1 EIA kit 900-020, Assay Designs, Inc. Ann Arbor). Because ET-1 concentrations in the samples were often below the ET-1 sensitivity of this assay (0.14pg/ml) based on a pilot study, collected effluent samples were concentrated 4-fold by evaporation of solvent (i.e. the KH solution) at room temperature under a stream of dry nitrogen. Subsequently, the actual ET-1 concentration was calculated as $1/4^{th}$ of the measured ET-1 level in the concentrated sample. This concentration procedure did not diminish the accuracy of the ET-1 measurements, as tested by using known concentrations of ET-1 standards and control measurements without ET-1.

Enzyme immunoassays (EIA) were performed in duplicate by adding 100 µL of human ET-1 standards or concentrated samples onto 96-well microplates. After 1 hour of incubation at 37°C, the microplates were washed seven times with wash solution. The microplates were further incubated at 37°C for 30 min after the addition of 100 µL labeled antibody to each well, except the blank. The microplates were then washed nine times before adding 100 µL of the substrate solution to each well. Thereafter, the microplates were incubated for 30 min at room temperature, followed by the addition of 100 µL stop solution to each well. The plates were read at 450 nm and the values of the unknowns were expressed as picograms ET-1 per milliliter effluent. The samples were coded (as was the case for all assays in this study) and the investigator responsible for ET-1 assays was blinded until the completion of the assay.
5.3.4. Measurement of CK-MB

Measurement of CK-MB was performed using a commercially available enzyme immunoassay kit (Catalog number: BC-1121, BioCheck, Inc, Burlingame, CA). The EIA measurements were performed in duplicate by dispensing 20 μL of human CK-MB standard or samples into appropriate wells of a microplate followed by the addition of 200 μL enzyme conjugate reagent into each well. The plate was incubated at 4 °C for 20 hours and was then washed five times with double-distilled water. After the addition of 100 μL TMB reagent into each well, the plate was incubated at room temperature for 20 min. The reaction was terminated by adding 100 μL of stop solution to each well. The plate was read within 15 min at 450 nm and the values of the unknowns were expressed as nanograms CK-MB per milliliter effluent.

5.3.5. 15-F₂t-IsoP Assays

Enzyme immunoassay of free 15-F₂t-IsoP was performed according to the methods provided by the manufacturer (Cayman Chemical, Ann Arbor) as previously described 213. In brief, 50 μL standards and samples were added in duplicate to the 96-well plate provided in the kit, followed by addition of 15-F₂t-IsoP acetylcholinesterase tracer and antibody. The prepared plates were then incubated overnight at room temperature. On the next day, the plates were washed 5 times with wash buffer, followed by addition of Ellman’s reagent. After optimal color development, the plates were read at
405 nm, and the values of the unknowns were expressed as picograms 15-F2t-IsoP per milliliter effluent.

5.3.6. Myocardial Infarct Size Measurement

The measurement of infarct size was essentially identical to that described by Downey\textsuperscript{270} except for the method of quantification. After the 2,3,5-triphenyl-tetrazolium chloride (TTC) reaction, the hearts were sectioned transaxially, and size of infarct was evaluated as percentage of sectional area of infarcted tissue to the sectional area of the whole heart in 1 mm layers (five layers, LG scanner). Morphometric measurements of infarct size were performed with a LG scanner and 6.0 CE software. The histogram counts of the red (viable tissue) and white (infarcted tissue) were recorded. The percent infarction was calculated as white counts divided by the sum of the red plus white counts.

5.3.7. Statistical Analysis

All data are presented as means ± SEM. Hemodynamic variables and chemical assay parameters were compared by two-way analysis of variance (ANOVA) with repeated measures. One-way ANOVA was used to test for differences in infarct size between groups. The correlation was evaluated by the Pearson test. P<0.05 was considered statistically significant.

5.4. Results

5.4.1. Endothelin-1 Release and its Relation with 15-F2t-IsoP
Baseline effluent ET-1 concentrations did not differ among the experimental groups (Fig 5.1A). Effluent ET-1 did not significantly change over time in the sham group. ET-1 increased in the control group during ischemia (Fig 5.1A, P < 0.001 vs baseline) and increased further in the Iso-P group compared to control (P<0.05). ET-1 increased approximately 20% at Re-1 and 32.8±26.9% at Re-30 compared to baseline (BS10) in the control group. These increases did not reach statistical significance (P>0.1). Effluent ET-1 concentration in the IsoP group was significantly higher than that in the control group (P<0.05) at reperfusion 60min (Re-60). Effluent ET-1 concentrations in both the IsoP-SQ and the SQ groups did not differ from those found in the control during ischemia and reperfusion. A weak but significant positive correlation (r = 0.77, P =0.04, Fig 5.1B) was noted between effluent concentrations of 15-F2t-IsoP and ET-1 during ischemia, but not during reperfusion, in the control (i.e., untreated) group.

5.4.2. 15-F2t-IsoP Generation During Ischemia-reperfusion

Effluent 15-F2t-IsoP release in the sham group did not change over time during the 120 min perfusion period (data not shown). As shown in Fig 5.2, effluent 15-F2t-IsoP levels increased during ischemia (P < 0.001 vs BS10) and remained elevated at Re-1 (P<0.05 or P<0.01 vs BS10) in the control and the SQ groups. Effluent 15-F2t-IsoP release during early reperfusion (Re-1 and Re-5) in the SQ group tends to be higher than that in the control group, but the difference did not reach statistical significance (P>0.2).
**Figure 5.1.**

A. Effluent Endothelin-1 (ET-1) concentrations during myocardial ischemia-reperfusion. BS10 and isch indicate 10 min after stabilization and 30 min during global myocardial ischemia, respectively; Re-1, Re-5, Re-30 and Re-60 indicate 1, 5, 30 and 60 min after reperfusion, respectively. * P<0.001 vs BS10; # P<0.05 or P<0.01 vs control. (n=7 for each group).

B. Relationship between 15-F_{2t}-isoprostane (15-F_{2t}-isoP) and ET-1 concentration during the first 30 min of ischemia in the control group. ET-1 release is positively correlated with 15-F_{2t}-isoP concentration (r = 0.7695, 95% CI: 0.0389 – 0.9640, P (two-tailed) = 0.043).
Figure. 5.2. Effect of SQ 29548 (SQ) on 15-F_{2\alpha}-isoprostane (15-F_{2\alpha}-isoP) release during myocardial ischemia-reperfusion. BS10 and isch indicate 10 min after stabilization and 30 min during global myocardial ischemia, respectively; Re-1, Re-5, Re-30 and Re-60 indicate 1, 5, 30 and 60 min after reperfusion, respectively. * P<0.001 or P<0.05 vs BS10. P >0.05 SQ vs control.
5.4.3. CK-MB Release During Ischemia-reperfusion

Baseline CK-MB release was detectable in this model and did not differ among groups (Fig 5.3). Effluent CK-MB release did not significantly change over time in the sham group.

During ischemia, CK-MB increased relative to its baseline value in control hearts, but the increase did not reach statistical significance (P>0.05). CK-MB release was significantly higher than its baseline value (P<0.05) during ischemia in the IsoP group, but unchanged in IsoP-SQ and SQ (P>0.05).

During reperfusion, CK-MB in the control group increased gradually and was significantly higher than the baseline value at Re-30 (P<0.01). Effluent CK-MB concentration in the IsoP group increased more rapidly during reperfusion and was significantly higher than its baseline value at Re-5 (P<0.05). The CK-MB level at Re-5 was greater in the IsoP group than in controls (P<0.05). CK-MB levels in IsoP-SQ did not differ from untreated control during ischemia and reperfusion. CK-MB level in SQ was higher than in the control group and the IsoP group at Re-1 (<0.05). It decreased quickly thereafter and was lower than the corresponding values in control and IsoP groups at Re-30.

A positive correlation (r = 0.89, P =0.02) existed between effluent 15-F2t-IsoP and CK-MB levels measured in the control group during ischemia.(data not shown).
Figure 5.3. Effluent CK-MB concentration during myocardial ischemia-reperfusion.

BS10 and isch (ischemia) indicate 10 min after stabilization and 30 min during global myocardial ischemia, respectively; Re-1, Re-5, Re-30 and Re-60 indicate 1, 5, 30 and 60 min after reperfusion, respectively. IsoP and SQ indicate 15-F_{2\alpha}-isoprostane and SQ 29548, respectively. *P < 0.05 vs BS10; #P < 0.05 vs control; +P < 0.05 vs isoP group. (n = 7 for each group)
Figure 5.4. A: Development of left ventricular end-diastolic pressure (LVEDP), reflecting myocardial contracture, during ischemia. B: Ischemic contracture onset time. The onset of contracture is defined as elevation of LVEDP ≥ 2.5 mmHg vs baseline value. # $P<0.05$ vs control; $^+P<0.05$ or $P<0.01$ vs IsoP (15-F$_2$-isoprostane) group; *$P<0.05$ or $P<0.01$ vs ischemia 30 min within the same group.
**Figure 5.5.** Variations of left ventricular end-diastolic pressure (LVEDP), reflecting myocardial stiffness, during reperfusion. BS10 and Pre-isch indicate 10 min after stabilization and the time immediately prior to ischemia, respectively; Re-1, Re-5, Re-30 and Re-60 indicate 1, 5, 30 and 60 min after reperfusion, respectively. *P<0.05 or P<0.01 vs BS10; #P<0.05 or P<0.01 vs control; †P<0.05 or P<0.01 vs IsoP (15-F₂-isoprostane) group.
5.4.4. Contracture Development during Ischemia

The LVEDP increased progressively during ischemia in the control group (Fig 5.4A). LVEDP in the IsoP group increased more quickly than that in the control group. At 30 and 35 min of ischemia, the magnitude of LVEDP in the IsoP group was significantly higher than that in the control group (P<0.05). SQ 29548 attenuated the effect of 15-F$_2$-IsoP in augmenting LVEDP. The magnitude of LVEDP in the IsoP-SQ group was significantly lower than that in the IsoP group at ischemia 30 min and onwards. The magnitude of LVEDP in the IsoP-SQ and the SQ group did not differ from that in the control group during ischemia.

Time to the onset of ischemic contracture, defined as elevation of LVEDP $\geq$2.5 mmHg vs baseline value, was significantly shorter in the IsoP group (11.4±1.9 min) than in the control group (17.4±1.6 min, P<0.05, Fig 5.4B). The latency to ischemic contracture in the IsoP-SQ (20.0±1.5 min) and the SQ (18.1±1.5 min) groups was significantly increased as compared to that in the IsoP group (P<0.01 or P<0.05), but did not differ from that in the control group (P>0.05, Fig 5.4B).

5.4.5. Functional Response to Ischemia-reperfusion

During reperfusion, LVEDP in the control group was significantly higher than that at baseline (Fig 5.5). 15-F$_2$-IsoP augmented the increase of LVEDP during
reperfusion. At Re-30 and Re-60, LVEDP values in the IsoP group were higher than those in the control group (P<0.01). SQ 29548 attenuated the 15-F_{2t}-IsoP-induced increase in LVEDP. The magnitude of LVEDP in the IsoP-SQ and the SQ groups did not significantly differ from that in the control group during reperfusion.

The LVDP in the sham group did not change significantly over time during the experimental period. The LVDP in the control group recovered to a maximum of 87.0±11.6 % of its baseline value at Re-30 (P>0.05 vs BS10, Fig 5.6) and decreased thereafter. The LVDP in the IsoP group recovered to a maximum of 56.5±13.5% of its baseline value at Re-30 (P<0.05 vs BS10) and decreased quickly thereafter. At Re-60, LVDP in the IsoP group was lower than that in the control group. The LVDP values in the IsoP-SQ and the SQ group did not differ from those in the control group at Re-60. SQ 29548 exacerbated 15-F_{2t}-IsoP induced reduction in LVDP relative to control group at Re-10.
Figure 5.6. Recovery of left ventricular developed pressure (LVDP), reflecting effective myocardial contractility, during reperfusion. BS10 and Pre-isch indicate 10 min after stabilization and the time immediately prior to ischemia, respectively; Re-1, Re-5, Re-30 and Re-60 indicate 1, 5, 30 and 60 min after reperfusion, respectively. *P<0.05 vs BS10; #P<0.05 vs control; +P<0.05 or P<0.01 vs IsoP (15-F$_2$-isoprostane) group.
Figure 5.7. Myocardial infarct size. Top: representative images showing myocardial infarction (white) in the control (A), 15-F_{2\alpha}-isoprostane (IsoP, B), 15-F_{2\alpha}-isoprostane plus SQ 29548 (IsoP+SQ, C) and SQ 29548 (SQ, D) groups. Bottom: Percentage infarction (Mean ± SEM): *P<0.05 vs control; +P<0.05 or P<0.01 vs IsoP group.
5.4.6. Coronary Perfusion Pressure

Neither 15-F$_2$-IsoP, SQ 29548, nor their combination affected CPP before ischemia. CPP did not increase significantly until after 60 min of reperfusion in the untreated control group (80.4±11.0 mmHg at Re-60 vs 51.3±1.1 mmHg at BS10, P<0.05). CPP in the IsoP group increased more quickly during reperfusion relative to the control group. At Re-30, the CPP value in the IsoP group (100.4±13.9 mmHg) was higher (P<0.05) than its baseline value (52.1±3.9 mmHg) and higher (P<0.05) than the corresponding value in the control group (65.6±4.6 mmHg). SQ 29548 did not significantly affect CPP as compared to the control group. At Re-60, CPP values did not significantly differ among the control (80.4±11.0 mmHg), the IsoP (110.4±14.9 mmHg), the IsoP+SQ (115.4±14.9 mmHg) and the SQ (84.8±13.5 mmHg) groups (P>0.05).

5.4.7. Myocardial Infarct Size

As shown in figure 5.7, myocardial infarct size in the IsoP group is significantly larger than that of the control (untreated) group (P<0.05). The myocardial infarct sizes in the IsoP-SQ and SQ groups are significantly smaller than those in the IsoP group (P<0.05 or P<0.01). Infarct sizes in the SQ group and IsoP-SQ groups were somewhat smaller than those in the control group, but the differences did not attain statistical significance.
5.5. Discussion

To our knowledge, this is the first study providing evidence that 15-F$_{2\pi}$-IsoP may play a causative role in exacerbating myocardial IRI in the isolated perfused rat heart. Our findings include the following: (1) 15-F$_{2\pi}$-IsoP(100 nM) did not affect pre-ischemic cardiac mechanics and coronary perfusion pressure but did reduce cardiac tolerance to ischemic insult, as manifested by an early onset and higher magnitude of ischemic contracture; (2) 15-F$_{2\pi}$-IsoP stimulated the release and/or production of ET-1 during ischemia which was accompanied by increased severity of myocardial cellular damage evidenced by increased CK-MB release; (3) 15-F$_{2\pi}$-IsoP increased myocardial infarct size and exacerbated post-ischemic myocardial dysfunction, which may be attributable, in part, to stimulation of ET-1 production and/or release during reperfusion.

Endothelin-1 has potent vasoconstrictor properties and is known to reduce myocardial contractility and contribute to the progression of the heart failure process\textsuperscript{263}. Plasma levels of ET-1 increase during cardiac operations requiring cardiopulmonary bypass (CPB)\textsuperscript{205; 271}. A high plasma ET-1 level during the early postoperative period has been associated with prolonged pharmacologic management, longer intensive care unit stay, and complicated recovery\textsuperscript{205; 272}. The present study clearly demonstrates that 15-F$_{2\pi}$-IsoP, whose formation increased in the myocardium and coronary artery during CPB surgery\textsuperscript{19}, can increase the release and/or production of ET-1 during myocardial ischemia-reperfusion. This might be a mechanism whereby 15-F$_{2\pi}$-IsoP exacerbates myocardial IRI. The positive correlation between effluent concentrations of 15-F$_{2\pi}$-IsoP
and ET-1 during ischemia in the control (untreated) group suggests that endogenous 15-F2t-IsoP may act to stimulate increased ET-1 release during ischemia.

We observed a reduction in ET-1 concentration at Re-30, but a significant increase by Re-60 compared to control in IsoP group (Fig 1A). 15-F2t-IsoP may have triggered an increased formation of ET-1 during late reperfusion. In the IsoP group, the infusion of 15-F2t-IsoP was terminated at 15 min of reperfusion. Sequestration of a significant amount 15-F2t-IsoP in the heart tissue 45 min after the termination of exogenous 15-F2t-IsoP infusion is unlikely in this study, since the 15-F2t-IsoP decay half-life in this model is about 4 min, as we observed in a preliminary study. 15-F2t-IsoP triggered increased formation of ET-1 during late reperfusion could represent an important mechanism responsible for post-ischemic myocardial dysfunction in the clinical setting. Whereas we previously found that plasma free 15-F2t-IsoP levels increased during ischemia-reperfusion for approximately 30 min during cardiac surgery, the 15-F2t-IsoP decay pattern during early reperfusion correlated with early postoperative cardiac recovery. Plasma ET-1 levels may remain elevated at least 24 hours after cardiac surgery. We postulate that high levels of 15-F2t-IsoP during ischemia and/or early reperfusion induces ET-1 gene expression resulting in increased ET-1 production during late reperfusion.

Our study allows the postulation that 15-F2t-IsoP may increase the secretion of ET-1 into the coronary circulation relative to the myocardial tissue during ischemia. The study of isolated perfused rats has shown that the ratio of ET-1 secretion to the interstitial transudates versus secretion to coronary effluent is about 6.6 at baseline. However, the ratio of ET-1 secretion to the interstitial transudates versus coronary
Effluent is reduced to about 2.5 during the period of low-flow ischemia and the first 30 min of reperfusion. The relative reduction of ET-1 concentration observed in the IsoP group at 30 min of reperfusion, 15 min after the termination of 15-F₂t-IsoP infusion, indicates 15-F₂t-IsoP may have primarily stimulated ET-1 release rather than its production during ischemia and early reperfusion.

Despite 15-F₂t-IsoP's bioactivity as a vasoconstrictor, reduction of coronary flow is not likely a major mechanism of 15-F₂t-IsoP action during myocardial IRI, at least in this model. In the current study, hearts were perfused at a constant flow rate. In addition, CPP at Re-60 did not differ significantly among experimental groups although LVDP in the IsoP group was significantly lower than that in the control, IsoP-SQ and the SQ groups. It is possible that 15-F₂t-IsoP aggravates myocardial IRI by a complex mechanism involving the activation of Na⁺-H⁺ exchange indirectly through the action of ET-1. Alternatively, 15-F₂t-IsoP may act by reducing the intrinsic activity of nitric oxide, an endogenous vasodilator. This may explain why the CPP value at Re-30 was higher in the IsoP group relative to control irrespective of the similar effluent levels of ET-1 at this time point.

Despite abolishing the deleterious effects of high concentration exogenous 15-F₂t-IsoP, SQ 29548 did not confer any beneficial effect in attenuating myocardial IRI compared to the control in this model. This is in keeping with previous findings describing the effect of exogenous 15-F₂t-IsoP on the isolated guinea pig heart. The relatively high concentration of CK-MB at Re-1 in the SQ group is likely due to rapid release of CK-MB from the ischemic tissue rather than the result of more intense tissue damage, since the infarct size of the SQ group is comparable to that in controls. The
inability of SQ 29548 to attenuate myocardial IRI in the isolated perfused heart model may suggest the following: (1) 15-F_2t-IsoP production in the myocardium during ischemia and reperfusion is relatively low, and it is mainly a marker rather than a mediator of oxidative damage; (2) TXA_2 may play little role in myocardial IRI in rat, a finding similar to that found in gene knock-out mice.  

The low LVDP at Re-10 in the IsoP-SQ group, although transient, is possibly a consequence of concomitant antagonism of the action of TXA_2 by SQ 29584. ET-1 can exert positive inotropic effect in the isolated rat heart. This effect could be potentiated via the action of TXA_2. The SQ 29548 blockade on TXA_2 action may be enhanced in the presence of 15-F_2t-IsoP, an alternative ligand of the TXA_2 receptor.

Our finding that 15-F_2t-IsoP can increase myocardial infarct size and exacerbate myocardial IRI may have important clinical implications. During cardiac surgery, systemic production of ROS occurs during CPB and may exceed production arising from reperfusion of the ischemic heart. Recent studies have found that the plasma level of 15-F_2t-IsoP dramatically increased shortly after the start of CPB. These high levels of 15-F_2t-IsoP could enter the heart either before aortic cross-clamping (the beginning of global myocardial ischemia) or at the time of aortic declamping, triggering and/or exacerbating myocardial IRI. The findings of the current study combined with our previous work on the effect of antioxidant supplementation with propofol suggest that combined therapy with antioxidant and 15-F_2t-IsoP antagonism during ischemia and early reperfusion could offer a promising approach to attenuate myocardial IRI.
5.6. Conclusion

In summary, our study has demonstrated that 15-F_{2t}\text{-IsoP}, a specific maker of oxidant damage, can exacerbate myocardial IRI as measured by elevated myocardial enzyme release, increased infarct size, and concomitant cardiac dysfunction. 15-F_{2t}\text{-IsoP}, applied before, or present in high concentration during, ischemia produced an increase in effluent ET-1 concentration during reperfusion in the isolated rat heart. This may provide a mechanism whereby 15-F_{2t}\text{-IsoP} mediates myocardial IRI. The 15-F_{2t}\text{-IsoP} – ET-1 relationship in the pathogenesis of IRI requires further evaluation in the laboratory and clinical setting.
CHAPTER 6

GENERAL SUMMARY AND CONCLUSIONS

6.1. Summary

Ischemic heart disease is a major cause of death and/or disability for adult men and women living in industrialized societies. Its morbidity and mortality increase with aging, a process known to be associated with decreases in endogenous antioxidant capacity.

Myocardial ischemia-reperfusion injury (IRI) is a major pathophysiologic factor contributing to post-operative cardiac dysfunction in patients undergoing coronary artery bypass surgery utilizing cardiopulmonary bypass (CPB). Reactive oxygen species (ROS)-mediated lipid peroxidation plays a critical role in mediating myocardial IRI. Using 15-F2t-isoprostane as reliable measure of lipid peroxidation, we have demonstrated for the first time that significant in vivo lipid peroxidation occurs early during myocardial ischemia and continues during reperfusion (as described in Chapter 2) rather than primarily during reperfusion in patients undergoing cardiac surgery utilizing CPB. This finding is confirmed by results of a concomitant study by Ulus and colleagues who submitted their work for publication in December 2002, shortly after the submission of our work in October 2002. Our results provide direct evidence that effective antioxidant intervention should be initiated and reinforced early during myocardial ischemia in order to maximally attenuate post-ischemic myocardial injury.
In Chapter 3, a unique therapeutic regimen of propofol was developed to best utilize its antioxidant properties in order to attenuate ROS generation during myocardial ischemia and early reperfusion using an isolated heart model. Propofol provides better cardiac protection when applied at the clinically achievable high concentration of 67 µM for 10 min before ischemia, during global myocardial ischemia, and continued during early reperfusion, followed by a relatively lower concentration during the later phase of reperfusion. This specific propofol treatment regimen has proven to be clinically promising (effective) in facilitating post-operative cardiac functional recovery in adult patients undergoing CABG surgery or heart valve(s) replacement surgery and in pediatric patients undergoing open heart surgery for congenital ventricular septum defect repair (personal communication from Dr. David M. Ansley, Clinical Associate Professor of Anaesthesiology, Vancouver General Hospital, The University of British Columbia, Canada; Dr. Zhiyong Hunag, Clinical Associate Professor of Anesthesia, Sun Yat-sen Cardiovascular Hospital, Shenzhen, China; and Dr. Jiazhen Gu, Professor of Anesthesiology, Renmin Hospital, Wuhan University, Wuhan, China). Of particular relevance is our identification, using the isolated perfused heart model, that 15-F_2r-isoprostane is produced in situ during global myocardial ischemia. This finding provides evidence to support the use of antioxidant intervention during ischemia which would target the coronary endothelium and/or the cardiomyocytes.

The study in Chapter 4 investigated whether or not aging could be a factor adversely affecting the cardiac protective effect on myocardial IRI. The results showed
that propofol, when applied at 67 μM before, during ischemia and during early reperfusion, equally preserved myocardial endogenous antioxidant capacity in the young and middle-age rat hearts and, more significantly, enhanced post-ischemic myocardial functional recovery in the middle-aged rat hearts relative to young rat hearts. This finding provides evidence to support the notion that drug(s) with antioxidant properties (such as propofol, in our study) could be more effective in attenuating myocardial IRI in populations suffering from insufficient or decreased endogenous antioxidant capacity, such as the elderly. In our study, we identified a strong inverse correlation between myocardial 15-F₂-isoprostane and post-ischemic cardiac function in the isolated rat heart. This is similar in nature to the finding described in Chapter 2 showing an inverse relation between the decay pattern of plasma 15-F₂-isoprostane during early reperfusion and postoperative cardiac function, suggesting 15-F₂-isoprostane itself could be a factor mediating myocardial IRI.

During myocardial ischemia and early reperfusion, apoptosis of coronary endothelial cells precedes myocyte apoptotic cell death in ischemia/reperfusion injury. 

Apoptosis spreads radially to the surrounding cardiac myocytes. 

To investigate whether propofol's protective effect against myocardial IRI involves the attenuation of vascular endothelial cell apoptosis, we examined the effects of propofol on tumor necrosis factor-alpha (TNFα)-induced human umbilical vein endothelial cell (HUVEC) apoptosis (Appendix I). We found that propofol, in the concentration range from 12.5 to 100 μM, reduced TNFα-induced HUVEC apoptosis in concentration-dependent fashion. The most apparent reduction in apoptosis being seen at propofol concentrations ≥ 50 μM.
This finding is in keeping with the results described in Chapter 3 concerning the dose-dependent protection by propofol against myocardial IRI and provides the first direct evidence that vascular endothelial cells are an important target of propofol action.

The strong correlation between plasma or coronary effluent 15-F_{2t}-isoprostane and post-ischemic myocardial function as described in Chapters 2 and 4 suggests (but does not prove) a causative effect of 15-F_{2t}-isoprostane in mediating myocardial IRI. Taken together with the fact that propofol attenuated, but did not completely prevent, the increase of 15-F_{2t}-isoprostane during myocardial ischemia and early reperfusion, it would be important to know if 15-F_{2t}-isoprostane can directly mediate myocardial IRI and if 15-F_{2t}-isoprostane antagonism could be a potential adjunct therapy. In the study described in Chapter 5, we found that 15-F_{2t}-isoprostane, when applied during myocardial ischemia and early reperfusion, exacerbated myocardial IRI as evidenced by the increased myocardial infarct size, cellular damage and reduced post-ischemic myocardial function. 15-F_{2t}-isoprostane antagonism with SQ 29548 abolished 15-F_{2t}-isoprostane deleterious effects. We also found evidence that 15-F_{2t}-isoprostane may mediate myocardial IRI, at least in part, by enhancing the release of ET-1 during ischemia and increasing ET-1 production during later reperfusion.

It is hoped that the studies described in the thesis have enhanced knowledge concerning the role of 15-F_{2t}-isoprostane in the pathogenesis of myocardial IRI, from its mechanism(s) of action to its clinical relevance. It is hoped that our findings can aid in the development of novel and effective therapeutic interventions that would favourably
influence the unacceptably high mortality and morbidity associated with myocardial ischemia-reperfusion injury.

6.2. Conclusions

Role of 15-F2t-isoprostane in the pathogenesis of myocardial IRI

(1) 15-F2t-isoprostane release is increased during myocardial ischemia and reperfusion in isolated perfused rat hearts and in clinical settings using CPB, indicating increased lipid peroxidation during both myocardial ischemia and reperfusion.

(2) 15-F2t-isoprostane is a potential mediator of myocardial IRI, its mechanism of action appears to involve the stimulation of ET-1 release during ischemia and ET-1 production during reperfusion. 15-F2t-isoprostane antagonism can abolish the deleterious effects of 15-F2t-isoprostane.

Protective effect of propofol against myocardial IRI

(1) The concentration-dependent protection by propofol against myocardial IRI is associated with a reduction of ROS-mediated lipid peroxidation and attenuation of 15-F2t-isoprostane production during ischemia and early reperfusion.
(2) Propofol may exert its cardiac protection by attenuating vascular endothelial cell apoptosis induced by noxious stimuli.

(3) Propofol, at clinically relevant (high) concentrations (up to 67 μM), cannot completely prevent the increased production of 15-F_{2t}-isoprostane during myocardial ischemia and reperfusion in the isolated rat heart model in the current study.

6.3. Future directions for research

Ischemic preconditioning or anesthetic preconditioning-mediated cardiac protection appears to require the generation of ROS prior to ischemia; however, it is not known whether or not 15-F_{2t}-isoprostane could be acting as a mediator downstream of ROS generation. Understanding this would be important with regard to the development of optimal combination therapy to limit the severity of myocardial IRI. 15-F_{2t}-isoprostane antagonism should be most effective if applied during ischemia and reperfusion if its potential in mediating ischemic preconditioning is confirmed.

It is yet to be established if 15-F_{2t}-isoprostane primarily stimulates the release of ET-1 during myocardial ischemia, or if it also stimulates ET-1 production, or increases ET-1 gene expression. Studies incorporating exogenous 15-F_{2t}-isoprostane and specific endothelin-converting enzyme inhibitor(s) may help further address this question. Although vascular endothelium is considered the major source of ET-1, ET-1 can also be produced by cardiac myocytes \(^{277, 278}\). Therefore, it would be of interest to investigate
whether or not 15-F_{2\alpha}-isoprostane-stimulated ET-1 release/production during myocardial ischemia-reperfusion originates primarily from coronary endothelium or from cardiomyocytes, or both.

Mechanistically, it would be meaningful to address whether 15-F_{2\alpha}-isoprostane can directly affect myocardial contractility under ischemic/hypoxic conditions. In addition, given the fact that the decay patterns of plasma 15-F_{2\alpha}-isoprostane predict the recovery of post-operative cardiac function as described in Chapter 2, it would be interesting to know if 15-F_{2\alpha}-isoprostane can adversely affect human vascular endothelial cell integrity during reperfusion. Using the established human umbilical vein endothelial cell culture model as described in appendix I, we may investigate whether 15-F_{2\alpha}-isoprostane can directly, or indirectly by affecting the secretion of ET-1, induce umbilical vein endothelial cell death. One could further explore the molecular signaling pathway(s) (i.e., specific G-protein-coupled receptors or different protein kinase C isomers) whereby 15-F_{2\alpha}-isoprostane exerts its actions.

Endothelin-1 is one of the most potent vasoconstrictors thought to contribute to post-ischemic myocardial dysfunction. A recent study has shown that endothelin-1 can stimulate arachidonic acid release by cytosolic phospholipase A2 activation in rat vascular smooth muscle \(^{279}\). This would increase the substrate (i.e., arachidonic acid) for 15-F_{2\alpha}-isoprostane production. If this is the case during myocardial ischemia-reperfusion, then 15-F_{2\alpha}-isoprostane-induced ET-1 production during the late phase of reperfusion could in turn promote the formation of 15-F_{2\alpha}-isoprostane. This vicious cycle could potentially affect the patency of the grafted vessels after CABG surgery. A high plasma
ET-1 level during the early postoperative period has been associated with prolonged pharmacologic management, longer intensive care unit stay, and complicated recovery. Therefore, the interplay between 15-F_2t-isoprostane and ET-1 during myocardial ischemia-reperfusion would be an important issue to address in the future studies.

Following these studies, a larger scale randomized clinical trial utilizing a combination of propofol and 15-F_2t-isoprostane antagonism during ischemia and early reperfusion and/or ET-1 antagonism during late reperfusion is clearly merited.
References


2. Reimer KA, Jennings RB: The "wavefront phenomenon" of myocardial ischemic cell death. II. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. *Lab Invest* 1979;40:633-44


   *Br Heart J* 1989;61:4-8


34. Murphy MP, Packer MA, Scarlett JL, Martin SW: Peroxynitrite: a biologically significant oxidant. *Gen Pharmacol* 1998;31:179-86


36. Murad F: Nitric oxide signaling: would you believe that a simple free radical could be a second messenger, autacoid, paracrine substance, neurotransmitter, and hormone? *Recent Prog Horm Res* 1998;53:43-59; discussion 59-60


58. Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ 2nd: Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. 

*Proc Natl Acad Sci USA* 1992;89:10721-5

59. Kayganich-Harrison KA, Rose DM, Murphy RC, Morrow JD, Roberts LJ 2nd: 
Collision-induced dissociation of F2-isoprostane-containing phospholipids. 

*J Lipid Res* 1993;34:1229-35


62. Morrow JD, Minton TA, Badr KF, Roberts LJ 2nd: Evidence that the F2-isoprostane, 8-epi-prostaglandin F2 alpha, is formed in vivo. *Biochim Biophys Acta* 1994;1210:244-8


72. Montine TJ, Montine KS, Reich EE, Terry ES, Porter NA, Morrow JD:
   Antioxidants significantly affect the formation of different classes of
   isoprostanes and neuroprostanes in rat cerebral synaptosomes. *Biochem
   Pharmacol* 2003;65:611-7

73. Dietrich M, Block G, Hudes M, Morrow JD, Norkus EP, Traber MG, Cross CE,
   Packer L: Antioxidant supplementation decreases lipid peroxidation
   biomarker F(2)-isoprostanes in plasma of smokers. *Cancer Epidemiol
   Biomarkers Prev* 2002;11:7-13

74. Takahashi K, Nammour TM, Fukunaga M, Ebert J, Morrow JD, Roberts LJ 2nd,
   Hoover RL, Badr KF: Glomerular actions of a free radical-generated novel
   prostaglandin, 8-epi-prostaglandin F2 alpha, in the rat. Evidence for

75. Fukunaga M, Makita N, Roberts LJ 2nd, Morrow JD, Takahashi K, Badr KF:
   Evidence for the existence of F2-isoprostane receptors on rat vascular

76. Mobert J, Becker BF, Zahler S, Gerlach E: Hemodynamic effects of isoprostanes
   (8-iso-prostaglandin F2alpha and E2) in isolated guinea pig hearts. *J
   Cardiovasc Pharmacol* 1997;29:789-94

77. Kromer BM, Tippins JR: The vasoconstrictor effect of 8-epi prostaglandin F2alpha
   in the hypoxic rat heart. *Br J Pharmacol* 1999;126:1171-4
78. Mallat Z, Philip I, Lebret M, Chatel D, Maclouf J, Tedgui A: Elevated levels of 8-
iso-prostaglandin F2alpha in pericardial fluid of patients with heart failure: a
potential role for in vivo oxidant stress in ventricular dilatation and
progression to heart failure. *Circulation* 1998;97:1536-9

Tamaddon F, Grimm M, Glogar HD, Sinzinger H: The isoprostane, 8-epi-
PGF2 alpha, is accumulated in coronary arteries isolated from patients with

J: Immunological characterization of urinary 8-epi-prostaglandin F2 alpha


82. Itoh S, Lu R, Bao Y, Morrow JD, Roberts LJ, Schuster VL: Structural determinants
of substrates for the prostaglandin transporter PGT. *Mol Pharmacol*
1996;50:738-42


84. Roberts LJ 2nd, Moore KP, Zackert WE, Oates JA, Morrow JD: Identification of
the major urinary metabolite of the F2-isoprostane 8-iso-prostaglandin


**Notes:** CORPORATE NAME: PPP Collaborative Group on the antioxidant effect of vitamin E.


100. Hess ML, Manson NH: Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. *J Mol Cell Cardiol* 1984;16:969-85


137. Magarey JM: Propofol or midazolam--which is best for the sedation of adult ventilated patients in intensive care units? A systematic review. *Aust Crit Care* 2001;14:147-54


140. Bao YP, Williamson G, Tew D, Plumb GW, Lambert N, Jones JG, Menon DK:
   Antioxidant effects of propofol in human hepatic microsomes: concentration
   effects and clinical relevance. *Br J Anaesth* 1998;81:584-9

141. Murphy PG, Myers DS, Davies MJ, Webster NR, Jones JG: The antioxidant

142. Ko SH, Yu CW, Lee SK, Choe H, Chung MJ, Kwak YG, Chae SW, Song HS:
   Propofol attenuates ischemia-reperfusion injury in the isolated rat heart.
   *Anesth Analg* 1997;85:719-24

143. Kokita N, Hara A: Propofol attenuates hydrogen peroxide-induced mechanical and
   metabolic derangements in the isolated rat heart. *Anesthesiology*
   1996;84:117-27

144. Musacchio E, Rizzoli V, Bianchi M, Bindoli A, Galzigna L: Antioxidant action of
   propofol on liver microsomes, mitochondria and brain synaptosomes in the

145. Cheng DC, Karski J, Peniston C, Raveendran G, Asokumar B, Carroll J, David T,
   Sandler A: Early tracheal extubation after coronary artery bypass graft
   surgery reduces costs and improves resource use. A prospective,
   randomized, controlled trial. *Anesthesiology* 1996;85:1300-10


165. Sorescu D, Griendling KK: Reactive oxygen species, mitochondria, and NAD(P)H oxidases in the development and progression of heart failure. *Congest Heart Fail* 2002;8:132-40


246. Barry WH, Peeters GA, Rasmussen CA Jr, Cunningham MJ: Role of changes in 
[Ca\(^{2+}\)]\(_i\), in energy deprivation contracture. *Circ Res* 1987;61:726-34

**Notes:** i in energy deprivation contracture


249. Javadov SA, Lim KH, Kerr PM, Suleiman MS, Angelini GD, Halestrap AP:
Protection of hearts from reperfusion injury by propofol is associated with inhibition of the mitochondrial permeability transition. *Cardiovasc Res* 2000;45:360-9


255. Zaugg M, Lucchinetti E, Spahn DR, Pasch T, Schaub MC: Volatile anesthetics mimic cardiac preconditioning by priming the activation of mitochondrial K(ATP) channels via multiple signaling pathways. *Anesthesiology* 2002;97:4-14


270. Downey JM: Measuring infarct size by the tetrazolium method. Available at: 


APPENDIX 1: Propofol inhibition of TNF-alpha-induced vascular endothelial cell apoptosis: effects on Bcl-2 and Bax protein expression

Introduction

The vascular endothelial monolayer serves as a barrier between the bloodstream and the vascular wall. Apoptotic endothelial cell death may critically disturb the integrity of endothelial monolayer and thereby contribute to vascular injury and atherosclerosis. Apoptosis has become increasingly recognized as a mechanism of cell death during myocardial ischemia reperfusion injury (IRI), although the relative contribution of necrosis and apoptosis to total cardiac cell loss during IRI remains controversial. Recently, endothelial cell apoptosis was shown to precede myocyte cell apoptosis in the setting of myocardial IRI. The latter study suggests that circulatory pro-apoptotic inflammatory cytokines (such as tumor necrosis factor-alpha, TNF-alpha) and reactive oxygen species (ROS), that are elevated during myocardial IRI and atherosclerosis, promote myocyte apoptosis subsequent to the induction of endothelial cells apoptosis.

Propofol, 2,6-diisopropylphenol, an intravenous anesthetic agent frequently used during cardiac surgery and in postoperative sedation, enhances red blood cell and tissue antioxidant capacity both in vitro and in vivo. We recently demonstrated that propofol enhances myocardial antioxidant capacity and results in improved post-ischemic cardiac function in the isolated rat heart, in a dose-dependent manner. In addition,
recent studies show that aging enhances the sensitivity of human endothelial cells toward apoptotic stimuli.\textsuperscript{283} Interestingly, propofol, when applied at a clinically achievable high concentration, enhances the ischemic tolerance of middle-aged rat hearts.\textsuperscript{227} This finding prompted us to postulate that propofol may produce a cardioprotective effect that is attributable to its ability to enhance endothelial cells resistance toward apoptotic stimuli.

We hypothesized that propofol could inhibit TNF-alpha induced human umbilical vein endothelial cells (HUVECs) apoptosis by resuming a proper ratio of the anti-apoptotic Bcl-2 protein over the pro-apoptotic Bax protein expression and that the propofol anti-apoptotic effect is related to its antioxidant capacity and its ability to enhance the generation of nitric oxide (NO), an important endothelial cell survival factor.\textsuperscript{284, 285}

\section*{Methods}

\subsection*{Cell Culture}

Human umbilical vein endothelial cells (HUVECs) were isolated according to the method of Jaffe et al.\textsuperscript{286} Cells were cultured in a medium of DMEM (Gibco) supplemented with 20\% bovine calf serum (Sigma), maintained at 37 °C in 5\% CO\textsubscript{2}, and used at passage 2-3 to avoid "age-dependent" variations in levels of apoptosis.\textsuperscript{287}

\textit{Study 1.} Dose-dependent effect of propofol on TNF-alpha induced HUVECs apoptosis

When the cells were at 70\% confluence, the cultured HUVECs were divided into seven groups: HUVECs in untreated group (control) and propofol treatment control (P25) group were further cultured at 37 °C for 24 hours, respectively, in the absence
(control) or presence of 25 μM propofol (Zeneca, Ltd.) in the medium; HUVECs in the
TNF-alpha (TNF) group and TNF-alpha plus propofol treatment groups were initially
cultured for 30 min in the presence of zero (TNF), 12.5 (P_{12.5}+TNF), 25 (P_{25}+TNF), 50
(P_{50}+TNF) and 100 (P_{100}+TNF) μM propofol, respectively. Cells were then cultured for
24 hours with TNF (40 ng/mL). The concentration of TNF used to induce apoptosis in
the present study was chosen on the basis of previously published literature in addition
to preliminary studies.

**Study 2.** Synergistic effect of H\textsubscript{2}O\textsubscript{2} and TNF in inducing HUVECs apoptosis

When the cells were at 70% confluence, the cultured HUVECs were divided into
three treatment groups: (1) HUVECs were cultured in the presence of 10 μM /L H\textsubscript{2}O\textsubscript{2}
(H), (2) 10 μM /L H\textsubscript{2}O\textsubscript{2} plus 40 ng/mL TNF (TNF+H), or (3) 10 μM /L H\textsubscript{2}O\textsubscript{2} plus 40
ng/mL TNF and 50 μM propofol (TNF+H+P\textsubscript{50}) in the medium, respectively, at 37 °C for
24 h. Study results were compared with those obtained from the control and TNF-alpha
groups of study 1.

**Detection of Apoptosis**

Apoptosis was detected using DNA in situ terminal deoxynucleotidyl transferase
(TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling (TUNEL)
staining as per the manufacturer's protocol (Boshide Biotech Ltd, Wuhan, China). In
brief, after equilibration, end-labeling with digoxigenin-11-dUTP by TdT enzyme in
buffer was carried out for 1 hour at 37°C in a humidifying chamber. After treatment with
stop/wash buffer, sections were incubated with anti-digoxigenin antibody–peroxidase
conjugate, rinsed, and stained with diaminobenzidine tetrahydrochloride. Negative controls were incubated with PBS instead of TdT enzyme, and positive controls were treated with DNase1. Sections were counterstained with Mayer's hematoxylin and mounted. All experiments were repeated on at least six independent occasions with consistent results.

**Immunocytochemistry**

Cells were washed in DMEM without BSA, and cytospins were performed (650 rpm for 6 minutes) on saline-coated slides at $1 \times 10^6$ cells/mL. Slides were fixed in 2% paraformaldehyde for 15 minutes at room temperature and washed 5 times in PBS. Cells were permeabilized for 10 minutes at room temperature in blocking buffer (3% BSA in PBS) plus 0.1% Triton X-100 followed by blocking of nonspecific binding in blocking buffer for 1 hour at room temperature. They were then incubated with the primary antibody (anti-Bcl-2 1:50)(Boshide Biotech Ltd, Wuhan); anti-nitrotyrosine 1:1000 diluted in phosphate-buffered saline (PBS) containing 1.5% goat serum(Boshide Biotech Ltd, Wuhan). Following overnight incubation at 4°C, cells were washed with PBS and incubated for 30 min at RT with the biotinylated secondary antibody. After washing, the cells were incubated for 30 min in Vectastain Elite ABC reagent (Boshide Biotech Ltd, Wuhan). After another wash, cells were incubated in peroxidase substrate solution until desired stain intensity is developed. The cells were then washed, dehydrated in increasing concentrations of ethanol, and cover-slipped using permount. Control sections were either incubated with the secondary antibody alone or immunoabsorbed with an excess of blocking peptide. Random fields (20-30 per slide) were examined at a high magnification
(×400) to calculate the prevalence of DNA fragmentation and \textit{Bcl-2/Bax} expression. The percentage of TUNEL positive cells (termed apoptotic index, AI) was determined by dividing the number of positive-staining nuclei by the total number of nuclei of the cell and multiplying that value by 100. The percentage of \textit{Bcl-2/Bax} expression was also determined. The density of Bcl-2 and Bax protein expression were determined using an automatic computer-assisted image analyzing system (IBAS-2000 Kontron, Germany), which automatically measure the density of 100 HUVECs from 4 to 6 random fields. The density of Bcl-2 and Bax protein expression were expressed in arbitrary units.

\textbf{Electron Microscopy}

Electron microscopy was performed to confirm that the ultrastructural features of apoptosis were present in cells exposed to TNF in the present study. Endothelial cells exposed to the conditions outlined above were fixed in 2.5% glutaraldehyde (pH 7.3) buffered with 0.1 mol/L sodium cacodylate overnight at 4°C and then washed with 0.1 mol/L sodium cacodylate buffer for 15 min before post-fixation with 1% osmium tetroxide buffered with 0.1 mol/L sodium cacodylate for 1 h on ice. After another wash with 0.1 mol/L sodium cacodylate buffer for 15 min, cells were dehydrated with increasing concentrations of alcohol. Next, cells were infiltrated with propylene oxide for 15 min, followed by 1:1 propylene oxide:epoxy resin for 1 h, 1:2 propylene oxide:epoxy resin for 2 h, and finally 100% epoxy resin for 2 h. Cells were embedded with fresh epoxy resin into molds and placed in a 60°C oven for 2 h. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined with the use of a Hitachi H-600 electron microscope (Hitachi, Japan).
Nitric Oxide (NO) content

Media from study 1 was collected 24 h after their respective treatments. The concentration of nitrites (NO₂⁻) and nitrates (NO₃⁻), stable end products of nitric oxide (NO), was determined by the Griess reaction, as follows. After deproteination by a solution of zinc sulfate, samples were incubated with cadmium granules to reduce nitrate to nitrite. The total nitrite was measured at 540 nm absorbance by diazotization with Griess reagent (Boshide Biotech Ltd, Wuhan). Nitrite concentrations were calculated by comparison with a standard calibration curve with sodium nitrite.

Statistical Analysis

Results are expressed as mean ± SEM. Significance was evaluated using ANOVA followed by Tukey’s post test. The correlation relationships were evaluated by the Pearsons test. P <0.05 was considered significant.

Results

Endothelial cell apoptosis

TUNEL staining was rare in control (3.1±0.5%) and propofol (P25) treated HUVECs (3.0±0.6%) (Fig. A-1, A, B). Stimulation of HUVECs with TNF resulted in a dramatic increase in the AI to 45.5±1.2% (Fig.A-1,C, E). Propofol dose-dependently reduced TNF-induced apoptosis. More profound reduction in AI was seen in P25 (35.0±0.7%, Fig.A-1, D,E) and P50 (25.2±0.8%). P100 did not significantly further decrease AI (22.6±0.5%) compared to that of P50.
**Immunohistochemical analysis of Bcl-2 and Bax protein expression**

As shown in figure A-2 and A-3, stimulation of HUVECs with TNF leads to a significant reduction in Bcl-2 protein expression and a significant increase in Bax protein expression as compared to untreated controls. Propofol (25 µM) did not affect either Bcl-2 or Bax protein expression in the absence of TNF stimulation. However, propofol, at ≥12.5 µM, significantly and dose-dependently attenuated TNF induced reduction in Bcl-2 protein expression (Fig.A-2, E). The maximal effect was seen at P_{100}. The Bcl-2 density in the P_{100} plus TNF (P_{100}+TNF) group was significantly higher than that in the P_{50} plus TNF (P_{50}+TNF) group. Propofol also significantly attenuated TNF induced elevation in Bax protein expression in a dose-dependent manner at the range 12.5 to 50 µM. There was no difference in effect between P_{50} and P_{100}. TNF reduced the ratio of Bcl-2 expression over Bax expression (Bcl-2/Bax) as compared to control (Fig.A-4, A). Propofol dose-dependently attenuated the TNF induced reduction in Bcl-2/Bax ratio (Fig. A-4, A).

**NO production**

As shown in figure A-5, stimulation of HUVECs with TNF leads to significantly increased production of NO as compared to untreated control. Interestingly, addition of propofol (at 25 µM) to the culture medium also results in a significantly increased production of NO in the absence of a noxious stimulus (such as TNF). P_{12.5} and P_{25}, in a dose-dependent manner, further increased TNF induced release of NO. However, P_{50} and P_{100} did not result in more profound increase in HUVECs NO release in the presence of TNF, as compared to P_{25}. 
Electron Microscopy

By qualitative electron microscopic analysis, typical features of apoptosis could hardly be seen in HUVECs from control and P25 groups (Fig. A-6), but they were apparent in HUVECs from TNF group (Fig A-6, C). Apoptotic morphologic changes can also be seen in HUVECs from TNF plus propofol treatment groups (Fig A-6, D shows a typical endothelial cell from P25+T group), but to a much less degree in terms of severity.

Effect of H2O2 on TNF induced endothelial cell apoptosis, Bcl-2 and Bax expression

We then investigated whether H2O2 at a relatively low concentration (10 μmol/L), could further augment the potential of TNF to increase apoptosis in HUVECs. As shown in Fig.A-7, H2O2 itself could independently increase the HUVEC apoptotic index (approximately 2-fold as compared to untreated control), but to a much less degree as compared to TNF which increased HUVEC apoptotic index by approximately 14-fold. Stimulation of HUVECs concomitantly with H2O2 and TNF lead to about 20-fold increase in HUVECs apoptotic index comparing to control (AI =62.7±1.4% in T+H group, vs 3.1±0.5% in control, Fig.A-7, F), showing apparent synergistic effects. Coincidentally, cellular morphological changes are more severe in the T+H group than in the TNF group (data not shown).

As expected, stimulation of HUVECs concomitantly with H2O2 and TNF led to a further significant decrease in Bcl-2 protein expression (Fig.A-8, A). It is of interest that H2O2 did not exaggerate TNF induced increase of Bax protein expression (Fig.8B).
Propofol (50 μM) significantly attenuated the joint effect of H$_2$O$_2$ and TNF in increasing HUVECs apoptotic index and in decreasing Bcl-2 protein expression (Fig A-7 and A-8).

**Correlation analysis**

A tight inverse correlation exists between the ratio of Bcl-2/Bax protein expression and apoptotic index in untreated control HUVECs, and TNF treated HUVECs with or without concomitant administration of propofol ($r$=-0.9520, $P=0.0009$, Fig.A-4, B). There was no relation between NO production and apoptotic index in HUVECs from control and the propofol (25 μM) treated groups. This is because propofol increased NO production without affecting the AI in the absence of TNF stimulation. A trend of inverse relation between NO production and AI was seen in HUVECs stimulated with TNF alone or TNF with varying concentrations of propofol, but this is not statistically significant ($r$ =-0.85, $P =0.07$). However, a weak but significant positive correlation exists between NO production and the rate of Bcl-2/Bax protein expression ($r=0.93$, $P =0.02$) in HUVECs stimulated with TNF with and without propofol treatments.
Figure A-1. TNF-induced apoptotic cell death in cultured HUVECs is confirmed with TUNEL staining. TUNEL positive (TP) cells are stained brown. The percentage of TP cells is termed apoptotic index (AI). Representative photomicrographs of HUVECs from untreated control (column A), propofol (25 μM, P25) treated (column B), TNF (T) treated (column C), and (column D) TNF and P25 co-treated (P25+T) groups. Original manifestations: ×200. Column E summarized the average AI of HUVECs from control, P25 or TNF (T) treated groups, or HUVECs co-cultured with T plus varying concentrations of propofol ranging from 12.5 (P25), 25 (P25), 50 (P50) and 100 (P100) μM. TUNEL-positive nuclei were counted and expressed as the percentage of total nuclei. A total of 1000 nuclei were counted in 10 random fields (n=10) on slides from each group.

* P<0.001 vs control; † P<0.001 vs T; ‡ P<0.001 or P<0.05 vs P50+T.
**Figure A-2.** The expression of Bcl-2 protein evaluated by immunoperoxidase technique (PAP). Bcl-2 immunostaining positive cells display brown to deep brown particles (stained proteins) in the cytoplasm. Columns A through D show representative photomicrographs of HUVECs from untreated control (A), propofol (25 µM, P25) treated (B), TNF (T) treated (C), and (D) TNF and P25 co-treated (P25+T) groups. Original manifestations: ×200. Column E summarized the Mean (and SEM) levels of Bcl-2 protein (in arbitrary densitometry units) of HUVECs from control, P25 or TNF (T) treated groups, or HUVECs co-cultured with T plus varying concentrations of propofol ranging from 12.5 (P25), 25 (P25), 50 (P50) and 100 (P100) µM. * P<0.001 vs control; + P<0.001 vs T; # P<0.001 or P<0.05 vs P50+T. (n=100 cells per group).
Figure A-3. The expression of Bax protein evaluated by immunoperoxidase technique (PAP). Bax immunostaining positive cells display brown to deep brown particles (stained proteins) in the cytoplasm. Columns A through D show representative photomicrographs of HUVECs from untreated control (A), propofol (25 μM, P25) treated (B), TNF (T) treated (C), and (D) TNF and P25 co-treated (P25+T) groups. Original manifestations: ×200. Column E summarized the Mean (and SEM) levels of Bax protein (in arbitrary densitometry units) of HUVECs from control, P25 or TNF (T) treated groups, or HUVECs co-cultured with T plus varying concentrations of propofol ranging from 12.5 (P25), 25 (P25), 50 (P50) and 100 (P100) μM. * P<0.001 vs control; † P<0.001 vs T; # P<0.001 or P<0.05 vs P50+T. (n=100 cells per group).
Figure A- 4. A. Bcl-2/Bax ratio. The Bcl-2/Bax ratio is significantly decreased in endothelial cells treated with TNF (T). Propofol at 12.5 (P12.5), 25(P25), 50 (P50) and 100 µM (P100) dose-dependently increased Bcl-2/Bax ratio when administered with TNF. *P<0.001 vs control; †P <0.001 vs T; ‡P < 0.001 or P< 0.01 vs P50+T. B. An significant inverse correlation exists between endothelial cell apoptotic index (AI) and the ratio of bcl-2 over Bax proteins expression (r=-0.9520, 95% CI: -0.9931 to -0.7029; P =0.0009).
Figure A-5. Nitric oxide (NO) concentration in the culture medium. Cultured endothelial cells were either untreated (control), treated with propofol (25 μM, P25) alone, with TNF (T) or T plus varying concentrations of propofol ranging from 12.5 (P25), 25 (P50), 50 (P50) and 100 (P100) μM. * P<0.001 vs control; † P <0.001 vs T; # P <0.001 vs P50+T. (n=6 measures per group).
Figure A- 6. Representative electron microscopy of endothelial cells (ECs). A (control) and B (ECs treated with propofol alone at 25 μM): Normal ECs. C (EC treated with TNF): a typical apoptotic EC from TNF group. Local cytomembrane break, cytoplasm condensation and vesicle formation, chromatin condensation and margination, nucleolus can not be seen. D (EC treated with TNF and propofol at 25 μM): a typical EC in TNF+P25 group undergoing apoptosis. Cytomembrane intact, localized cytoplasm vesicle formation, nucleolus margination. E. a typical EC from TNF + P50 group. Original magnification: ×5000.
**Figure A-7.** H$_2$O$_2$ and TNF synergistically induced apoptotic cell death in cultured HUVECs. TUNEL positive cells are stained brown. Representative photomicrographs of HUVECs from untreated control (column A), H$_2$O$_2$ (H) treated (column B), TNF (T) treated (column C), H$_2$O$_2$ and TNF co-treated (H+T), H$_2$O$_2$ and TNF and propofol (50 μM, P) co-treated (T+H+P) groups. Original manifestations: ×200. Column E summarized the average apoptotic index (AI) of the individual group. TUNEL-positive nuclei were counted and expressed as the percentage of total nuclei. A total of 1000 nuclei were counted in 10 random fields on slides from each group. * P<0.001 vs control; † P<0.001 vs T; # P<0.001 vs T+H.
**Figure A-8.** Effects of $H_2O_2$ on TNF-mediated changes in Bcl-2 (A) and Bax (B) expression. The expression of Bcl-2 and Bax protein were evaluated by immunoperoxidase technique (PAP). Levels of Bcl-2 and Bax protein expression (in arbitrary densitometry units) were expressed as mean ± SEM. HUVECs cultures was either untreated (control), treated with $H_2O_2$ (H)25 or TNF (T), co-cultured with T plus H, or co-cultured with T+H plus propofol at 50 µM (T+H+P50). * P<0.001 vs control; † P<0.001 vs T; ‡ P<0.001 vs T+H. (n=100 cells per group).
Discussion

TNF stimulation resulted in a reduced Bcl-2/Bax ratio in HUVECs. Propofol dose-dependently enhances the ratio of the anti-apoptotic Bcl-2 protein over the pro-apoptotic Bax protein expression in this system. This was associated with graded suppression of TNF-induced apoptosis as assessed by TUNEL assay and confirmed by characteristic apoptotic morphologic changes. At a dose range from 12.5 to 50 μM, propofol enhancement of Bcl-2/Bax ratio is achieved through an increase in expression of Bcl-2 and a decrease in the expression of Bax. However, this effect appears dose limited since the highest concentration of propofol (P_{100}) does not significantly reduce Bax expression more than P_{50}. However, P_{100} did increase Bcl-2 expression more than P_{50}. This could be explained on the basis that P_{100} completely abolished TNF-induced increases in Bax expression while maintaining baseline levels (Fig. A-3, E). This is likely an important mechanism of protection. Like other important molecules, notably nitric oxide (NO), Bax also has a dual role. Under pathological conditions, Bax overexpression may induce mitochondrial depolarization and cytochrome c release, resulting in the downstream activation of executioner caspases to augment apoptosis. The formation Bax-Bax homodimer serves to induce apoptosis, while the Bax-Bcl-2 heterodimer formed under physiological conditions, is an important inhibitor of apoptosis.

It is noteworthy that propofol treatment primarily restores the expression of Bcl-2 and the Bcl-2/Bax ratio towards normal values and does not result in an overexpression of the anti-apoptotic Bcl-2 protein. This effect is not what we expected to see, but may represent a promising therapeutic approach. Bcl-2 is localized to intracellular sites of
ROS generation including mitochondria and may function in an antioxidant pathway to prevent apoptosis.\textsuperscript{292} Following an apoptotic signal, cells sustain progressive lipid peroxidation. Overexpression of Bcl-2 functions to suppress lipid peroxidation.\textsuperscript{292} However, in lymphocytes the level of Bcl-2 expression may determine the balance between apoptosis and necrosis, but does not prevent cell death induced by oxidized low density lipoproteins (oxLDL).\textsuperscript{293} In cells expressing relatively high levels Bcl-2, oxLDL induced mainly necrosis. In cells expressing relatively low levels Bcl-2, the rate of oxLDL-induced apoptosis was higher than that of primary necrosis.\textsuperscript{293} Recent studies demonstrate that overexpression of Bcl-2 paradoxically exerted a pro-apoptotic effect in the reperfused liver.\textsuperscript{294} Taken together, these studies, together with ours, suggest that stabilizing Bcl-2 and Bax expression, rather than induction of the "anti-apoptotic" Bcl-2 and/or suppression of the "pro-apoptotic" Bax protein, represents a more meaningful approach.

Propofol (P\textsubscript{25}) treatment, in the absence of TNF, enhances the production and release of NO from HUVECs. This is similar in nature to a previous report that application of propofol stimulates the production of NO from cultured porcine aortic endothelial cells.\textsuperscript{295} Interestingly, TNF enhances the production of NO to a similar degree as propofol (Fig. A-5), but, in contrast, this is accompanied by an increase in apoptosis in HUVECs. It seems plausible that the TNF-induced NO overproduction relative to control levels (in the absence of antioxidant intervention) would result in increased production of peroxynitrite which may promote apoptosis by increasing Bax expression.\textsuperscript{296} Co-culture of HUVECs with TNF and propofol led to profound overproduction of NO compared to TNF or propofol alone. This is associated with reduced Bax expression and enhanced
Bcl-2 production compared to the TNF group. This suggests that overproduction of NO by endothelial cells in response to TNF stimulation is initially aimed to protect the cells, rather than produce cell injury. This is manifested by the significant positive correlation between NO production and the rate of Bcl-2/Bax protein expression in HUVECs stimulated with TNF, with and without propofol in increasing concentrations. Indeed, study shows that NO confers resistance to apoptosis in HUVECs, likely secondary to the attenuation of caspase activity by nitrosylating caspase 3 and stabilizing Bcl-2. The question arises “How does propofol confer its protective effect on endothelial cells?”

TNF stimulates upregulation of NO synthase activity and NO production in HUVECs which can be accompanied by a burst in production (three- to fourfold increase) of intracellular ROS including superoxide anion and H_2O_2. The reaction of NO with superoxide anion can increase the generation of peroxynitrite (ONOO'). Exposure of eNOS to oxidants (including peroxynitrite) causes increased enzymatic uncoupling and the generation of superoxide anion rather than NO, resulting in increased oxidant stress and a net decrease in NO production. Propofol can directly scavenge ROS including ONOO', therefore, blocking the vicious cycle. Our finding that the addition of H_2O_2 exaggerates TNF induced apoptosis supports the interplay between ROS and TNF. The H_2O_2 concentration (10 umol/L) used in this study is in the lowest range that induces endothelial cell apoptosis but not necrosis. Since H_2O_2 is not a direct source of ONOO' (which increases Bax expression), its use in this study helps to elucidate the relative roles of ROS in inducing endothelial cell apoptosis.

The release of cytochrome c from mitochondria into the cytosol is considered as an important event leading to irreversible cell death and the opening of mitochondrial...
transition pore (MPTP) may be an important mechanism for mitochondrial cytochrome c release.\textsuperscript{305} Inhibition of MPTP reduced TNF induced release of mitochondrial cytochrome c in HUVECs.\textsuperscript{306} Further, it has been suggested that propofol may offer myocardial protection by inhibiting MPTP,\textsuperscript{249} at concentrations low as 11-22 \textmu M (2-4\textmu g/ml), likely acting indirectly through its antioxidant properties since ROS is a major mediator of MPTP opening.\textsuperscript{253} Propofol, however, may directly inhibit myocyte MPTP at concentrations \geq 50 \textmu M.\textsuperscript{252} Interestingly, the most profound inhibition of TNF induced HUVECs apoptosis is manifested at propofol concentrations \geq 50 \textmu M (P_{50} and P_{100}) in this study, suggesting that inhibition of MPTP in HUVECs as one of the mechanisms of propofol protection.

**Conclusions and clinical implications**

Our study provides evidence for the first time that propofol, a commonly used anesthetic agent for cardiac surgery and for postoperative sedation, attenuates TNF induced HUVECs apoptosis in a dose-dependent manner. Given the fact that the release of inflammatory cytokines (including TNF) is increased during and after cardiac surgery and that serum from patients with acute coronary syndromes displays a pro-apoptotic effect on endothelial cells,\textsuperscript{307} application of propofol, in high concentrations (~50 to 60 \textmu M),\textsuperscript{125} may prove to be a promising approach in reducing peri- and post-operative IRI and related cardiovascular symptoms.