Abstract

Heart transplantation is often the only therapeutic option for patients with end stage heart disease. Allograft organs are in short supply. Thus, preserving the life of a grafted organ is extremely important. Cardiac allograft vasculopathy (CAV) is an expression of chronic rejection that accounts for the greatest loss of graft function in transplanted hearts. Peri-transplant ischemia/reperfusion (I/R)-injury occurs during transplantation when blood flow is stopped to remove the heart from the donor and then is reinstated upon implantation of the donor heart into the recipient. This oxidative injury contributes to vascular dysfunction and CAV. In this dissertation, I hypothesize that prevention and/or reduction of I/R during transplantation reduces post-transplant vascular dysfunction and CAV. In this regard, myself and my colleagues, examined the roles of apoptosis repressor with caspase recruitment domain (ARC) and cytochrome p450 (CYP) 2C enzymes in I/R-induced vascular dysfunction and CAV.

ARC expression was detected in endothelial cells (ECs) and smooth muscle cells (SMCs); however, increased levels of ARC do not protect against oxidant injury. ARC overexpression did protect against oxidant-induced cell death in H9c2 rat embryonic myoblasts. We observed that ARC-overexpression prevented H9c2 differentiation into muscle cells. With our focus on vascular injury, we turned our attention to the CYP 2C enzymes. Both endothelium-dependent and independent vascular function was impaired following I/R. Pre-treatment with the CYP 2C inhibitor sulfaphenazole (SP) restored endothelial sensitivity to acetylcholine, but did not restore sensitivity to endothelium-independent vasodilators. Rat heterotopic heart transplants were performed with rats being
treated with SP or vector control prior to surgery. Rats treated with SP showed significantly reduced luminal narrowing and had decreased SMC proliferation, oxidant and interferon-γ levels. No differences were detected in immune infiltration or apoptosis. Complementary studies in cultured vascular cells revealed that CYP 2C9 expression decreased viability and increased ROS production following hypoxia and re-oxygenation in ECs but not in SMCs.

In summary, we did not detect protection of vascular cells by ARC, but did discover a novel role for ARC in differentiation. CYP 2C contributes to post-ischemic vascular dysfunction and CAV through increased oxidative stress and endothelial dysfunction.
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<th>Symbol</th>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>AA</td>
<td>Arachidonic acid</td>
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<tr>
<td></td>
<td>ACh</td>
<td>Acetylcholine</td>
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<tr>
<td></td>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td></td>
<td>AM</td>
<td>Acetoxymethyl</td>
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<td></td>
<td>AMR</td>
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<td>ARC</td>
<td>Apoptosis repressor with caspase recruitment domain</td>
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<tr>
<td>C</td>
<td>[Ca$^{2+}$]_c</td>
<td>Intracellular calcium levels</td>
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<td>CARD</td>
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<td></td>
<td>CAV</td>
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<tr>
<td></td>
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<td></td>
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<td></td>
<td>DHE</td>
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<td></td>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
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<td>E</td>
<td>E(B/G)M</td>
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<td></td>
<td>EC</td>
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<td></td>
<td>EDHF</td>
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<tr>
<td></td>
<td>EGF</td>
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<tr>
<td></td>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td></td>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
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<tr>
<td>F</td>
<td>F344</td>
<td>Fisher 344 rat</td>
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<tr>
<td></td>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<td></td>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>G</td>
<td>GA-1000</td>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<td>Growth-related oncogene</td>
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<td>H/R</td>
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<td>Hydrogen peroxide</td>
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<td>HAR</td>
<td>Hyperacute rejection</td>
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<td></td>
<td>HS</td>
<td>Horse serum</td>
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<tr>
<td></td>
<td>HUVEC</td>
<td>Human umbilical venous endothelial cell</td>
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<tr>
<td>I</td>
<td>I/R</td>
<td>Ischemia and reperfusion</td>
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</table>
ICAM - Intracellular adhesion marker
IFN-γ - Interferon gamma
IHC - Immunohistochemistry
IL - Interleukin
iNOS - Inducible nitric oxide synthase
iPLA₂ - Inducible phospholipase A₂
ISHLT - International society for heart and lung transplantation
IVUS - Intravascular ultrasound
LOX - Lipoxygenase
LT - Leukotrienes
MAPK - Mitogen activated protein kinase
MCP - Monocyte chemoattractant protein
MHC - Major histocompatibility complex
MI - Myocardial infarction
MTS - CellTiter96 AQueous assay
Neo - Neomycin
NF-κB - Nuclear factor kappa B
NK - Natural killer
NO• - Nitric oxide
O₂• - Superoxide
ONO0- - Peroxynitrite
OxLDL - Oxidized low density lipoprotein
PARP - Poly (ADP-ribose) polymerase
PECAM - Platelet endothelial cell adhesion molecule
PG - Prostaglandin
PGI₂ - Prostacyclin
PKC - Protein kinase C
PLA₂ - Phospholipase A₂
PVS - Perivascular space
RNS - Reactive nitrogen species
ROS - Reactive oxygen species
RT-PCR - Reverse transcriptase - polymerase chain reaction
SERCA - Sarco/endoplasmic reticulum calcium ATPase
Sm(B/G)M - Smooth muscle basal/growth medium
SMC - Smooth muscle cell
SNP - Single nucleotide polymorphism
SNP - Sodium nitroprusside
SOD - Superoxide dismutase
SP - Sulfaphenazole
TAT - Transactivator of transcription
TNF-α - Tumour necrosis factor alpha
TX - Thromboxane
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Firstly, I would like to thank my primary supervisor and mentor, Dr. David Granville. Dave’s drive towards discovery, creativity and productivity has encouraged me to set my bar higher and push to obtain my goals. He has also challenged me to think and work independently and to take the lead in my research. As a result, Dave has put me on my way towards becoming an innovative and productive researcher. He has also encouraged me to always keep my eyes open, never to discount unexpected findings and, of course, has imparted his wisdom about the importance of a good lunch. I would also like to thank my co-supervisor Dr. Bruce McManus. Bruce’s immense understanding of inflammatory cardiovascular diseases, including cardiac allograft vasculopathy, has been a tremendous asset to this project. In addition, Bruce’s fire and determination has been inspirational.

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Co-Authorship Statement

Chapter 2 is based on the manuscript "Apoptosis repressor with caspase recruitment domain (ARC) inhibits myogenic differentiation." It was published in FEBS Letters, 2007, volume 581(6), pages 879-84. This manuscript was co-authored with Jingchun Zhang, Shirley C. Chen, Dr. Xiaoning Si, Brian Wong, Dr. Daryoush Ekhterae, Dr. Honglin Luo and Dr. David J. Granville. I worked with the senior authors on this paper to develop the research plan for this paper. I was assisted by Jingchun Zhang and Dr. Xiaoning Si with the adenoviral infection experiments and quantitation of multinucleation. Shirley Chen was a co-operative education student under my supervision and assisted with collection of cell lysates and Western blotting analyses on experiments related to stably transfected cells. Brian Wong provided valuable assistance in experiments related to immunofluorescence staining. Dr. Daryoush Ekhterae provided the stably transfected cell lines for this paper. Drs. Luo and Granville are co-senior authors on this publication. I was primarily responsible for writing the manuscript and assisted the senior authors with the editing and response to reviewers.

Chapter 3 is based on the manuscript "Cytochrome p450 2C inhibition reduces post-ischemic vascular dysfunction" published in Vascular Pharmacology, 2005, volume 43(4), pages 213-9. This manuscript was co-authored with Dr. Ni Bai, Dr. Ismail Laher and Dr. David Granville. Dr. Ni Bai was co-first author on this paper and was primarily responsible for heart isolation, isolation of septal arteries and artery cannulation. Drs. Laher and Granville are co-senior authors on this publication. I, along with the two senior authors, designed the experiments described in the paper. I was responsible for the majority of writing...
of the manuscript. I performed all assays assessing reactive oxygen species production and assisted with assays related to induction of ischemia and reperfusion and measurement of vascular function.

Chapter 4 is based on the manuscript “Cytochrome p450 2C enzymes contribute peri-transplant ischemic injury and cardiac allograft vasculopathy” in revisions for The American Journal of Transplantation. This manuscript was co-authored with Dr. Alexandra Kerjner, Katelyn Mueller, Dr. Bruce McManus and Dr. David Granville. Dr. Kerjner performed the surgical aspects of the rat heterotopic cardiac transplantation. Katelyn Mueller was a co-operative student under my supervision who assisted with some of the immunohistochemical studies. Dr. McManus provided insight into the grading of the immune infiltration. Dr. Granville is the senior author on this publication. I developed the experimental approach together with the senior author and helped to write the grant that funded this research. I conducted the bench work and wrote the paper which was reviewed and edited by the senior author.

Chapter 5 is based on a manuscript currently in preparation. Co-authorship is held by me, Paul Hiebert and Dr. David Granville. Paul Hiebert is a co-operative education student under my supervision that assisted with measurements of arachidonic acid metabolites. Dr. Granville is the senior author on this publication. I, along with the senior author, developed the research plan for this project and carried out the experimental protocols described in this chapter.
Chapter 1: Introduction

1.1 Cardiac Transplantation

1.1.1 Historical perspective

Although organ transplantation became a viable therapeutic strategy only in the past twenty-five years, the concept of exchanging organs and tissues between individuals has existed for millennia. Early references to organ transplantation include the Chinese physician Pien Ch’iao reportedly exchanged hearts between a man of weak will and a man of strong will in 500 B.C. and in the third century A.D. the Roman Catholic saints Damian and Cosmas reportedly replaced the gangrenous leg of a Roman Deacon with the leg of a recently deceased Ethiopian.\(^1\) While there were many documented, and likely many undocumented, attempts at organ transplantation prior to the 20\(^{th}\) century, it was not until this time that major advancements in the field of transplantation occurred. This section does not aim to provide a complete history of transplantation but simply to highlight major findings that furthered the advancement of the field and underline those areas for which significant research is needed.

The first systemic study of transplantation occurred in 1908 when Alexis Carrel performed double kidney exchanges between cats. This study was made possible due to the development, by Carrel with Charles Guthrie, of the technique of artery and vein anastomoses.\(^2\) This technique, still used today, laid the groundwork for solid organ transplantation and many other vascular procedures and won Alexis Carrel the Nobel Prize in Physiology or Medicine in 1912. Although none of the cats in Carrel’s study survived, some
Chapter 1

were able to maintain urinary output for up to 25 days, thus demonstrating that organ transplantation was viable at the surgical level. The discovery of human ABO blood groups by Karl Landsteiner in 1900 combined with the hypothesis of Peter Medawar that transplant rejection was an immunological process lead to the first successful solid organ transplant; performed in 1954 by Joseph Murray, involving a kidney transplant between identical twins.

Unlike other solid organ transplants, cardiac orthotopic transplantation was not surgically viable until the development, in the early 1950s, of the heart-lung bypass machine, credited to John Gibbon. James Hardy attempted the first documented human heart transplant in 1964. Unfortunately the recipient’s heart failed prior to a human donor becoming available so Hardy proceeded using a chimpanzee heart which quickly failed due to hyperacute rejection (described in section 1.2.1). In 1967, Christiaan Barnard performed the first successful heart transplant in South Africa with the recipient surviving for eighteen days following transplantation before dying as a result of pneumonia. This lead to over 100 heart transplants being performed in the late 1960s. Unfortunately the results were disappointing with a mean survival of only 29 days and many centres discontinued their cardiac transplantation programs. However, during this time Dr. Norman Shumway, at Stanford University, continued programs in both transplantation research and clinical transplantation. His team developed techniques in simplified orthotopic surgical procedures, organ preservation by hyperthermia and rejection monitoring by electrocardiography and serial biopsy. By the end of the 1970s the Stanford transplantation program had improved their 1 year survival level from 22% to 65%. For his efforts, Dr. Shumway is widely considered the father of modern clinical cardiac transplantation.
The discovery of potent immunosuppressive drugs was equally as important in the history of transplantation as the aforementioned surgical advances. As early as 1951, Peter Medawar, working for the National Institute for Medical Research, suggested immunosuppressive drugs could be used in transplantation. However, the drugs available at the time; namely, cortisone and azathioprine, were not strong enough immunosuppressors for most types of transplantation. In 1980, a sufficiently potent immunosuppressor was discovered in cyclosporin. Since that time, many other important immunosuppressive drugs have been used in transplantation including: prednisone, tacrolimus, rapamycin, azothioprine and mycophenolic acid.

As of 2006, The International Society for Heart and Lung Transplantation (ISHLT) published that approximately 3,000 heart transplants are performed and reported to the society annually. Of those transplants reported, heart transplant recipients can now expect a 1-year survival rate approaching 90% and an average graft life of 10.3 years (Figure 1.1). Despite these impressive achievements, the field of cardiac transplantation still has a long way to go. Malignancies and infections due to immunosuppressive regimes is currently the largest cause of death amongst transplant recipients and the largest impediments to long term graft survival is chronic heart transplant rejection in the form of cardiac allograft vasculopathy (CAV, described in detail in section 1.1.3). The pathogenesis of CAV is the central focus of this thesis.
Figure 1.1 ISHLT Kaplan-Meier survival curves for adult heart transplantation by era. Survival of adult heart transplant recipients as calculated using the Kaplan-Meier method as illustrated by the ISHLT registry slides. Graph incorporates information from all transplants where follow-up is available with unavailable data being estimates rather than exact rates. The half-life is defined as the time point at which 50% of all of the recipients have died. Comparisons were made using log-rank test statistic.
1.1.2 Hyperacute and acute forms of cardiac transplant rejection

Transplant rejection is the process by which the transplant recipient’s immune system recognizes the transplanted organ, tissue or cells as foreign and attempts to destroy it/them. The main types of transplant rejection are hyperacute, acute cellular rejection, acute antibody-mediated rejection and CAV. This section aims to provide a brief overview of the hyperacute and acute forms of allograft rejection. As CAV is central to this thesis, a more detailed discussion of its pathological features and aetiology is provided in section 1.1.3.

1.1.2.1 Hyperacute rejection

Hyperacute rejection (HAR), also termed antibody-mediated rejection, describes the process of graft destruction within minutes to hours following transplantation. HAR is caused when the recipient has pre-formed circulating antibodies to endothelial antigens present on the graft; most commonly ABO blood group antigens or major histocompatibility complex (MHC) antigens. The formation of antigen-antibody complexes activates the complement system causing mass neutrophil infiltration, endothelial damage and subsequent micro-thrombi. HAR is now rarely observed in allografts (i.e., grafts between two members of the same species) but can occur if recipients have previously been exposed to MHC-antigens present on the graft from prior pregnancies, blood transfusions or transplants or when errors in ABO blood type matching occur. An interesting exception to this happens in infants where ABO blood type matching may not be required. Infants do not produce isohemagglutinins or serum anti-A or anti-B antibodies until 12-14 months of age. Heart transplants have been successfully performed in infants across the ABO barrier without
incidence of HAR. Unlike with allotransplantation, HAR remains a major hurdle in xenotransplantation (i.e. transplantation between two members of different species) and remains an area of intense research in this field.

1.1.2.2 Acute cellular rejection

Acute cellular rejection is a process that can occur starting only days following transplantation but can also occur at any time during the life of the graft. Between 40 and 50% of all transplant recipients will be treated for at least one acute rejection episode within a year of receiving their transplants. Although increasing levels of immunosuppressive drugs are able to combat the majority of acute rejection episodes, over-immunosuppressing patients can lead to malignancies and infections and therefore must be kept in balance.

Acute cellular rejection requires alloreactive T-lymphocytes (either CD4$^+$ or CD8$^+$) to recognize alloantigens expressed on the graft resulting in their activation and subsequent proliferation. Immune infiltration then leads to graft cell necrosis and or vessel thrombosis and eventually graft function loss. The endomyocardial biopsy was first described by Caves et al. in 1973 as a method of monitoring cellular transplant rejection. It remains the ‘gold standard’ for monitoring cardiac transplants for signs of rejection. The ISHLT first created a grading scale for histological diagnosis of acute rejection in 1990. This grading scale remained unchanged until it was updated by the Society in 2004. The criteria for the updated grading scale are shown in Table 1.1.

Immunosuppressive strategies for rejection vary per transplant centre and often among patients within each centre. Most commonly, triple-drug therapy and cytolytic
### Grade code

<table>
<thead>
<tr>
<th>Grade code</th>
<th>Grade criteria</th>
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<tbody>
<tr>
<td><strong>Cellular rejection</strong></td>
<td></td>
</tr>
<tr>
<td>Grade 0 R</td>
<td>No rejection</td>
</tr>
<tr>
<td>Grade 1 R, mild</td>
<td>Interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage</td>
</tr>
<tr>
<td>Grade 2 R, moderate</td>
<td>Two or more foci of infiltrate with associated myocyte damage</td>
</tr>
<tr>
<td>Grade 3 R, severe</td>
<td>Diffuse infiltrate with multifocal myocyte damage ± edema, ± hemorrhage ± vasculitis</td>
</tr>
<tr>
<td><strong>Antibody-Mediated Rejection (AMR)</strong></td>
<td></td>
</tr>
<tr>
<td>AMR 0</td>
<td>Negative for acute AMR</td>
</tr>
<tr>
<td></td>
<td>No histological or immunopathological features of AMR</td>
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<tr>
<td>AMR 1</td>
<td>Positive for AMR</td>
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<tr>
<td></td>
<td>Histological feature of AMR</td>
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<tr>
<td></td>
<td>Positive immunofluorescence or immunoperoxidase staining for AMR (positive CD68, C4d)</td>
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**Table 1.1 2004 Revised ISHLT heart biopsy grading categories for cellular and antibody-mediated rejection.**

Standardized cardiac biopsy grading for acute cellular rejection and acute antibody-mediated rejection as modified from Stewart et al.27 ‘R’ denotes revised grade to differentiate these grades from the 1990 criteria.26
therapy are utilized during the peri-operative period. Triple-drug therapy includes prevention of lymphocyte differentiation by interleukin-2 (IL-2) reduction through cyclosporine or tacrolimus, purine synthesis inhibition by azathioprine or mycophenolate mofetil, and lympholytic treatment with corticosteroid therapy. Cytolytic agents employed include OKT3 and ATG/ALG. To reduce the negative impact of the various side effects of each of these treatments doses are decreased following the peri-operative period constituting maintenance immunosuppression. (Reviewed in 28)

1.1.2.3 Antibody-mediated rejection

Antibody-mediated rejection (AMR), also termed ‘biopsy-negative rejection’, ‘vascular rejection’ and ‘humoral rejection’, is a form of vascular inflammation or damage resulting in hemodynamic compromise where there is minimal evidence of cellular rejection. Surprisingly, patients in this category have worse outcomes than patients with higher ISHLT biopsy scores. AMR does not typically respond well to increased immunosuppressive therapy and increases risk of graft loss, CAV and mortality.

AMR is characterized by prominent capillaries in the biopsy that have endothelial swelling and deposition of immunoglobulin and complement. Endothelial swelling of the capillaries results from macrophage influx into injured capillaries and thus can be detected using macrophage markers, most commonly CD68. AMR is typically diagnosed as present or absent based on the ISHLT guidelines shown in Table 1.1. Current treatment regimes for AMR are limited and usually involve high-dose corticosteroids with more severe cases also requiring cytolytic agents, such as OKT3, and thymoglobulin or gamma globulin,
heparin and antiproliferative agents.\textsuperscript{37} Other immunosuppressive drugs including tacrolimus, mycophenolate mofetil and sirolimus have also been used with some success against AMR.\textsuperscript{38}

1.1.3 Chronic cardiac transplant rejection by cardiac allograft vasculopathy (CAV)

CAV is the greatest cause of graft loss for heart transplant recipients surviving 1 year following transplantation.\textsuperscript{17} Because transplanted hearts are largely denervated, transplant patients do not experience typical sensations associated with myocardial ischemia or infarction. Therefore, the first clinical indications of CAV can be arrhythmias, congestive heart failure or even sudden death.\textsuperscript{40} Using the traditional detection method of angiography, CAV can be detected in up to 42\% of patients 5-years post-transplant.\textsuperscript{41} Intravascular ultrasound is able to detect much higher levels of CAV with transplant related intimal thickening being detected in 75\% of transplant recipients only 1 year following transplantation.\textsuperscript{42}

1.1.3.1 Pathological characteristics of CAV

CAV is a form of arteriosclerosis characterized by diffuse and obliterative intimal thickening. Although CAV is classically described as concentric, fibrous plaques, there are a wide array of abnormal phenotypes including lesions resembling complicated native atherosclerosis.\textsuperscript{43} In the latter stages of development allograft arteriosclerosis often involves lipid deposition and calcification.\textsuperscript{44, 45} Further complicating histological phenotyping is that CAV can occur in regions with existing atheromatous disease. This phenotype is likely to
become more common with transplant programs accepting hearts from donors over 55 years of age, considered marginal donors. CAV involves large and small epicardial and intramural arteries as well as venous structures of the graft.\textsuperscript{46,47} The recipients native blood vessels are not affected.\textsuperscript{40} Both focal plaques and diffuse intimal thickening have been observed in CAV. Contrary to what happens in the intima, the medial layer of the vessels do not thicken and may experience thinning.\textsuperscript{48} Figure 1.2 shows the classical phenotype and some of the common pathological characteristics of CAV.

1.1.3.2 Pathobiology of CAV

The pathogenesis of CAV, shown in Figure 1.3, is not yet fully elucidated but is believed to involve a chronic allogenic response to the transplanted organ propagated by non-immunological factors.\textsuperscript{49} The importance of the immune system to the development of CAV is evident by the absence of CAV development in isografts.\textsuperscript{40} The endothelium of the grafted heart serves as an interface between the allograft and the recipient. Endothelial cells are the first cells to be recognized and are the primary target of the hosts immune system.\textsuperscript{49} Non-immunological factors, as well as, prior episodes of acute and antibody-mediated rejection can contribute to endothelial activation early following transplantation. Although endothelial activation remains a loosely defined term it generally involves increased expression and/or presentation of MHC antigens, adhesion and co-stimulatory molecules, along with altered secretion of cytokines and chemokines.\textsuperscript{50} Endothelial activation enhances entry of immune cells, decreases endothelial adhesiveness, contributes to impaired vasomotor function, described in section 1.1.4, and intimal thickening.\textsuperscript{51} Both cell-mediated and
Figure 1.2 Histology of CAV.
Distal left anterior descending artery from a 16 year old male, 20 months post-transplant from a 47 year old male donor. The artery shows a narrowed lumen (L), a recent, nearly occlusive thrombus (T), a concentrically thickened neointima (NI) and a largely intact media (M). This vessel also has areas of fatty deposits (FD), as well as, areas of collagen (C) and proteoglycan (PG) deposits. The adventitia (A) has multiple enlarged vaso vasora (examples shown with arrows).
Figure 1.3 Pathogenesis of CAV.
Upon propagating endothelial injury induces both denuding and non-denuding injury of the endothelium. Denuding injury increases endothelial permeability allowing increased entry of T-lymphocytes (T), monocytes and macrophages (M) and lipids further contributing to vascular damage. Non-denuding injury induces MHC expression and presentation of endothelial antigens (EA). Immunogenic endothelium allows antigen expression by dendritic cells (D) and consequent activation and differentiation of T-cells. Non-denuding injury also leads to decreased nitric oxide (NO•) production increasing SMC and myofibroblast migration and proliferation in the neo-intima.
humoral responses have been found to play roles in CAV.

Cell-mediated responses primarily involve recognition of MHC antigens through antigen presenting cells or directly by circulating T-lymphocytes. Recipient dendritic cells (DCs) are believed to be the first cell type to recognize foreign MHC molecules on donor endothelial cells. These professional antigen presenting cells can then activate large numbers of T-lymphocytes. Endothelial cells constitutively express both class I and class II MHC antigens and are therefore able to activate CD8\(^+\) and CD4\(^+\) lymphocytes, respectively, through indirect alloreognition. The degree of MHC antigen mismatch has been found to correlate with the development of CAV.\(^53,\ 54\) During allograft rejection MHC class II is upregulated further enhancing recognition by CD4\(^+\) lymphocytes.\(^55\) CD4\(^+\) lymphocytes upon activation release IL-2 stimulating the generation of CD8\(^+\) cytotoxic lymphocytes, activate alloantibody-producing B cells and macrophages. In a rat heterotopic heart transplant model, depletion of CD4\(^+\) but not CD8\(^+\) T-cells was able to prevent CAV.\(^56\) Further research into the mechanisms of CD4\(^+\) induced allograft intimal thickening have indicated that a Th1-type response contribute to,\(^57,\ 58\) and a Th2-type response may protect against\(^59\) CAV.

Although MHC molecules are important contributors to CAV development, they are not required for its pathogenesis. CAV has been shown in MHC identical grafts and MHC 'knockout' animals.\(^60\) The rat heterotopic transplant model, described in section 1.1.5.1, utilized in this thesis does not contain MHC class I or II differences yet the allografts develop CAV.\(^61\) Antibodies against multiple and diverse endothelial antigens have been detected in transplant recipients and have been linked to graft vasculopathy.\(^62,\ 64\) Vimentin has been found as the most prominent antigen in CAV and patients with high levels of anti-vimentin antibodies are at increased risk of graft arteriopathy.\(^64,\ 66\) Antibodies against intracellular
adhesion marker (ICAM)-1 has also been linked to CAV and polymorphisms in ICAM-1 may be protective against rejection.\textsuperscript{67}

Upon entry of immune cells, the graft vessel wall sustains chronic immune injury. Intimal thickening in CAV is largely due to modified smooth muscle\textsuperscript{46} and myofibroblast\textsuperscript{68} proliferation, fibrosis and infiltration of macrophage/monocytes and T-lymphocytes.\textsuperscript{40} These cells are also able to alter cytokine and chemokine levels and augment extracellular matrix synthesis.\textsuperscript{24} Endothelial cells in healthy vessels produce nitric oxide (NO\textsuperscript{-}) and anti-thrombotic proteins.\textsuperscript{69} NO\textsuperscript{-} plays a vital role in vascular homeostasis, described in section 1.1.4, but also prevents pro-inflammatory cytokine production by inhibiting nuclear factor κB (NF-κB) and smooth muscle cell (SMC) proliferation.\textsuperscript{70-73} CAV causes dysregulation of the NO\textsuperscript{-} synthase pathway leading to impaired NO\textsuperscript{-} production and activity.\textsuperscript{51} Endothelial NO\textsuperscript{-} synthase (eNOS) deficient mice have accelerated allograft vasculopathy in aortic transplants.\textsuperscript{74}

Several cytokines and chemokines are involved in the pathogenesis of CAV. Interferon-gamma (IFN-γ) is thought to be a key regulator of graft arteriosclerosis. (Reviewed in \textsuperscript{75}) IFN-γ is primarily produced by Th1 CD4\textsuperscript{+} T-cells following induction by IL-12 but can also be produced by CD8\textsuperscript{+} T-cells, natural killer (NK) cells, NK T-cells, DC and macrophages.\textsuperscript{75} As mentioned above, transplants in mice with a deficient Th1-type response and in IFN-γ knockout mice have reduced CAV.\textsuperscript{57, 58} IFN-γ regulates hundreds of genes including pro-inflammatory cytokines and chemokines, growth factors, transcription factors and membrane receptors.\textsuperscript{76} IFN-γ enhances expression of MHC class I and II molecules\textsuperscript{77, 78} and induces leukocyte independent SMC proliferation in vascular transplants.\textsuperscript{79} Th1 CD4\textsuperscript{+} T-cells also produce IL-2 involved in T-cell expansion. RANTES is an IFN-γ-induced
chemokine which increases monocyte adhesion of activated graft endolum.\textsuperscript{80} Monocyte chemoattractant protein (MCP)-1, MCP-3 and IL-8 have also been detected in CAV lesions.\textsuperscript{50} IL-10 and other Th2-type response cytokines, including IL-4, 5, 6, 9, and 13 appear to be protective against CAV development.\textsuperscript{59}

Non-immunological factors known to contribute to CAV development include hyperlipidemia, viral infection, immunosuppressive drug toxicity and ischemia and reperfusion (I/R) injury. Hyperlipidemia promotes development of fibrotic intimal hyperplasia and lipid deposition in the native vasculature and, at an accelerated rate, in the vasculature of the allograft.\textsuperscript{81} Prospective assessment of simvastatin therapy in transplant recipients has demonstrated that statins increase the survival of transplant patients and decrease the development of CAV by more than 45\% 11 years post-transplant.\textsuperscript{82} Cytomegalovirus (CMV) is the most common viral infection amongst transplant patients.\textsuperscript{50} CMV infection has been linked with CAV development though activation of NF-\kappa B and subsequent production of pro-inflammatory cytokines and SMC proliferation.\textsuperscript{83-86} Anti-CMV therapy with CMV hyperimmune globulin and ganciclovir reduced both CMV titres and coronary artery luminal narrowing in heart and heart-lung transplant recipients.\textsuperscript{87} Most immunosuppressive drugs have little protective benefit against CAV and their side effects such as hyperlipidemia, glucose intolerance and hypertension may actually contribute to its development.\textsuperscript{88} I/R injury and its relationship to CAV development are discussed in detail in section 1.2 and 1.2.2, respectively.
1.1.3.3 Treatment of CAV

Treatments to control risk factors, such as CMV infection and hyperlipidemia, may help prevent CAV. Unfortunately, there are relatively few treatments for established CAV. In patients where the CAV is focal and has not spread throughout the vascular tree, percutaneous and surgical interventions may be useful. Percutaneous transluminal coronary angioplasty and coronary artery stenting are used by some transplant programs to extend graft life. Unfortunately, transplant patients have very high levels of restenosis (30-60%) following angioplasty. Restenosis levels were lower in a small study of transplant patients that received stents. Coronary artery bypass grafting in patients with CAV carries abnormally high risk of perioperative death with death rates between 33.3% and 40% at various transplant centers. For patients with diffuse CAV, retransplantation is often the only option. Among immunosuppressive drugs, sirolimus appears to offer the most protection against CAV development and progression by reducing smooth muscle proliferation and migration, increased NO• production, decreased angiogenesis, and inhibition of fibrosis and extracellular matrix production. Ultimately, preventing the factors that propagate CAV development would offer the greatest advantage to transplant recipients.

1.1.4 Vasomotor function following transplantation

Post-transplant abnormalities in endothelium-dependent vasomotion can be detected early following transplantation in both macro and micro vessels. Some of these abnormalities can be indicative of peri-transplant endothelial denudation or dysfunction. Transplanted organs often have partial vasoconstrictory, rather than a vasodilatory, responses
Chapter 1

to acetylcholine. This phenomenon may resolve in the months following transplantation. However many groups have detected impairments in vasodilatory responses to acetylcholine, substance P, exercise, serotonin, and cold-pressor testing both immediately following transplantation and years post-transplant. Reduced eNOS expression post-transplant is reported to contribute to impaired acetylcholine-induced vasodilation. Endothelium-independent vasodilation is also impaired following heart transplantation. This impairment may be caused by cytotoxic damage to the vascular SMCs leading to medial thinning and impaired SMC contractile responses.

1.1.4.1 Early vasomotor dysfunction as a predictor of CAV

Although some researchers have not found a correlation between early vascular dysfunction and CAV development, other studies have found that early vascular dysfunction is predictive of CAV. Davis et al. found that quantitative angiography measurements of acetylcholine-induced vasodilation correlated with CAV development 1-year following transplantation as detected by intravascular ultrasound (IVUS). Hollenberg et al. also found a correlation between impaired acetylcholine responses and CAV development detected using angiography. In the latter study, responses to adenosine and nitroglycerine were also assessed but were not found to correlate with future CAV diagnoses.
1.1.5 Animal models of CAV

Animal models for CAV include orthotopic arterial grafting and heterotopic and orthotopic cardiac transplantation. All of these models expose the grafted tissue to the recipient’s immune system and have provided valuable insight into CAV’s aetiology. The advantages and disadvantages of these models are briefly described below.

Orthotopic arterial grafting involves harvesting a section of a major artery, typically the aorta or carotid artery, from a donor and inserting the artery into the identical position in the recipient’s arterial system using end-to-end anastomoses.\textsuperscript{108} This model has many advantages, not the least of which is that it is surgically less challenging and can be used in small animal models with lower post-surgical morbidity or mortality (< 2%)\textsuperscript{108} than cardiac transplantation. Arterial grafts develop classical features of CAV including intimal thickening, smooth muscle proliferation and immune infiltration.\textsuperscript{108} These arteries are maintained at near physiological conditions, unlike with heterotopic transplantation; however, they lack the parenchymal factors that can contribute to rejection. This technique precludes measurements of organ function and associative studies of acute rejection and CAV development.

Heterotopic cardiac transplantation involves grafting of a donor heart into a non-physiological position, most commonly the abdominal cavity, of the recipient. A detailed surgical protocol for abdominal rat heterotopic cardiac transplantation is provided in Appendix I. Similarly to the orthotopic arterial graft model, heterotopic cardiac grafts can be performed in small animal models. This allows researchers to study CAV in inbred and genetically altered populations. This model is advantageous over arterial grafting as it allows
evaluation of acute rejection and graft survival can be monitored easily and non-invasively by abdominal palpation. The disadvantage of heterotopic heart transplantation is that the hearts are not physiologically loaded and perfusion of the myocardium by the recipient’s circulatory system is retrograde through the ostia and into the coronary circulation.

Orthotopic cardiac transplantation parallels human orthotopic heart transplantation and offers the advantages that the hearts are physiologically loaded and that parenchymal effects and acute rejection can also be examined. Unfortunately, the surgical aspects of this procedure are inhibitory in most cases. Specifically, a heart-lung bypass machine is required and thus this operation is generally restricted to canine, swine and other large mammal models. The expense of using large animal models and the lack of genetic and inbred populations makes use of this model rare.

1.1.5.1 Lewis to Fisher 344 rat model of heterotopic heart transplantation

We selected the Lewis to Fisher 344 (F344) rat heterotopic heart transplant model for these studies. As mentioned in section 1.1.3.2, Lewis and F344 rats have identical class I and II antigens. Minor histocompatibility differences in the Qua-like RT1.C locus, as well as, erythrocyte and lymphocyte antigens do differ between the strains. The Lewis-F344 transplant model was originally described as a CAV model in 1993 by Adams et al. This model was ideal for this study as it is a well-established model allowing for comparisons with previous studies. It also utilizes commercially available rat strains, has long-surviving grafts and has a high incidence of graft arteriosclerosis. This model produces CAV lesions in >90% of arteries by 3 weeks post-transplant. These lesions closely resemble human CAV
lesions consisting largely of intimal SMC accumulation. A slight increase in monocellular infiltration and necrosis are observed compared to human CAV.\textsuperscript{112}

\section*{1.2 Ischemia and Reperfusion (I/R) Injury}

I/R is integral in the pathophysiology of myocardial infarction and is a contributing complication to multiple surgical procedures including cardiac transplantation and coronary bypass.\textsuperscript{113-118} I/R results in apoptosis and necrosis in the myocardium. The vascular endothelium is even more susceptible to ischemic damage.\textsuperscript{119} I/R is associated with decreased endothelium-dependent vasodilation, decreased NO- levels, increased expression of MHC, adhesion molecules and leukocyte adhesion\textsuperscript{120}, and adverse contractile modulatory effects.\textsuperscript{121}

Ischemia is insufficient or absent blood flow and reperfusion is restoration of blood flow flowing an ischemic period. During ischemia, ATP supplies are depleted leading to an increase in cytosolic calcium ([Ca\textsuperscript{2+}]\textsubscript{c}). Elevated [Ca\textsuperscript{2+}]\textsubscript{c} can be prolonged due to reperfusion injury because of reactive oxygen and nitrogen species (ROS and RNS), such as peroxynitrite (ONOO-) can damage the sarco/endoplasmic reticulum (ER) calcium ATPase (SERCA) inhibiting sequestration of intracellular calcium back into the ER.\textsuperscript{122} Elevation of [Ca\textsuperscript{2+}]\textsubscript{c} in turn causes an increase in mitochondrial calcium ([Ca\textsuperscript{2+}]\textsubscript{m}) levels associated with increased production of ROS linked to damage of the respiratory chain.\textsuperscript{123}
1.2.1 Reactive oxygen and nitrogen species in I/R

ROS and RNS are central players in the pathogenesis of I/R. ROS and RNS can be broadly divided into free radicals (1-electron donors) and non-radical oxidants (2-electron donors). Free radical oxidants, such as superoxide (O$_2^-$) and NO•, can be highly reactive and can act as both oxidizing and reducing agents as they are capable of both donating and accepting a single electron. Nitric oxide (NO−) is produced by eNOS under basal conditions. Under stressed states NO• can also be produced by macrophages and SMCs through inducible NO synthase (iNOS). As described above, NO• plays important roles in vascular homeostasis through its induction of endothelium dependent vasodilation, platelet aggregation, and in controlling smooth muscle growth and differentiation. However, when NO• is produced in the presence of O$_2^•$− the two rapidly react in the formation of ONOO•, a non-radical oxidant. Non-radical oxidants are capable of accepting two electrons. Other examples of such oxidants include: hydrogen peroxide (H$_2$O$_2$), ozone and hypochlorous acid. Non-radical oxidants, like ONOO•, are highly chemically reactive and known to cause cell damage through lipid peroxidation, tyrosine nitration, and reactions with sulfhydryl groups. In addition to its ability to chemically alter many cellular components, ONOO• can also cause cellular dysfunction through the activation of multiple signalling pathways. ONOO• has been shown to increase integrin-dependent adhesion of human neutrophils to human coronary artery endothelial cells through activation of the Raf-1/ extracellular signal-regulated kinases (ERK) pathway. It is also known to activate the ERKs, c-Jun NH$_2$-terminal kinase, calcium-dependent protein kinase C (PKC), and p38 mitogen activated protein kinase (MAPK). ONOO• can also induce apoptosis. Previous research by our centre has shown that acute cardiac rejection and apoptosis is attenuated when mouse cardiac
allografts are transplanted into iNOS knockout recipients compared to iNOS+/+ recipients.\textsuperscript{134} Furthermore, O$_2^-\cdot$ and ONOO$-\cdot$ can disrupt ER calcium ATPases and Ca$^{2+}$ regulation in coronary arteries.\textsuperscript{122,135-141} ONOO$-\cdot$ is also implicated in smooth muscle cell damage through DNA damage and the activation of poly(ADP)ribose synthetase which results in energy depletion.\textsuperscript{142}

ROS and RNS are generated through multiple pathways including: NAD(P)H oxidase, xanthine oxidase, myeloperoxidase, lipoxygenase, mitochondrial respiration, transition metals, and nitric oxide synthase (NOS). Although they are largely unstudied outside of the hepatic system, cytochrome p450 enzymes (CYPs) can also generate ROS and lead to the production of RNS in cardiovascular tissues. Production of ROS by CYPs is discussed in section 1.4. Antioxidants are capable of significantly preventing or delaying the oxidative damage of substrates which are present at higher concentrations than the antioxidants themselves. Enzymatic antioxidants are perhaps the most well known and are largely responsible for maintaining a reducing intracellular environment in cells. These antioxidants include superoxide dismutases (SODs), catalases and peroxidases.

1.2.2 I/R and transplantation

I/R injury plays a significant role in endothelial dysfunction and the pathophysiology of CAV.\textsuperscript{113-118,143} The transplant organ is vulnerable to I/R injury induced by graft ischemia time, quality of graft preservation during transport, hemodynamic status of the donor, catecholamines used for inotropic support, and reperfusion itself.\textsuperscript{116} Three sequential phases of graft ischemic time contribute to graft injury during transplantation: (1) the episode of
warm ischemia upon removal of the heart from the donor, (2) the cold ischemic interval associated with storage and preservation of the heart, and (3) the period of warm ischemia during engraftment. Paradoxically, although reperfusion is required to restore tissue oxygenation, much of the damage that ensues during transplantation is associated with the oxidative burst that occurs during reperfusion.

Compelling evidence supports a molecular and cellular basis for a causal relationship between I/R injury during transplantation and the onset and progression of CAV. I/R injury to endothelial cells may provide the initial trigger for atherogenesis by stimulating platelet adhesion, release of growth factors, upregulation of MHC Class I and II expression, release of donor antigens, expression of adhesion molecules, and proliferation of vascular smooth muscle cells. (Reviewed in 113-118, 143)

Several experimental models using superoxide dismutase and antioxidants have demonstrated the importance of ROS in the pathophysiology of I/R injury. However, the development of effective treatments to alleviate reperfusion injury remains elusive. Furthermore, several candidate pathways have been proposed to produce ROS during I/R including mitochondria, NADPH oxidases, xanthine oxidase and eNOS. However, the data supporting a role for these systems in I/R injury remain inconclusive. For example, targeted deletion of P47^phox, an essential component of NADPH oxidase, abrogates NADPH-dependent superoxide generation in endothelial cells. However, I/R studies in p47-null mice reveal no significant difference in infarct size. Similarly, xanthine oxidase inhibitors have failed to protect against I/R while eNOS may play a protective role.
Recently, apoptosis repressor with caspase recruitment domain (CARD), described in section 1.3, and cytochrome p450 2C enzymes, described in section 1.4, have been found to play roles in myocardial I/R injury. The role of these proteins in vascular I/R injury and CAV are unknown.

1.3 Apoptosis Repressor with Caspase Recruitment Domain (ARC)

ARC, apoptosis repressor with caspase recruitment domain, was first identified by Gabriel Nunez’s group in 1998. It is a 23 kDa protein with an N-terminal CARD domain and a C-terminal proline/glutamic acid rich domain. Its expression was originally thought to be confined to terminally differentiated skeletal and cardiac muscle. More recently, ARC has been shown to be expressed in cancer cells. Caspases and other CARD containing proteins are known to bind to one another through this domain. ARC was found to bind and inhibit caspase-2 and 8 indicating important implications in apoptosis. ARC is also able to inhibit potassium efflux associated with apoptosis induction and cell shrinkage.

Apoptosis is a tightly controlled form of cell death that is characterized by cell shrinkage, DNA fragmentation and membrane blebbing resulting in the packaging of the cell into membrane-enclosed vesicles. These vesicles are then engulfed by surrounding ‘professional’ (macrophages) or ‘non-professional’ phagocytes. Endothelial cell (EC) and SMC apoptosis and necrosis have been identified as important factors in the progression of CAV. As described above, numerous factors such as oxidative stress, immune cells and cytokines participate in the induction of cell death in CAV. Therefore multiple death-inducing pathways must be inhibited in order to attenuate vascular damage. ARC is one of
the first known multifactorial apoptosis inhibitors and was shown to inhibit both apoptosis and necrosis in cardiac myoblasts.\textsuperscript{152} Therefore, ARC may provide a unique method of inhibiting the multiple apoptotic and necrotic pathways that are triggered in this disease.

### 1.3.1 ARC in I/R injury

ARC is protective against ischemic injury and oxidative stress in cardiomyocyte and neuronal cells. Ekhterae \textit{et al.}\textsuperscript{152} were the first to link ARC with protection against oxidative injury. They demonstrated that ARC overexpression was able to protect against hypoxia and re-oxygenation (H/R) induced caspase-3 activation, Poly (ADP-ribose) polymerase (PARP) cleavage and cytochrome c release.\textsuperscript{152} Gustafsson \textit{et al.}\textsuperscript{147} found that human immunodeficiency virus (HIV) transactivator of transcription (TAT)-fusion protein transduction (described in section 2.1) of ARC was protective against oxidative injury induced by H\textsubscript{2}O\textsubscript{2} in cultured embryonic myocytes and was protective against I/R injury in Langendorff perfused rat hearts. In the latter set of experiments, TAT-ARC transduction reduced both infarct size and creatine kinase (CK) release following I/R.\textsuperscript{147} Chatterjee \textit{et al.}\textsuperscript{153} found similar results using adenoviral transfer of ARC in a rabbit model of regional cardiac ischemia. Treated animals maintained left ventricular geometry, had higher ejection fractions and less border zone fractional shortening that control groups.\textsuperscript{153} ARC-deficient mice demonstrate reduced contractile function, cardiac enlargement, and myocardial fibrosis following aortic banding.\textsuperscript{154} These mice also show increased infarct areas following I/R.\textsuperscript{154} Studies in hippocampal neurons showed that hypoxia downregulates ARC expression in the hippocampus and that overexpression of ARC protects against hypoxia-induced death in
There are currently no data indicating whether or not ARC is protective against ischemic injury in the vasculature or whether that protection could reduce CAV development.

1.4 Cytochrome p450 Enzymes (CYPs)

CYPs are membrane-bound, heme-containing terminal oxidases that are found in organisms ranging from archaebacteria to humans. These enzymes are responsible for the metabolic activation or inactivation of most types of drugs as well as toxins. CYPs oxidize, peroxidize, and/or reduce steroids, arachidonic acid (AA), cholesterol, vitamins and other foreign substances in an oxygen and NADPH-dependent manner. The majority of CYPs isoforms are mono-oxygenases that catalyze the incorporation of a single atom of oxygen into a substrate. CYPs are critical mediators of drug metabolism. Thus, considerable attention has been given to these enzymes by the pharmaceutical industry with respect to their role in drug-drug interactions, drug bioavailability and toxicity.¹⁵⁶

There is substantial inter-individual variation in the activities of various CYP isoforms in humans resulting in differential metabolism, detoxification and/or clearance of xenobiotics. Much of this inter-individual variability can be attributed to polymorphisms in CYP genes resulting in altered activity or expression of the encoded enzyme. However, CYP activity is also heavily influenced by other factors such as drugs, hormones, development, diet and cytokines.¹⁵⁷,¹⁵⁸ Thus, both genetic and epigenetic components determine the ability of an individual to metabolize a particular drug or toxic substance. To add further complexity, the sequencing of the mouse, rat and human genomes has revealed substantial
differences in the CYP makeup of these animals. For instance, the 2J subfamily, which has been shown to be abundantly expressed in the heart, has one member in humans, 4 members in rats and 8 members in mice. Furthermore, mice contain 84 different CYP isoforms versus only 63 isoforms in humans. Thus, caution must be used when assessing drug metabolism or the activation/deactivation of other toxins in rodents with respect to translating this research to humans as rodents possess CYP isoforms that are not present in humans and vice-versa. This may be one explanation as to why many therapeutics are effective in mice, but fail in humans.

The liver expresses the highest levels of CYP and plays a dominant role in the first-pass clearance of ingested xenobiotics and in the regulation of systemic levels of drugs and other chemicals. However, extra-hepatic tissues also possess CYP and contribute not only to first-pass clearance, but may also influence tissue burden of foreign compounds or bioavailability of therapeutics. Many substances require CYP-mediated metabolic activation to form toxicants or carcinogens. The reactive intermediates that are produced are for the most part unstable and unlikely to be transported from the liver to other tissues to exert toxicity. Thus, chemical toxicity in extra-hepatic tissues may be regulated by CYP-mediated in situ metabolic activation in the target organ itself.

1.4.1 CYP 2C enzymes

CYP 2C enzymes are mono-oxygenases that catalyze the transfer of a single oxygen molecule to their substrates. This process requires electron transfer from NADPH to
Figure 1.4 The CYP mono-oxygenase reaction cycle

$O_2^-$ is generated during the CYP reaction cycle when the electrons for the reduction of the central heme iron are transferred on the activated bound oxygen molecule. $O_2^-$ is then readily converted to other ROS and RNS through reactions such as those shown for $H_2O_2$ and ONOO-. 

$O_2^- + 2H^+ \rightarrow H_2O_2$

$O_2^- + NO^{-} \rightarrow ONOO^-$
cytochrome p450 through electron carriers.\textsuperscript{163} This process is shown in Figure 1.4. Unfortunately, this process is relatively inefficient and poorly coupled in eukaryotes, compared to protoplasmic microbial mono-oxygenase systems, leading to the production and release of ROS.\textsuperscript{163} CYP mono-oxygenases produce superoxide during three stages of their reaction cycle and can produce superoxide by NADPH consumption even in the absence of substrate.\textsuperscript{163} Substrate availability further increases the catalytic activity of CYPs and results in an increase in superoxide production.

CYP 2C9 was mapped to chromosome 10 in humans\textsuperscript{164}, to chromosome 7 in mice and chromosome 1 in rats.\textsuperscript{165} In the heart CYP 2C9 was found to be predominantly expressed on the right side, more specifically in the right ventricle and also in the vasculature.\textsuperscript{166} Much of what we know about CYP 2C9 comes from studies related to the metabolism of the many drugs that it metabolizes. Tolbutamide, used in the treatment of type 2 diabetes, is metabolized by CYP 2C9. An uncommon variant of the CYP 2C9 gene seems to be associated with a reduced ability to metabolize tolbutamide. This same variant, CYP 2C9*3 in which isoleucine at position 359 is mutated to leucine, is also associated with both reduced clearance of the anti-inflammatory drug, celecoxib as well as reduced clearance of warfarin, an anticoagulant.\textsuperscript{167} X-ray crystallography studies by Williams et al.\textsuperscript{168} elucidated the structure of CYP 2C9 and discovered a binding pocket in which the anticoagulant, warfarin, binds. Poor metabolizing variants occur at a higher frequency in the white population compared to the black population.\textsuperscript{169} Two CYP 2C9 variants have been identified as poor metabolizers of warfarin, CYP 2C9*2 (arg144→cys) and CYP 2C9*3 described above. In a retrospective cohort study of patients being treated with warfarin, individuals with poor metabolizing CYP 2C9 variants were associated with an increased risk of bleeding events.\textsuperscript{170}
Although the rodent equivalent to human CYP 2C9 has not been fully characterized, CYP 2C6 and CYP 2C11 are recognized as its putative orthologs.\textsuperscript{171} We and others have detected an isoform corresponding to a similar sized protein in rat heart protein extracts using an antibody for human CYP 2C9 and have demonstrated that CYP 2C9 inhibitors reduce post-ischemic superoxide generation in rat hearts.\textsuperscript{146, 172} Rat CYP 2C6, but not CYP 2C11, has previously been shown to be selectively inhibited by sulfaphenazole in rat liver preparations.\textsuperscript{173}

1.4.1.1 CYP 2C in vascular homeostasis

Although the majority of CYP are most abundantly expressed in the liver, CYP are also expressed in extra-hepatic tissues including the heart and the vasculature.\textsuperscript{174} The human AA-metabolizing epoxygenases of the 2 gene family; namely 2B, 2C8, 2C9, 2C10, and 2J2, are expressed in the vasculature and have been implicated in vascular homeostasis.\textsuperscript{174} These epoxygenases generate epoxyeicosatrienoic acids (EETs), ROS, and other products.

Vascular tone and homeostasis is modulated by numerous vasoactive signals and compounds produced by the autonomic nerves, the tissue, and the endothelium.\textsuperscript{174} Vasodilators include vascular flow, the well-known autacoids, NO\textsuperscript{•} and prostacyclin (PGI\textsubscript{2}), and several less well characterized receptor-mediated agonists.\textsuperscript{174} NO/PGI\textsubscript{2}-independent pathways make a significant contribution to vasodilation, particularly in the renal, mesenteric, and coronary arteries.\textsuperscript{174} Endothelium-derived hyperpolarizing factor (EDHF) is an agonist that causes the hyperpolarization of endothelial and smooth muscle cells though both Na-K-ATPase and calcium-dependent K\textsuperscript{+} channels.\textsuperscript{175, 176} CYP have been linked to EDHF activity.
because CYP specific inhibitors, such as 6 (2-proparglyoxyphenyl) hexanamide, can prevent NO/PGI2-independent vasodilation.\textsuperscript{177} Furthermore, an antisense approach against the CYP 2C family was able to demonstrate an attenuation of bradykinin-induced EDHF-mediated vascular responses without affecting NO-mediated vascular responses.\textsuperscript{178} Moreover, sulfaphenazole, a selective inhibitor of CYP 2C9, was able to inhibit EDHF-mediated vasodilation in porcine coronary arteries.\textsuperscript{179} This research implicates CYP 2C9 as a putative EDHF synthase and 11,12-EET as the putative EDHF.

CYP products such as EETs and hydroecosatraenoic acids (HETEs) as well as their degradation products have been associated with both the induction and inhibition of vasodilation. For example, CYP 2J2 is localized to the endothelium of large and small coronary arteries and is able to generate not only EET from AA, but also epoxyeicosatraenoic acids from eicosapentaenoic acid.\textsuperscript{180} Both EET and epoxyeicosatraenoic acid are known dilators of the microvasculature.\textsuperscript{180} The diol products of EETs, dihydroxyeicosatrienoic acids, can be taken up by ECs and cardiac myocytes and incorporated into phosphatidylcholine, phosphatidylinositol, and to a lesser extent other phospholipids.\textsuperscript{181} Even when EETs are released into the extracellular environment they are believed to incorporate into circulating lipoproteins through esterification.\textsuperscript{182} It is hypothesized that the incorporation of EETs into phospholipids serves as a means of storing these molecules, but it is not known if EETs are also active in this form.\textsuperscript{174} Unfortunately, the effects of EETs on vasodilation have typically been measured in the presence of inhibitors of NO-dependent vasodilation. This is a concern because NO• is an inhibitor of CYP. For that reason, it is unknown how much of an effect EETs have on vasodilation in the presence of NO•. This is further complicated because CYP also generate ROS during their reaction cycle.
as electrons are transferred from the central heme iron to the activated bound oxygen molecule.\textsuperscript{179} In fact, CYP make a significant contribution to the cellular production of ROS such as $\text{O}_2^{-}$, $\text{H}_2\text{O}_2$ and hydroxyl radicals.\textsuperscript{179} Through the production of free radicals, CYP may also contribute to vascular homeostasis because ROS are known participants in the maintenance of vascular tone and homeostasis.\textsuperscript{183} Unlike the EET products of CYP, ROS are implicated in the inhibition of NO-mediated relaxation. $\text{O}_2^{-}$ reacts with NO• to form ONOO$^-$ thus reducing the bioavailability and vasoactivity of NO•.\textsuperscript{179}

1.4.1.2 CYP 2C in I/R injury

Yasar \textit{et al.} (2003)\textsuperscript{184} examined correlations between genetic variants of CYP 2C8 and 2C9 and risk of acute MI. An increased risk of acute MI has been associated with the genetic CYP variants CYP 2C9*2 and *3 in female patients, and CYP 2C8*3 in both males and females.\textsuperscript{184} These variants have reduced activity compared to their wild-type counterparts.\textsuperscript{185, 186} Recent studies from our group suggest that the rat CYP 2C9-equivalent is an important mediator of I/R injury.\textsuperscript{146} In the latter study, several CYP inhibitors were tested for their ability to protect against cardiac I/R injury. Three structurally-unrelated CYP mono-oxygenase inhibitors (chloramphenicol; multi-CYP inhibitor, cimetidine; 1A2, 2C6/9, 2D6, 3A4 inhibitor and sulfaphenazole; 2C6/9 inhibitor) were highly protective against I/R injury. The one commonality between these inhibitors was their ability to suppress rat CYP 2C6 or human CYP 2C9. Thus, it became apparent that CYP 2C6/9 may be a key player in cardiac I/R injury. In rat hearts perfused in Langendorff mode, the CYP inhibitors reduced infarct
size, ROS production and CK release compared to that of controls.\textsuperscript{146} Similar results were found in a rabbit model of left anterior descending coronary artery constriction. CYP 2C9 inhibitors also increased post-ischemic coronary flow suggesting that increased vasodilation and/or reduced post-ischemic vascular dysfunction plays a role in the cardioprotective effect.

The observation that CYP inhibitors attenuate I/R injury is significant. Many risk factors for heart attacks, such as tobacco smoke and cocaine, are potent inducers of CYPs in the heart, while cardioprotective factors, such as resveratrol (found in red wine) and statins, inhibit CYPs.\textsuperscript{187-192} Although there are more deaths associated with smoking-induced cardiovascular disease than cancer, the mechanism by which smoking contributes to cardiovascular disease is poorly understood. However, there is evidence to suggest CYP might be responsible. The role of CYP in smoking-related cancer is well-established and recent findings indicate that certain CYP isoforms are involved in atherogenesis. Polymorphisms in CYP 1A1, one of the key detoxifying enzymes catabolizing cigarette smoke-derived toxins, are associated with smoking-induced atherogenesis. CYP 1A1 polymorphisms have been associated with susceptibility to severe coronary artery disease and type 2 diabetes in smokers.\textsuperscript{189} Further evidence for a role of CYP in MI stems from studies of cocaine-induced heart attacks. Cocaine can induce acute MI in young adults\textsuperscript{190} and has been reported to be a potent inducer of CYP in cardiac tissues.\textsuperscript{193} Conversely, although several mechanisms have been forwarded to explain the cardioprotective effects of polyphenolic compounds found in red wine and other foods\textsuperscript{191,194-197}, it is of interest to note that these substances are also known CYP inhibitors.\textsuperscript{191,194-197} In summary, there is powerful and accumulating indirect evidence supporting a role for CYP in tissue-specific cytotoxicity and cardiovascular disease.
1.4.1.3 CYP 2C in atheromatous disease

Thum and Borlak\textsuperscript{198} have implicated oxidized low density lipoprotein (oxLDL) in the downregulation of CYP mono-oxygenases in coronary arterial endothelial cells. This study linked oxLDL to increased ROS production and consequent loss in nuclear factor 1 (NF-1) activity.\textsuperscript{198} NF-1 is an important regulator of CYP mono-oxygenase expression.\textsuperscript{199} A significant decrease in the expression of CYP 1A1, 2A6/7, 2B6/7, 2C8, 2C9, 2E1, and 2J2 was detected in coronary arterial endothelial cells treated with oxLDL, but not in cells treated with normal LDL.\textsuperscript{198} Fichtlscherer et al.\textsuperscript{200} showed that CYP 2C9 inhibition via sulfaphenazole is associated with increased endothelium-dependent vasodilation in human patients with coronary artery disease.\textsuperscript{200} This effect was attributed to a decrease in ROS production by CYPs, as well as a consequent increase in NO\textsuperscript{•} bioavailability and NO\textsuperscript{•}-mediated vasodilation. This work suggests inhibition of CYP 2C9 as a possible therapeutic intervention to maintain blood flow and protect against ischemic damage in patients with established coronary artery disease.\textsuperscript{200}

1.4.1.4 CYP 2C in other cardiovascular diseases

CYPs have been speculated to play a significant role, both in the onset of and the protection against a broad spectrum of cardiovascular diseases. While CYPs have been studied extensively in drug metabolism in the liver, studies into their roles in xenobiotic metabolism and the production of biologically active metabolites and toxins in the heart requires further elucidation. CYPs have recently been implicated in the induction of
angiogenesis. Furthermore, endothelial cell proliferation, associated with angiogenesis, is linked with CYP 2C9 expression. Human umbilical vein endothelial cells infected with adenovirus to overexpress CYP 2C9 demonstrated a 50% increase in proliferation over antisense infected cells as well as a 3-fold increase in cyclin D1 expression. This increased endothelial proliferation was prevented with the addition of a CYP 2C9 specific inhibitor sulfaphenazole. Administration of 11,12-EET to chick choriollantoic membranes was able to induce angiogenesis to a similar degree as known pro-angiogenic factors such as endothelial growth factor (EGF) and vascular endothelial growth factor. Again, the induction of angiogenesis by 11,12-EET was inhibited using AG1478 as well as an EGF neutralizing antibody. Similar experiments in a human lung microvascular cell lines showed a more than 25% increase in proliferation with overexpression of CYP 2C9. In this study 14,15-EET, the most abundant EET product of CYP 2C9, was applied to a Matrigel and infused subcutaneously on the dorsal midline of a rat. Angiogenesis was subsequently measured as indicated by haemoglobin content and by immunostaining of platelet endothelial cell adhesion molecule-1 (PECAM). After one week 14,15-EET treated Matrigel showed a 1.6-fold increase in haemoglobin over control as well as positive PECAM staining. The relative contributions of 11,12- and 14,15-EET and the mechanism of EGF receptor involvement in CYP 2C9 induced angiogenesis are currently unknown and require further experimentation to be fully elucidated. CYP 4A1 was also shown to induce angiogenesis in renal interlobar arteries in a smooth-muscle cell dependent manner.

Hypertension, or high blood pressure, is a leading cause of death, MI, stroke, and other illness in North America. It is typically asymptomatic and the great majority of patients have essential hypertension, in which the cause of blood pressure elevation is
Hypertension is largely regulated by the cardiac output of the heart, the systemic resistance controlled by blood vessel tone, and the intravascular tone regulated by the kidneys. CYPs have often been considered when treating hypertensive patients due to their interactions with anti-hypertensive drugs such as candesartan, warfarin, phenytoin, and tolbutamide. Recently, due to the ability of CYPs and their metabolites, EETs and HETEs, to modulate vascular tone and alter renal blood vessels, as described above, they have been linked to the development of hypertension. There have been several reports, however, describing conflicting roles for CYP in hypertension. Fenofibrate, a drug known to induce expression of CYP 4A and elevate production of 20-HETE, has been shown to reduce blood pressure in stroke prone spontaneously hypertensive rats. On the other hand, the use of 17-octadecynoic acid, an inhibitor of EET and 20-HETE production, was also able to reduce blood pressure in Lyon hypertensive rats.

Single nucleotide polymorphisms (SNP) of CYP 2C9 were studied to determine if it was possible to predict the efficacy of treatment with irbesartan, a drug used to treat hypertension, with SNP information. The results indicated that the rate of irbesartan metabolism is indicative of the CYP 2C9 genotype expressed, providing a valuable use for genotyping before treatment of hypertension.

1.5 Arachidonic Acid (AA) Metabolism

AA is metabolized by three major pathways; the cyclooxygenase (COX) pathway, the lipoxygenase (LOX) pathway, and the CYP epoxygenase pathway. These pathways are shown in Figure 1.5.
Figure 1.5: Overview of the three pathways of arachidonic acid metabolism. AA is metabolized by three main pathways; the cyclooxygenase pathway, the lipoxygenase pathway and the cytochrome p450 epoxygenase pathway. The main products of these pathways are shown as described in 211, 212.
1.5.1 AA metabolism by cyclooxygenase

The COX pathway results in the cyclization and oxidation of AA, hence its name. Alternatively, the COX enzymes are known as prostaglandin (PG) H synthases because they lead to formation of PGH. There are three types of COX. COX-1 and COX-3 are constitutively expressed and are present in the stomach, kidney and thrombocytes and the brain, respectively. COX-2 is the inducible form of the enzyme and is present in multiple tissues including the heart and vasculature. However, COX-2 is thought to be constitutively expressed in some tissues including gastric tissues and endothelial cells. Initially COX oxidizes AA into the endoperoxides PGH$_2$ and PGB$_2$ which are precursors of the prostaglandins PGE$_2$, PGF$_2$, PGI$_2$ and of thromboxanes (TX). The production of these metabolites differs in different tissues with TX formation dominating in blood platelets and prostaglandin and prostacyclin formation dominating in vascular cells.

1.5.2 AA metabolism by lipoxygenase

The LOX are dioxygenases that metabolize AA into HPETEs (hydroperoxyeicosatraenoic acids) and DEA (dihydroxyeicosatraenoic acid). These products are then converted to HETEs, leukotrienes, and lipoxins by peroxidases, hydrase and glutathione S-transferase, and lipoxygenases, respectively. There are two main LOX enzymes 5-LOX and 12-LOX, defined by the carbon atom on which the oxygen is fixed. 5-LOX is present in many cell types and leads to the formation of leukotrienes (LT) A4, B4 and subsequently through modifications LTC4, LTD4 and LTE4. 12-LOX is more restricted in its expression being present in skin, thrombocytes and some tumours.
1.5.3 AA metabolism by CYPs

The epoxygenase pathway employs CYP epoxygenases in the formation of EETs and HETEs.\textsuperscript{211} CYP 2C are the primary epoxygenases involved in AA metabolism by the third pathway.\textsuperscript{215} Unfortunately, in addition to the production of EETs and HETEs, CYP 2C also make a significant contribution to the cellular production of ROS such as $\text{O}_2^-$, $\text{H}_2\text{O}_2$ and hydroxyl radicals.\textsuperscript{179,216}

AA metabolism is increased during myocardial ischemia and to an even greater degree during reperfusion.\textsuperscript{212} The COX and CYP pathways are largely responsible for the increase in AA metabolism during I/R. This increase is the result of an increase in intracellular calcium levels during I/R which activates phospholipase A\textsubscript{2} (PLA\textsubscript{2}). PLA\textsubscript{2} catalyses the hydrolysis of AA from membrane phospholipids thus increasing the concentration of free AA in the cytosol.\textsuperscript{217-220} AA metabolism, on the whole, is detrimental during I/R. Several studies, in multiple cell types, have demonstrated that inhibition of AA metabolism during I/R by inhibition of PLA\textsubscript{2} or inducible PLA\textsubscript{2} (iPLA\textsubscript{2}) is cardioprotective.\textsuperscript{32-35,219,221,222} AA has been shown to induce oxidative stress in multiple cell types\textsuperscript{223-226} and AA causes CYP-mediated superoxide production in isolated renal microsomes.\textsuperscript{216} We have previously demonstrated that CYP 2C contributes to vascular and cardiac post-ischemic $\text{O}_2^-$ production,\textsuperscript{146,227} likely as a result of increased AA metabolism.
1.5.4 AA metabolites and cardiovascular disease

COX-2 is constitutively expressed endothelial cells, however, COX-2 levels are also known to be induced by cytokines, growth factors, lipopolysaccharides, prostanoids and substrates. COX-2 is also known to be bound to PGI₂ synthase in endothelial cells resulting in PGI₂ being the predominant AA metabolite in these cells. PGI₂ is anti-thrombotic and vasodilatory and plays a central role in vascular homeostasis.

Coxibs, selective COX-2 inhibitors, have been associated with increased cardiovascular events. The first studies were related to rofecoxib, Vioxx, and eventually led to its withdrawal from the market. The VIGOR study, of rofecoxib, showed a nearly 5-fold increased risk of myocardial infarction in those patients that received rofecoxib and a correlation between myocardial infarction and rofecoxib was also found by the APPROVe (Adenomatous Polyp PRevention On Vioxx) study of rofecoxib. Studies related to celecoxib and paracoxib/valdecoxib have also shown an association with increased cardiovascular risks. These later studies resulted in warnings related to increased cardiovascular risks for patients taking celecoxib and the voluntary withdrawal or paracoxib/valdecoxib from the market. Although coxibs have been associated with increased risk of cardiovascular events, and it is likely that decreased PGI₂ synthesis contributes to these events, the underlying mechanisms and other contributing factors have not been fully examined.

During ischemia increased intracellular calcium levels induces the activation of PLA₂ and the subsequent hydrolysis of AA from membrane phospholipids. Several AA metabolites, including COX-2 derived PGI₂, are known to be cardioprotective. The negative
cardiovascular effects of COX-2 inhibitors have been largely attributed to decreased prostacyclin production. However, the overall metabolism of AA during I/R has been implicated as a key contributor in the progression of ischemic injury. In support of this notion, inhibition of PLA$_2$ or iPLA$_2$ protects against I/R. Therefore, blocking prostacyclin production upstream of COX-2 at the point of AA hydrolysis does not have the same negative cardiovascular effects. This implies that the effects of COX-2 on the cardiovascular system are more complex than inhibition of PGI$_2$ production alone.

CYPs, primarily the CYP 2C epoxygenases, are often referred to as the third pathway of AA metabolism (LOX and COX being the other two). We have previously demonstrated that CYP 2C contributes to vascular dysfunction and myocardial injury following I/R. In the latter study, inhibition of CYP 2C by sulfaphenazole reduced myocardial infarction by nearly 60%. CYP 2C undergoes substrate-induced activation. As AA can be metabolized by one of 3 possible mechanisms, it is logical that if one of these pathways were blocked, that this may result in a shift towards the other 2 pathways and increased activity of these pathways. However, the effect of COX inhibition on AA metabolism by LOX and CYP epoxygenases has not been examined.

1.6 Thesis objectives and hypotheses

The overall objective of this thesis is to examine potential mechanisms to reduce peritransplant ischemic injury in the vasculature and to assess the relationship between this form of injury and the development of CAV.
To this end we examined the potential of the anti-apoptotic protein ARC to prevent oxidant cell death in the vasculature. We hypothesized that inhibition of apoptotic and necrotic cell death in the donor heart through increased ARC protein levels would attenuate I/R and thus immune-mediated cell death and chronic transplant rejection caused by CAV. Results from these experiments are described in Chapter 2.

We also examined the contribution of the CYP 2C enzymes to peri-transplant ischemic injury and CAV development. We hypothesized that CYP 2C enzymes play a key role in the pathogenesis of CAV through the production of reactive oxygen species that contribute to inflammation, endothelial damage and dysfunction. Our initial examinations, described in Chapter 3, determined the influence of the rodent CYP 2C9-equivalent on I/R-mediated vascular dysfunction in coronary arteries isolated from Langendorff-perfused hearts. We then assessed the contribution of rodent CYP 2C on peri-transplant ischemic injury and CAV using a rat heterotopic heart transplant model of chronic rejection. Results from these studies are described in Chapter 4. Finally, we assessed the effects of CYP 2C9 on hypoxia/re-oxygenation (H/R) induced cell death in EC and SMC and examined questions related to altered oxidative stress and AA metabolism following CYP 2C9 inhibition.

Results described in this thesis demonstrate that ARC does not have similar protective effects against oxidant induced injury in vascular cells as it does in myocytes. Further we serendipitously discovered a novel role for ARC in myogenic differentiation. We have demonstrated that CYP 2C contributes to endothelium-dependent vascular dysfunction and vascular ROS generation following I/R. Inhibition of CYP 2C during cardiac transplantation was found to be protective against CAV development and that expression of CYP 2C9 increases cell death and may alter AA metabolism in cultured human ECs.
1.7 Bibliography


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Chapter 1


Chapter 2: Apoptosis Repressor with Caspase Recruitment Domain in Vascular Cell Death and Myogenic Differentiation

2.1 Introduction

Apoptosis is a tightly controlled form of cell death that is characterized by cell shrinkage, DNA fragmentation and membrane blebbing resulting in the packaging of the cell into membrane-enclosed vesicles. These vesicles are then engulfed by surrounding ‘professional’ (macrophages) or ‘non-professional’ phagocytes. Endothelial cell and smooth muscle cell apoptosis and necrosis have been identified as important factors in the progression of ischemic injury and CAV. Numerous factors such as oxidative stress, immune cells and cytokines participate in the induction of cell death in CAV as reviewed in section 1.1.3.2. Therefore, multiple death inducing pathways must be inhibited in order to attenuate vascular damage.

ARC was originally discovered as a caspase-2 and -8-interacting, anti-apoptotic protein that is expressed primarily in the heart and skeletal muscle.\(^1\) ARC is capable of preventing both apoptotic and necrotic cell death by preserving mitochondrial function.\(^2\) More recently, work by Nam \textit{et al.}\(^3\) has suggested that ARC is a unique protein that is capable of intersecting with both the intrinsic and extrinsic apoptotic pathways. Overexpression of ARC inhibits ischemia-induced apoptosis in cardiomyoblast H9c2 cells by preventing

mitochondrial cytochrome c release and, in Langendorff-perfused rat hearts, TAT-transduction of ARC was shown to significantly reduce infarct size following ischemia and reperfusion.\(^5\)

ARC's ability to inhibit both apoptotic and necrotic forms of cell death when transfected into cardiac myoblasts\(^4\), may provide a unique method of inhibiting the multiple apoptotic and necrotic pathways involved in ischemic injury and CAV. However, little is known about the expression or activity of ARC in EC or SMCs.

HIV TAT-mediated protein transduction has been developed as a highly efficient method of transducing biologically active proteins into cells and tissues \(\textit{in vivo}\). The technology requires the synthesis of a fusion protein, linking the arginine-rich, 11 amino acid TAT protein transduction domain, to the protein of interest using a bacterial expression vector followed by purification of this fusion protein under soluble or denaturing conditions. TAT fusion proteins can be added directly to cells in culture or injected \(\textit{in vivo}\) into mice. TAT-mediated transduction of IP injected TAT-beta galactosidase (β-gal) has previously been shown to be detectable and functional in all tissues, including the heart.\(^6\) Protein transduction occurs with nearly equivalent concentrations in all cells in the transduced population within 15 min, in a dose-dependent manner.\(^7\)\(^9\)

### 2.2 Aim

As ARC has previously been shown to protect against cardiac I/R injury\(^5\) and I/R injury is associated with the development of cardiac allograft vasculopathy (discussed in section 1.2.2), we hypothesized that ARC may be protective against peri-transplant ischemic
injury and prevent the development of cardiac allograft vasculopathy. The aim of this chapter is to explore the potential for ARC to protect against oxidative damage in cardiovascular cell types in culture as a marker of ischemic injury. In this study, we examined the native expression of ARC in EC, SMC and cardiomyocytes. We examined the effects of altered ARC levels on protection against oxidative damage. In the course of these experiments we have found compelling evidence that ARC inhibits myoblast differentiation. We examined alterations in native ARC expression following the induction of differentiation as well as the effect of ARC overexpression on muscle cell differentiation using H9c2 rat myoblasts as a model. We demonstrate that ARC expression is increased in differentiated cells and we show, for the first time, that ARC overexpression prevents myoblast differentiation. Taken together, these results provide evidence of a novel bi-functional role for the apoptosis regulatory protein ARC in myoblast differentiation.

2.3 Materials and Methods

2.3.1 Cell culture

Pooled human umbilical venous endothelial cells (HUVECs) and human coronary artery smooth muscle cells (HCASMCs) were obtained from Cambrex (Baltimore, MD). HUVECs were cultured in complete endothelial growth medium (EGM: endothelial basal medium supplemented with 0.4% bovine brain extract, 0.1% human endothelial growth factor (hEGF), 0.1% hydrocortisone and 0.1% gentomycin-amphotericin B (GA-1000); Cambrex) plus 5% foetal bovine serum (FBS, Invitrogen). HCASMCs were cultured in complete smooth muscle growth medium (SmGM: smooth muscle basal medium...
supplemented with 0.1% insulin, 0.5% human foetal growth factor B, 0.1% GA-1000 and 0.1% hEGF; Cambrex) plus 5% FBS. The rat embryonic cardiac cell line H9c2 was obtained from the American Type Culture Collection (Manassas, VA). They were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS) up to passage 23. All cells were cultured using sterile technique.

2.3.2 RNA extraction and reverse transcriptase (RT)-PCR

RNA was extracted from cultured HUVEC and HCASMCs to assess native ARC expression. A minimum of 2.5 x 10^6 cells were trypsinized and collected and total RNA was extracted using the Qiagen RNeasy kit as per manufacturer’s instructions (Qiagen). Briefly, cells were lysed in RLT buffer, homogenized using the Quashredder spin column, and 1 volume of 70% ethanol was added. Samples were then centrifuged through the RNeasy mini column for 15 s at 10,000 rpm. The column was washed once with buffer RW1 and twice with buffer RPE. Samples were eluted in 50 µl of RNase free water (Qiagen).

Purified total RNA was then assessed for ARC and 18S (control) expression by RT-PCR. Purified RNA was subjected to DNase treatment to remove contaminating genomic DNA. Five micrograms of RNA was combined with 1X DNase I buffer, 5 mM MgCl₂, 1 mM dNTPs, 1X RNase inhibitor and DNase I. Samples were run at 37°C for 45 min, 99°C for 7 min and then cooled to 4°C. Following DNase treatment, samples were treated with Qiagen pre-mixed random primer RT reaction at 25°C for 10 min, 42°C for 50 min, 95°C for 5 min, and then cooled to 4°C. PCR reactions were carried out under the following conditions: 1X PCR buffer, 1 mM MgCl₂, 2.5 U/100µl Taq, 200 µM dNTPs, 1.0 µM primers. Primer
sequences were as follows: 18s forward (5'-GTAACCGGTGAACCCCATT-3'), reverse (5'-CCATCCAATCGGTAGTAGCG-3'), ARC forward (5'-GGAAACGCCTGGTCGAGAC-3') and reverse (5'-GCTTCAGCCTCGGGTTCC-3'). Thermocycler conditions utilized involved 30 cycles at 94°C, 52°C, 72°C for 1 min each. Products were separated by electrophoresis for 30 min at 100 V through a 1% agarose gel pre-stained with ethidium bromide. Gels were imaged using the Strategene EagleEye II ultraviolet imager (Stratagene, La Jolla, CA).

2.3.3 Cell lysis and Western blotting

Cells were washed two times with ice cold PBS and lysed in CellLytic M lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich, Oakville, ON). Protein concentrations were measured using the Bio-Rad protein assay which is a modified Bradford protein assay (Bio-Rad, Hercules, CA). This assay measures the change in absorbance of Coomassie Brilliant Blue G-250 to 595 nm upon binding to basic and aromatic amino acids in proteins. Equal amounts of protein were separated by sodium dodecyl sulphate – polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After blocking with 5% skim milk, the membranes were incubated for 1 h with primary antibodies (1:1000 anti-myogenin antibody and 1:200 anti-skeleton muscle troponin T antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or 1:1000 anti-ARC antibody (Alexis Co., Lausen, Switzerland)), followed by incubation for 1 h with 1:4000 IRDye800™ or 1:2000 IRDye700™–conjugated secondary antibodies (Rockland Inc. Gilbertsville, PA). Protein expression was detected by using the Odyssey Infrared Imaging System from LI-COR Biosciences (Lincoln, NE).
2.3.4 TAT protein expression, purification and Texas red staining

BL21(DE3)pLysS bacteria containing either the pTAT-HA-hARC plasmid or the pTAT-HA-β-gal plasmid were prepared as previously described\(^5\) and kindly provided by Dr. Roberta Gottlieb. The plasmids are modified from the pTAT-HA vector originally developed by Dowdy’s group.\(^{10}\) A map of this plasmid based on the pRSET vector (Invitrogen, Burlington, ON) is shown in Figure 2.1. It contains elements for ampicillin resistance, a T7 promoter, 6x-Histadine (His) and hemagglutinin (HA) tags, and an N-terminal TAT peptide fusion cassette.

Frozen glycerol stock cultures were transferred to Luria broth (LB) containing ampicillin (50 µg/ml) to select for transformed bacteria and isopropylthiogalactoside (IPTG, 100 µM) to induce expression and were cultured overnight at 37°C with shaking. Bacteria were centrifuged at 5000 rpm for 15 min, washed once with PBS, and resuspended in Buffer Z (8 M Urea, 100 mM NaCl, 20 mM HEPES pH 8.0) containing 20 mM imidazole. Bacterial solutions were then sonicated on ice 3 times for 15 s pulses with 30 s on ice in between pulses and then centrifuged at 11700 rpm for 30 min. Supernatants were collected.

Nickle-nitrilotriacetic acid (Ni-NTA) absorbent columns were used to purify the 6xHis tagged proteins. Ni-NTA is a tetrادentate chelating adsorbent which occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag. The NTA is able to stably bind metal ions and retain them under stringent wash conditions. The theoretical capability of this technique allows purification of proteins from less than 1% of the total protein preparation to more than 95% homogeneity in just one step.\(^{11}\) Ni-NTA columns (5 ml, Qiagen, Mississauga, ON) were
Figure 2.1 A Map of the pTAT-HA-fusion protein.

The pTAT-HA-fusion plasmid developed by Nagahara et al. based on the pRSET plasmid.\textsuperscript{10} Upon insertion of the desired gene sequence into the multiple cloning site (MCS) the plasmid produces a 6xHis, HA tagged TAT-fusion protein.
prepared by adding 10 ml of resuspended Ni-NTA resin and allowing excess fluid to run through by gravity. Columns were then pre-equilibrated with 50 ml of Buffer Z + 20 mM imidazole followed by the supernatants prepared above. Columns were then washed with 10 bed volumes (2 x 25 ml) of Buffer Z + 10 mM imidazole. TAT-fusion proteins were then eluted by adding 10 ml of 250 mM imidazole in Buffer Z. Elution fractions were analyzed by SDS-PAGE and Western blot.

Elution fractions containing TAT-fusion proteins were then desalted and concentrated. PD-10 desalting columns, purchased from Amersham Pharmacia (Piscataway, NJ), were drained and equilibrated with 25 ml of sterile PBS. Elution fractions were then added and samples were eluted in PBS. PD-10 elution fractions were again analyzed via SDS-PAGE, samples containing the highest protein levels were pooled and protein concentration was measured using the Bio-Rad modified Bradford assay described in section 2.2.3.

To assess subcellular localization some TAT-fusion protein preparations were stained with Texas red succinimidyl ester (Molecular Probes, Eugene, OR). Texas red (12 mM in DMSO) was added at a molar ratio of dye to protein of 5:1 in 0.1 M bicarbonate buffer (pH 8.3). The mixture was incubated for 60 min at 4°C followed by 30 min at room temperature. These conditions allow amide bonds to form with protein amines but prevent hydrolysis of the dye. Protein-dye mixtures were then desalted using PD-10 columns as described above.

2.3.5 TAT-fusion protein transduction and detection

Cells were grown to 70-90% confluency in complete media with 5% FBS. Media was removed and cells were washed with Dulbecco's PBS (DPBS). Media was then replaced with
serum free basal media. TAT-fusion proteins were added between 0-1 μM and were incubated for 1 h at 37°C. Cells were then washed twice with DPBS and were then utilized for further experimentation. To assess levels of TAT-fusion protein transduction we utilized proteins pre-stained with Texas Red. Following transduction media was replaced with phenol red free media and cells were imaged using the fluorescent microscope (595-605 nm excitation, 615 nm emission).

2.3.6 Cell viability

HUVECs and HCASMCs were seeded in 6-well plates, grown to 70-90% confluency, and were treated with 0, 62.5, 125, 250 or 500 nM of either TAT-ARC or TAT-β-gal as described in section 2.2.5. Cells were then treated with 0.6 mM H2O2 for 4 h. Viability was assessed using the CellTiter96™ AQueous Assay (MTS) (Promega, Madison, WI). MTS is a colorimetric assay involving a cell permeable novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and an electron coupling reagent (phenazine methosulfate) PMS. Upon entry in to viable cells, MTS is bioreduced to an aqueous soluble formazan product by dehydrogenase enzymes. The soluble formazan product is proportional to the number of viable cells and can be measured spectrophotometrically due to its absorbance at 490 nm. MTS was protected from light and was added at a 1:5 ratio of MTS: media and the reaction was allowed to proceed for 1 h at 37°C. Samples were transferred in triplicate to 96-well plates and scanned on the Tecan GENios Rainbow absorbance plate reader (Tecan, San Jose, CA). Data are shown as the mean ± standard deviation (SD) and represent 3 samples per experiment for 4 experiments measured in triplicate.
H9c2-Neo or ARC stable cell lines, L5 and L24, were treated with 0-500 μM H₂O₂ for 8 h. Cell viability was assessed using the calcein-acetoxymethyl (AM) cell viability assay which utilized non-fluorescent dye that is converted to a green-fluorescent calcein after AM ester hydrolysis by intracellular esterases. Calcein is retained in cells with intact membranes (Molecular Probes, Eugene, OR). Briefly, calcein-AM was added to cells at a final concentration of 5 μM. Fluorescence intensity was read following one hour (h) of substrate incubation (excitation 485 nm, emission 527 nm) using the TECAN GENios fluorescent plate reader (Tecan, San Jose, CA).

2.3.7 H9c2 stable and transient transfection

Stably transfected H9c2 cells with pcDNA3-Neo or pcDNA3-ARC were obtained from Dr. Daryoush Ekhterae. Cells were prepared using lipofectamine, as previously described, resulting in the production of Neo-transfected cells and two clones, L5 and L24, which express high levels of ARC.

An adenoviral vector expressing ARC (Ad-ARC) and an adenoviral vector expressing green fluorescent protein (Ad-GFP), control, were kindly provided by Dr. Roberta Gottlieb. Adenoviral infections were carried out by removal of media and addition of a 10:1 virus to cell ratio in low volume media. Cells were incubated with intermittent gentle rocking for 2 h, media levels were restored and cells were incubated overnight to allow for protein expression.
2.3.8 H9c2 myocyte differentiation

Muscle differentiation was induced by culturing cells to 100% confluency, removing media containing serum, washing cells twice with DPBS and replacing medium with DMEM containing 1% horse serum (HS). Media was changed daily for 5 days. Differentiation was assessed by measuring expression of differentiation markers by Western blot and was quantified by determining the number of cells that showed at least three nuclei. Multinucleation is expressed as a percentage of the total number of nuclei in ten randomly chosen microscopic fields.

2.3.9 F-actin and nuclear staining of H9c2 cells

H9c2 cells transfected with ARC or vector alone were grown on glass coverslips and differentiated as described in section 2.3.8. Cells were stained with AlexaFluor 488-labelled phalloidin and Hoechst 33342 (Molecular Probes, OR) to visualize F-actin and nuclei, respectively. Phalloidin is a water soluble bicyclic peptide derived from *Amanita phalloides* mushrooms that binds strongly to F-actin.\(^{13}\) Hoechst is a cell soluble, blue-fluorescent bisbenzimidazole derivative that binds to the minor groove of DNA.\(^ {14}\) Cells were washed twice with DPBS, fixed with 2% paraformaldehyde for 10 min at room temperature and rewashed twice with DPBS. Cells were then permeabilized in a 0.1% Triton X solution for 5 min and washed twice with DPBS. Cells were incubated with 1 μM AlexaFluor 488-labelled phalloidin and 1 μg/ml Hoechst 33342 for 30 min, washed three times with DPBS before imaging. Slides were imaged using a Nikon Eclipse T6300 microscope and Spot digital
camera. The excitation and emission wavelengths for AlexaFluor 488 and Hoechst 33342 are 350 nm and 461 nm, respectively.

2.3.10 DEVDase activity assay

DEVDase activity assays were performed to detect caspase-3/7-like activity, as described previously.\\textsuperscript{15, 16} At day 0, 1, 3 and 5 post-differentiation, H9c2 cells transfected with ARC or vector alone were lysed in whole cell lysis buffer (1% NP-40, 20 mM Tris, pH 8, 137 mM NaCl, 10% glycerol, 1 mM phenylmethyl sulfoxide, 0.15 U/ml aprotinin, and 1 mM sodium orthovanadate). Lysates (0.3 mg/ml) or buffer as control were plated in triplicate and incubated at 37°C for 15 min. Acetyl-DEVD-7-amino-4-methylcoumarin (Ac-DEVD-AMC) (37.5 mg/ml, Calbiochem) caspase-3 substrate was added and relative florescence units (RFU) were measured after 2 h at 37°C using the TECAN GENios fluorescence plate reader (ex: 380 nm, em: 460 nm).

2.3.11 Statistical analysis

All results are expressed as mean ± SE, and analyzed with \textit{GraphPad Prism 4} software using one-way analysis of variance (ANOVA) with multiple comparisons performed by Students’ T test. The results of statistical tests were considered statistically significant at p<0.05.
2.4 Results

2.4.1 Native ARC expression in endothelial and smooth muscle cell lines

Native levels of ARC expression were assessed in cultured HUVECs and HCASMCs by RT-PCR and Western blot. We were able to detect transcript for ARC in both cell lines. By comparing RNA levels to those of 18S rRNA we observed that ARC transcript levels are higher in HUVECs than in HCASMCs. A representative image of ARC transcript levels is shown in Figure 2.2(A). We then compared protein expression levels of ARC in these cell lines. Again we were able to detect ARC expression in both lines with higher expression levels observed in HUVECs. A representative Western blot is shown in Figure 2.2(B).

2.4.2 TAT-ARC purification and transduction in vascular cells

ARC is a splice variant of the Nop30 protein. Although they have poor homology at the protein level, as a result of a frame shift cause by an alternate splicing at exon 2, there is only one unique sequence of 10 nucleotides contained in ARC that is not in the sequence of Nop30. This sequence is unfortunately a poor target for siRNA. Thus we decided to examine the effect of increased ARC levels via TAT-fusion protein transduction. TAT-ARC and TAT-β-gal were expressed in BL21(DE3)pLysS bacteria, purified using Ni-NTA columns and were then desalted. We routinely obtained purified protein concentrations greater that 2 mg/ml. Texas-red conjugated TAT-ARC and TAT-β-gal were successfully transduced into both HCASMCs and HUVECs in culture (Figure 2.3). The transduced protein appears
Figure 2.2 HCASMCs and HUVECs express ARC. (A) Representative RT-PCR experiment (of n=3) showing detection of ARC transcripts in cultured HCASMCs (SMC) and HUVECs (EC). (B) Representative Western blot (of n=3) demonstrating ARC protein expression.
Figure 2.3 TAT-ARC fusion protein transduction into HCASMCs and HUVECs. Representative fluorescent images of HCASMCs and HUVECs were treated with Texas-red conjugated TAT-ARC fusion protein demonstrating successful protein uptake.
punctate (Figure 2.4(A)) and was taken up in a concentration-dependent manner (Figure 2.4(B)).

2.4.3 **ARC over-expression does not protect against H₂O₂ treatment.**

ARC’s ability to protect against H₂O₂ treatment was measured in both HUVECs and HCASMCs. Cells were pre-treated with TAT-ARC or TAT-β-gal (control) and were then exposed to 0.6 mM H₂O₂ for 4 h. Viability was measured using the MTS assay (see Figures 2.5 and 2.6). H₂O₂ treatment induced an average viability loss in HUVECs of 95.6 ± 1.8% compared to untreated cells and an average viability loss in HCASMCs of 90.8 ± 8.5% compared to untreated cells. In both cell lines TAT-ARC transduction was protective against H₂O₂ treatment; however, high levels of transduction (>100nM) were required to see any protective effect and TAT-ARC treatment was no more protective than treatment with the TAT-β-gal fusion protein.

2.4.4 **Functional overexpression of ARC in pre-differentiated H9c2 cells**

Since ARC has previously been shown to be protective in cardiomyocytes and skeletal muscle⁴,⁵,¹²,¹⁷ we turned to the rat embryonic myocyte cell line H9c2. H9c2 cell lines stably overexpressing ARC or Neo (control) were created by selecting clones from pcDNA3 transfected cells. Two clones, L5 and L24, were selected due to their high expression levels of ARC (Figure 2.7 (A)). Both clones were used in subsequent analyses in order to reduce the possibility that the effects observed are resulting from a gene disruption.
Figure 2.4 TAT-ARC uptake into HUVECs and HCASMCs is punctate and concentration-dependent.
(A) High magnification fluorescent image of Texas-red conjugated TAT-ARC internalization into HUVECs showing punctuate protein distribution. (B) Western blot of ARC protein following TAT-ARC transduction into HUVECs showing concentration-dependent uptake against β-actin protein control.
Figure 2.5 TAT-ARC does not confer greater protection against H$_2$O$_2$ in HUVECs than TAT-β-gal control

HUVECs were pretreated with increasing concentrations of TAT-ARC and TAT-β-gal and subjected to 0.6 mM H$_2$O$_2$ for 4 h. Data are shown as percent viability of untreated cells as measured by the MTS viability assay. Data represents the mean ± SD of 4 experiments, 3 repeats/experiment.
Figure 2.6 TAT-ARC does not confer greater protection against H$_2$O$_2$ in HCASMCs than TAT-β-gal control

HCASMCs were pretreated with increasing concentrations of TAT-ARC and TAT-β-gal and subjected to 0.6 mM H$_2$O$_2$ for 4 h. Data are shown as percent viability of untreated cells as measured by the MTS viability assay. Data represents the mean ± SD of 4 experiments, 3 repeats/experiment. Significance was calculated using a Student’s t-test, p-values > 0.1.
as a consequence of vector integration into the host cell genome rather than from the overexpression of ARC. The functionality of ARC was confirmed in these cells lines by examining cell viability following exposure to hydrogen peroxide (Figure 2.7 (B)). H9c2-Neo control cells demonstrated concentration-dependent loss in viability after 8 h of treatment with H2O2 concentrations between 0 and 500 μM. The H9c2-ARC clones, L5 and L24, demonstrated a significant anti-apoptotic effect at all H2O2 concentrations (p-values < 0.05) and were able to maintain cell viability; showing a slight decrease in viability at only the highest concentration of 500 μM (73.9 ± 4.2% for L5, 82.5 ± 12.5% for L24, compared to 41.1 ± 4.0% of Neo cells; expressed as mean ± SE).

### 2.4.5 Characterization of H9c2-ARC cell differentiation

We then wanted to examine the role of ARC overexpression in differentiated H9c2 cells. We induced myotube differentiation using standard protocols by reducing serum concentration from 10% FCS to 1% HS. Cells were visualized at 0, 3 and 5 days after the induction of differentiation. H9c2-Neo cells demonstrated myoblast elongation/differentiation; however, elongation was attenuated in H9c2-ARC cells (Figure 2.8). Elevated myotube disarray and disorganization were observed at day 3 and 5 in the ARC overexpressing cells compared to that of the H9c2-Neo cells. In addition to myotube elongation, multi-nucleation was also observed in the H9c2-Neo at a rate of 11.2 ± 5.1%, but was absent in the H9c2-ARC cells (Figure 2.9 and 2.10).

To further assess the influence of ARC overexpression on myoblast differentiation, the status of muscle specific proteins myogenin and troponin T were evaluated. Myogenin and troponin T were both highly expressed in differentiated (Day 3 and 5 post-differentiation)
Figure 2.7 Overexpression of ARC prevents H$_2$O$_2$-induced cell death.
(A) ARC stable cell lines, L5 and L24, express high levels of ARC. (B) H9c2-Neo and ARC cells were treated with various concentrations of H$_2$O$_2$ and cell viability was assessed using the calcein-AM cell viability assay at 8 h post-treatment. Values are expressed as the percent viability the test group compared to that of untreated H9c2-Neo cells. Bars represent the mean ± SE (n = 3).
Figure 2.8 Overexpression of ARC prevents myogenic differentiation.
Representative morphological changes of Neo control and ARC overexpressing stable cell lines, L5 and L24, at day 0, 3 and 5 following the induction of differentiation. Characteristic alignment and elongation of cells is observed in control cells but is not apparent in ARC overexpressing cell lines.
Figure 2.9 Overexpression of ARC prevents myogenic differentiation.
Fluorescent staining of ARC overexpressing H9c2 cells at day 5 post-differentiation. H9c2 cells transfected with ARC, or vector alone were stained with AlexaFluor 488-labelled phalloidin for F-actin (green). Cell nuclei were counterstained with Hoechst (blue). Arrows indicate multi-nucleated cells.
Figure 2.10 Overexpression of ARC prevents myogenic differentiation.
Myogenic differentiation was quantified by the number of differentiated cells which showed at least three nuclei and expressed as a percentage of the total number of nuclei in ten randomly chosen microscopic fields. The results shown are mean ± SD (n=10) and significance was determined by Student’s t-test. (*=p<0.05).
H9c2-Neo cells but were minimally detectable in the ARC-overexpressing, L5 and L24, H9c2 cell lines (Figure 2.11). Consistent with these findings, when H9c2-Neo cells were transiently transduced with an adenovirus ARC construct, the muscle differentiation markers were significantly reduced (Figure 2.12).

### 2.4.6 ARC expression during differentiation

We then examined the native expression levels of ARC throughout H9c2 cell differentiation. ARC was undetectable in undifferentiated H9c2 cells. However, ARC expression rose to detectable levels by day 3 following differentiation and by day 5 ARC levels had reached maximal and sustained expression levels (Figure 2.13).

### 2.4.7 Caspase-3 activation during H9c2 differentiation

To examine whether caspase-3 is activated during myotube differentiation, we measured caspase-3 activity by the cleavage of Ac-DEVD-AMC substrate. As shown in Figure 2.14, at day 0 post-differentiation, caspase-3 activity was not different between Neo and ARC overexpressing cells (Neo: 4400 ± 504 RFU, ARC: 4192 ± 2672 RFU; expressed as mean ± SD). However at day 1 post-differentiation caspase-3 activity significantly increased in the Neo cells, implying an important role of caspase activation in cell differentiation. Overexpression of ARC prevented caspase-3 activation at day 1 post-differentiation (16108 ± 1135 RFU for ARC cells compared to 39736 ± 1796 RFU for Neo cells). By day 3 post-differentiation caspase-3 activity levels decreased in both groups (Neo: 10505 ± 694 RFU, ARC: 14861 ± 1928 RFU) and were relatively stable through to day 5 (Neo: 10303 ± 624 RFU, ARC: 10390 ± 1707 RFU).
Figure 2.11 ARC stable overexpression prevents the expression of the muscle-specific markers troponin T and myogenin.
At day 0, 3 and 5 post-differentiation, cell lysates were collected and Western blotting was performed to determine troponin T, myogenin and ARC expression. The same membranes were blotted with an antibody against β-actin to illustrate equal protein loading. The data are representative of three different experiments.
Figure 2.12 Transient ARC overexpression prevents the expression of the muscle-specific markers troponin T and myogenin.
At day 0, 3 and 5 post-differentiation, cell lysates were collected and Western blotting was performed to determine troponin T, myogenin and ARC expression. The same membranes were blotted with an antibody against β-actin to illustrate equal protein loading. The data are representative of three different experiments.
Figure 2.13 ARC levels increase in H9c2 cells upon differentiation. Western blot of ARC expression at day 0, 3 and 5 following the induction of differentiation. The same blot was stained with an antibody against β-actin to illustrate equal protein loading.
Figure 2.14 ARC overexpression prevents caspase-3/7 activity during differentiation. At day 0, 1, 3, and 5 post-differentiation, cell lysates were harvested and caspase activity was determined. The data shown are mean ± SD (n=3) and significance was determined by Student’s t-test.
Chapter 2

2.5 Discussion

ARC expression was initially thought to be restricted to the highly differentiated tissues of skeletal and cardiac muscle.\textsuperscript{1} Since that time ARC has also been found to be expressed in other cell types, most notably cancer cells.\textsuperscript{18,19} Here we demonstrate that ARC is also expressed in endothelial cells and smooth muscle cells. Although ARC expression is usually associated with muscle phenotypes we observed greater levels of both ARC transcript and protein expression in endothelial cells compared to smooth muscle. Interestingly, protein transduction of ARC into these cell types did not confer greater protection than the control protein β-gal to treatment with H$_2$O$_2$. It is unclear why TAT-ARC is not exerting a protective effect in these cells. One possibility is that TAT-ARC is not being properly folded or transported and thus is not biologically active in these cells. TAT-ARC has been shown to be protective against oxidative injury in cardiac tissue\textsuperscript{5} and ARC overexpression has been shown to protect against H$_2$O$_2$ treatment in other cell types\textsuperscript{12} and as shown here in H9c2 cells.

We have also found that although ARC is expressed in differentiated skeletal and cardiac muscle that its expression must be repressed in these tissues to allow differentiation to occur. Apoptosis is a critical physiological process that is essential for normal tissue development and homeostasis. Dysregulation of this form of cell death is associated with numerous pathological conditions. Recent studies suggest that apoptosis and differentiation share common pathways in muscle cells.\textsuperscript{20-22}

Actin fibre disassembly and reorganization are conserved features of both apoptosis and myoblast differentiation. Similarly, caspase activity is a key component of both apoptosis and
skeletal muscle differentiation. Caspase-3 inhibition reduces myotube/myofibre formation as well as expression of muscle-specific proteins during myogenesis. Further, dysregulated myoblast differentiation and apoptosis in response to certain cytokines may be associated with increased muscle wasting in chronic disease states such as infection, Acquired Immune Deficiency Syndrome (AIDS) and cancer. Interestingly, tumour necrosis factor alpha (TNF-α), a principle cytokine associated with cachexia, is involved in the regulation of skeletal muscle differentiation and apoptosis. Thus, it is exciting to speculate that proteins which inhibit both differentiation and apoptosis could attenuate such degenerative diseases by preventing muscle differentiation thereby facilitating further replication.

As such, the role of ARC in the regulation of myocyte differentiation is interesting. H9c2 myoblasts, isolated from rat embryonic cardiomyocytes, proliferate under normal conditions and are mono-nucleated. This cell line is also a well-characterized model of differentiation. When H9c2 cells are exposed to reduced serum concentrations at confluence, they fuse and differentiate into elongated, multi-nucleated myotubes. Although this cell line exhibits some differences from primary cells, H9c2 cells share many of the properties of primary cardiomyoblasts and skeletal muscle and can be easily propagated and stably transfected, making them an ideal model for this study.

ARC is highly expressed in terminally differentiated cardiac and skeletal muscle cells. However, we were unable to detect ARC expression in undifferentiated H9c2 rat myoblast cells leading us to ask questions regarding the regulation of ARC expression during myoblast differentiation and the importance of this regulation on the differentiation process.
ARC expression increased by day 3 following differentiation and reached maximal and sustained levels by day 5. To understand whether regulation of ARC expression during differentiation is important to the process of differentiation itself, we examined the effect of ARC over-expression using pre-differentiated H9c2 cells. H9c2 cells overexpressing ARC were unable to differentiate as indicated by morphological characteristics such as myotube elongation and multinucleation.

Myogenic differentiation, characterized by cell growth arrest, myoblast alignment, elongation, and fusion of mono-nucleated myoblasts into multi-nucleated myotubes, is dependent on the expression of the MyoD family of basic helix-loop-helix (bHLH) transcription factors, which includes MyoD, Myf5, myogenin, and MRF4. Upon stimulation, myoblasts are induced to express muscle regulatory factors, which in turn leads to the expression of muscle-specific genes. To further assess the influence of ARC overexpression on myoblast differentiation, the status of muscle specific proteins myogenin and troponin T were evaluated. Myogenin and troponin T were both highly expressed in differentiated (day 3 and 5 post-differentiation) H9c2-Neo cells but were minimally detectable in the ARC-overexpressing, L5 and L24, H9c2 cell lines (Figure 2.11). These results were confirmed using H9c2-Neo cells that were transiently transduced with an adenovirus ARC construct. Thus, inhibition of ARC expression is vital for the proper differentiation of these cells. Whether premature ARC expression culminates in abnormal cardiovascular development is unclear and requires further investigation.

Recent studies have suggested that caspase-3 activity is required for skeletal muscle differentiation. As ARC inhibits caspase-mediated apoptosis, we examined whether caspase-3 is activated during myotube differentiation and whether overexpression of ARC
prevents that activation. Caspase-3 activity significantly increased at day 1 post-differentiation and ARC overexpression prevented caspase activation. As ARC does not interact directly with caspase-3, it is likely modifying caspase-3 activation through its interactions with caspase-2, 8 or the Bcl-2 protein Bax, as has previously been shown.\textsuperscript{3, 29}

These findings suggest that ARC expression must be attenuated or absent to allow for early differentiation events including caspase-3 activation and that it is at this early stage of differentiation during which ARC impacts the differentiation process. Our initial finding that ARC is endogenously expressed at undetectable levels until day 3 post-differentiation (i.e. after caspase-3 is activated) supports this hypothesis. Therefore, the inhibition of differentiation markers, such as myogenin and troponin-T, in the ARC overexpressing cell lines are likely a result of these upstream events. This hypothesis would explain why endogenous expression of ARC at day 3 and day 5 does not prevent differentiation. The induction of ARC following caspase-3 activation would likely be beneficial as it may protect properly differentiating cells from apoptosis.

It is becoming increasingly more apparent that ARC is a multi-faceted anti-apoptotic protein. Several mechanisms have been proposed as to how ARC attenuates apoptosis including: inhibition of caspases, mitochondrial membrane depolarization, DISC formation, Bax activation, and cytochrome c release or K+ currents.\textsuperscript{1-5, 12} The results of this study demonstrate a novel role for ARC in myoblast differentiation and suggest that ARC expression is tightly controlled throughout the differentiation process in order to allow for initiating events such as caspase-3 activity.

The challenge for future studies will be to further delineate the detailed mechanisms by which ARCs expression is regulated and contributes to myocyte differentiation as well as the
potential role of ARC in myocyte disarray and disease. Reduced troponin T expression, which we have shown can be induced by ARC expression during differentiation, contributes to myocyte disarray\textsuperscript{30} and mutations in this gene are associated with myocyte disarray and 15\% of all cases of familial hypertrophic cardiomyopathy.\textsuperscript{31} Myofibrillar disarray is also commonly associated with familial hypertrophic cardiomyopathy, the leading cause of sudden death in young athletes, indicating a potential role for ARC in its pathogenesis.

The studies shown here indicating ARC is unable to protect vascular cells against oxidant injury induced by $\text{H}_2\text{O}_2$ are preliminary in nature. It is possible that ARC is able to protect against oxidant stress induced through other pathways. Since we were able to detect ARC expression in both endothelial and smooth muscle cell lines it seems likely that ARC does play a role in these cells. One possibility is that the native levels of ARC are high enough to confer maximal protection via its many pathways. Although this is possible, other cell types, such as cardiomyocytes, that express much higher levels of ARC do still gain an additional protective effect upon TAT-ARC transduction.\textsuperscript{5} Our ultimate research goal is to examine the relationship between ischemic injury and CAV. Given the intense interest by other groups in examining the role of ARC in myocardial ischemic injury and our failure to obtain preliminary evidence demonstrating a protective role for ARC in vascular ischemic injury we selected to examine other proteins. One such group of proteins are the cytochrome p450 2C enzymes. The role of these enzymes in peri-transplant ischemic injury and CAV are examined in the subsequent chapters of this thesis.
2.6 Bibliography


Chapter 3: Cytochrome p450 2C Contributes to Post-Ischemic Vascular Dysfunction

3.1 Introduction

Cardiac I/R contribute to MI and is also a major complication in surgical treatments such as cardiac transplantation, balloon angioplasty and coronary bypass. I/R is associated with a burst of ROS which is thought to contribute to both vascular dysfunction and myocardial damage.

CYP enzymes, reviewed in section 1.4, are membrane-bound heme-containing terminal oxidases responsible for the oxidation, peroxidation and/or reduction of a large number of substances including cardiovascular drugs. Although the majority of CYP are found in hepatic tissues, these enzymes are also expressed in extra-hepatic tissues such as the intestine, kidney, lung, heart, and blood vessels. CYP enzymes contribute to the cellular generation of ROS. Superoxide anions (O$_2^-$), hydrogen peroxide and hydroxyl radicals are produced during the CYP reaction cycle when electrons for the reduction of the central heme iron are transferred to the activated bound oxygen molecule. Recent evidence indicates an important role for CYP in the pathogenesis of several cardiovascular diseases. We recently reported that CYPs are key generators of superoxide during I/R in the heart. Furthermore, we demonstrated that CYP 2C9 inhibitors significantly reduce infarct size in both a rat Langendorff perfusion model of global ischemia as well as in a rabbit coronary ligation

model of focal ischemia. In the latter study, CYP inhibition also reduced post-ischemic CK release and superoxide generation while restoring coronary flow.\(^3\) In a clinical setting, sulfaphenazole (SP) restored endothelium-dependent vasodilator responses in patients with manifest coronary artery disease.\(^2\) However, the role of CYP 2C9 in I/R-mediated vascular dysfunction has not been examined.

SP, a highly specific inhibitor of CYP 2C9 in humans, exerts its inhibitory effect by binding to the heme group of CYP 2C9. Its specificity is mediated through interactions between SP’s phenyl substituent and the phenyl group of Phe 114 on CYP 2C9.\(^9\) SP also inhibits CYP 2C6 in rats.\(^10\) SP is dose dependently specific for CYP 2C9 (humans) and CYP 2C6 (rats).

### 3.2 Aim

As CYP 2C6/9 inhibition increases endothelium-dependent vasodilation in patients with coronary artery disease and increases post-ischemic coronary flow we hypothesized that CYP 2C6/9 inhibition will decrease endothelium-dependent vasodilation following I/R. The aim of this study was to assess the role of the CYP 2C6/9 inhibitor SP on endothelium-dependent, NO-mediated vasodilation in post-ischemic vascular dysfunction. We demonstrate for the first time that SP restores endothelium-dependent vascular function following I/R.
3.3 Materials and Methods

3.3.1 Heart perfusion and vessel cannulation

Experimental protocols were approved by the Animal Care Committee of the University of British Columbia. A copy of the animal care certificate is provided in Appendix I. Male Sprague-Dawley rats (300-350 g, n=10) were injected with sodium pentobarbital (60 mg/kg) and heparin sulphate (1000 U/kg) intraperitoneally. After loss of reflexes in rats, hearts were removed and immediately placed in ice-cold modified Krebs’ buffer (composition in mM: NaCl 119, KCl 4.7, KH2PO4 1.18, NaHCO3 24, MgSO47H2O 1.17, CaCl2 1.6, and glucose 11.1). Hearts were perfused in the Langendorff mode as previously described.3 Hearts were randomly divided into three groups: i) I/R: hearts were perfused 20 min followed by 30 min no-flow global ischemia and 15 min reperfusion with modified Krebs’ buffer, ii) I/R with SP (I/R+SP): hearts prepared as in the I/R group but with the addition of SP (10 μM) in the perfusate, and iii) control: hearts were perfused for the total perfusion time of I/R groups without ischemic period (35 min) with modified Krebs’ buffer. After perfusion (control) or reperfusion (I/R, I/R+SP), hearts were removed from the Langendorff apparatus and placed in a dissection dish with ice-cold buffer. Septal coronary arteries (intraluminal diameter is between 190-290 μm at 20 mm Hg) were dissected and transferred to the chamber of a pressure myograph. A Video Dimension Analyzer (Living Systems Instrumentation, Burlington, VT, USA) was used to measure inner diameter as described elsewhere.11
3.3.2 Vasomotor responses in septal arteries

Septal coronary arteries were pretreated with U46619 (1 μM, Cayman Chemical, Ann Arbor, MI) at 20 mm Hg. After a sustained constriction, tissues were exposed to ACh (1 nM-10 μM) added to the external reservoir, and final maintained diameters were recorded. An identical protocol was used to study the vasodilator effects of sodium nitroprusside (SNP, 1 nM-10 μM), and isoproterenol (1 nM-10 μM). In addition, the constrictor responses to various concentrations of KCl (20, 35, 66, 84 mM) were examined. At the end of each experiment, Krebs’ buffer was substituted with Krebs’ buffer containing no CaCl₂ and 2.0 mM EGTA to achieve zero calcium and the maximal passive diameters.

3.3.3 Dihydroethidium (DHE) staining of coronary blood vessels

Hearts were flash frozen in liquid nitrogen following Langendorff perfusion, as described above, and stained for superoxide production modified from methods previously described (Miller et al., 1998). Frozen hearts were sectioned at 20 μm on a ThermoShandon cryostat. DHE (Molecular Probes, Eugene, OR) was prepared under N₂ gas by dissolving to 1 mg/ml in DMSO and then dilution in phosphate buffered saline (PBS). Sections were treated in 2 μM DHE for 30 min at 37°C under N₂ gas. Sections were washed twice with PBS, cover slips were applied and slides and were imaged immediately. Imaging was performed on an Eclipse TE300 fluorescent microscope (Nikon; excitation: 488 nm, emission: 610 nm) under identical exposure settings. Fluorescence density of arterial walls (n=6, control; n=5, SP) were quantified using Image-Pro Plus software. Values were normalized to the average of the arteries from the untreated hearts representing 100%.
3.3.4 Measurements of dityrosine in coronary effluents

Dityrosine measurements were performed utilizing the method developed by Yasmin et al.\textsuperscript{12} L-tyrosine (0.3 mM) was added to Kreb's buffer and Langendorff perfusions were carried out as described above. Coronary effluent fractions were collected. Upon reaction with peroxynitrite, L-tyrosine is converted to dityrosine which absorbs at 320 nm.

3.3.5 Statistical analysis

All results are expressed as mean ± SE, and analyzed with NCSS 2000 and PASS 2000 software using one-way analysis of variance (ANOVA) and/or repeated-measures ANOVA with multiple comparisons performed by Bonferroni's test. -LogEC\textsubscript{50} (pD\textsubscript{2}) was calculated by Graphpad Prism\textregistered{}, version 3.02. The results of statistical tests were considered statistically significant at p<0.05.

3.4 Results

3.4.1 Endothelium-dependent vasomotor responses

Tonic contractions for U46619 (1 μM), a stable analog of thromboxane A\textsubscript{2}, were not altered in either I/R or I/R+SP groups as compared to those obtained under control conditions. Reduced endothelium-dependent vasodilation was observed after I/R. Figure 3.1 shows
Figure 3.1. Sulfaphenazole (SP) restores post-ischemic endothelium-dependent NO-mediated vasodilation.
(A) Representative traces showing SP attenuates impaired endothelium-dependent vasorelaxation to ACh. (B) Concentration-response curves to ACh, showing that SP increased sensitivity and maximal responses to ACh (n=5, *p<0.05).
representative traces (A) and response curves (B) demonstrating a rightward shift in the acetylcholine (ACh) concentration-response curve following I/R. The pD₂ for ACh was 7.2 ± 0.1 (control) and 6.6±0.1 (I/R, n=5, p<0.005). Sensitivity to ACh was restored by SP (10 μM) with a pD₂ of 7.3±0.1 (n=5, p<0.005, I/R+SP vs. I/R).

### 3.4.2 Endothelium-independent vasomotor responses

Vasodilator responses to SNP were also reduced after I/R (Figure 3.2A, B). SP was not able to reverse this impairment. The maximal responses elicited by SNP in control, I/R, and I/R+SP were 85.7 ± 5.1%, 68.9 ± 3.2%, 67.9 ± 8.3%, respectively (n=4, p<0.01, control vs. I/R and I/R+SP). Likewise, SP (10 μM) failed to restore I/R-induced impairment of isoproterenol-mediated vasodilation (Figure 3.3A, B). The maximal responses elicited by isoproterenol in control, I/R, and I/R+SP were 93.2 ± 1.3%, 76.3 ± 2.6%, 75.5 ± 3.0%, respectively (n=4, p<0.01, control vs. I/R and I/R+SP). Vasoconstriction elicited by KCl demonstrated no significant differences in control (Figure 3.4), I/R and I/R+SP groups (maximal responses, which were produced at 66 mM KCl, were 57.8 ± 2.3%, 57.3 ± 5.4%, and 58.6 ± 5.7%, respectively).

### 3.4.3 Post-ischemic ROS production

DHE staining measures ROS production and is primarily reactive with superoxide. DHE staining was performed to assess superoxide production in arterial walls following I/R (Figure 3.5 and 3.6). Pre-treatment with 10 μM of SP caused a significant reduction in the
Figure 3.2. SP does not restore post-ischemic endothelium-independent vasodilation produced by sodium nitroprusside (SNP).

(A) Traces showing impaired vasodilation to SNP in I/R and I/R+SP groups. (B) Concentration-response curves to SNP (n=4) showing that SP does not improve endothelium-independent vasodilation.
Figure 3.3 SP does not restore post-ischemic endothelium-independent vasodilation produced by isoproterenol.
(A) Traces showing impaired vasodilation to isoproterenol in I/R and I/R + SP groups. (B) Concentration-response curves to isoproterenol (n=5), showing that SP does not improve endothelium-independent vasodilation.
Figure 3.4 Constrictor responses to KCl were unaffected by SP pre-treatment.
Response curves to KCl indicating that vasoconstriction was unaffected by I/R and SP had no added effect (n=5, p>0.95).
Figure 3.5 SP reduces ROS production following I/R.
Representative traces demonstrating that SP reduces relative fluorescent intensity of dihydroethidium staining following I/R.
Figure 3.6 SP reduces ROS production following I/R.
Mean ± SE of the fluorescent intensity of dihydroethidium staining following I/R in arterial walls (control n=6, SP n=5, * p<0.005).
Figure 3.7 Peroxynitrite measurements in post-ischemic coronary effluent.
Dityrosine conversion from L-tyrosine as measured by absorbance at 320 nm. L-tyrosine was added to the perfusate during Langendorff-perfusion induced I/R.
relative intensity of DHE staining to 40.2 ± 6.4% of untreated hearts 100.0 ± 5.9% (n=5, p<0.005, vs. control n=6).

3.5 Discussion

Increased intracellular calcium levels following I/R induces the activation of phospholipase A2 (PLA2) and the subsequent hydrolysis of AA from membrane phospholipids. AA is metabolized by CYP 2C9 into EETs, however, superoxide is also generated during this reaction cycle. Superoxide readily reacts with NO• to produce ONOO- and it has been proposed that NO• scavenging due to CYP 2C9-mediated superoxide production leads a reduction of NO• bioavailability that impairs endothelium-dependent vascular function in atherosclerosis. Inhibition of iPLA2 or PLA2 protects against I/R, further supporting the link between AA and superoxide generation. Thus, given that SP restores endothelium-dependent, NO-mediated vasodilation in patients with coronary artery disease in combination with our recent findings that SP significantly reduces infarct size caused by I/R, we hypothesized that SP attenuates post-ischemic endothelial dysfunction.

Although the rat equivalent of CYP 2C9 has not been fully characterized, a CYP 2C9-like isozyme that shares immunoreactivity and is selectively inhibited by SP has been detected in rat arteries. CYP 2C6 is a putative rat homologue for human CYP 2C9. In support of this, SP has been shown to inhibit rat CYP 2C6 but not other members of the rat CYP 2C family.
Here we showed that SP reduced the I/R-induced loss of endothelium-dependent, NO•-mediated vasodilation to ACh. However, SP was not able to improve the marked post-ischemic impairment of endothelium-independent vasodilation (SNP, isoproterenol). Fichtlsherer et al.² also observed a similar trend where SP had no effect on impaired responses to SNP in patients with coronary artery disease versus normal controls. Impairment of endothelium-independent vasodilation was not due to a loss in smooth muscle cell contractility as responses to KCl were similar in all three treatment groups. These results indicate that SP is acting through an endothelium-specific mechanism. SP’s inability to restore responses to either SNP or isoproterenol, indicate that it is not acting by altering guanylate or adenylate cyclase activities, but more likely acting to restore endothelium NO• bioavailability.

Our data suggests that inhibition of CYP 2C6/9 increases vasodilation through a reduction in superoxide formation and consequent increase in NO• bioavailability. Figure 3.8 shows a diagram of the proposed mechanism. Chloramphenicol, a potent of inhibitor of CYP 2C6/9, has previously been shown to reduce superoxide production in the heart following I/R. However, it was unclear what effect CYP 2C6/9 inhibition had on post-ischemic vessel wall ROS production. To examine this question, superoxide formation was assessed by staining with DHE following I/R with or without SP assessed. DHE is converted to ethidium in the presence of ROS and is most highly reactive with superoxide. Consequent ethidium staining is visible by fluorescent microscopy. DHE conversion was quantified in the area around vascular walls. There was a significant reduction (~60%) of superoxide in vessels of hearts pretreated with SP. These results indicate that CYP 2C9 contributes to I/R-induced vascular
Figure 3.8 Proposed mechanism of CYP 2C induced impaired post-ischemic vasodilation.
Upon I/R, $[\text{Ca}^{2+}]_c$ increases activating phospholipase A$_2$ hydrolysis of arachidonic acid from phospholipid membranes. AA is metabolized by CYP 2C leading to the production of EETs and 20-HETE and $\text{O}_2^-$. $\text{O}_2^-$ then reacts with NO• forming ONOO- and reducing the bioavailability of NO• and its ability to induce vasodilation. ONOO- can also inhibit vasodilation.
superoxide production and supports the hypothesis that CYP 2C9 mediates post-ischemic vascular dysfunction by reducing NO• bioavailability.

SP has previously been shown to have no effect on complex I, II, and IV of mitochondrial respiration and does not reduce superoxide generated via NADPH oxidase or xanthine oxidase.\textsuperscript{1,3} Therefore, SP is not acting as a general antioxidant and is likely acting through specific inhibition of CYP 2C6/9; a known producer of superoxide. Inhibition of ROS production following I/R has been examined using several experimental models that employ superoxide dismutase and antioxidants. Although these studies have demonstrated the role of ROS in I/R they have not resulted in the development of effective treatments to alleviate I/R injury. SP presents a promising alternative strategy at reducing ischemic injury as we have shown that it significantly decreases superoxide production and improves vascular function. Treatment with SP is particularly promising in the context of cardiac surgical procedures such as cardiac transplantation, balloon angioplasty and coronary bypass where I/R is predictable and SP could be administered prior to the ischemic period.

In summary, we report novel findings indicating that that i) I/R impairs both endothelium-dependent and independent vasodilation, ii) SP selectively restores post-ischemic endothelium-dependent, NO-mediated vasodilation and iii) SP reduces I/R-induced superoxide production. Our study indicates that SP confers a protective effect in post-ischemic vascular dysfunction through a reduction of CYP 2C6/9-mediated superoxide production. Thus, CYP 2C9 is a potentially important therapeutic target for patients with ischemic heart disease and those undergoing surgical procedure where I/R-injury is a factor.
3.6 Bibliography


Chapter 4: Cytochrome p450 2C Contributes to Cardiac Allograft Vasculopathy

4.1 Introduction

More than 3,000 heart transplants are performed worldwide annually. Current immnosuppressive regimens are very effective in preventing acute rejection. Unfortunately, chronic rejection associated with CAV remains a major hurdle to long-term graft survival of all vascularized organ transplants. CAV is an accelerated and diffuse form of arteriosclerosis that can be detected in up to 75% of heart transplant recipients following the first year of transplantation. Although immunological mechanisms clearly play an important role in the pathogenesis of CAV, non-immunological mechanisms, such as peri-transplant I/R injury, also contribute via direct damage or indirectly through cross-talk with immune responses associated with this type of vasculopathy.

The transplant organ is vulnerable to I/R injury induced by graft ischemia time, quality of graft preservation during transport, hemodynamic status of the donor, catecholamines used for inotropic support, and reperfusion itself. Compelling evidence supports a molecular and cellular basis for a causal relationship between I/R injury during transplantation and the onset and progression of CAV. I/R injury to endothelial cells may provide the initial trigger for atherogenesis by stimulating platelet adhesion, release of growth factors, upregulation of MHC Class I and II expression, release of donor antigens, expression of adhesion molecules, and proliferation of vascular smooth muscle cells. (Reviewed

Thus, attenuation of I/R injury would be of great benefit to transplant recipients not only through the inhibition of direct cellular injury, but also indirectly through the aforementioned factors that influence the allo-immune response. Several experimental models using superoxide dismutase and antioxidants have demonstrated the importance of ROS in the pathophysiology of I/R injury; however, the development of effective treatments to alleviate reperfusion injury remains elusive.

CYPs, as described in section 1.4, are membrane-bound heme-containing terminal oxidases that exist in a multi-enzyme system that includes a FAD/FMN-containing NADPH cytochrome p450 reductase and cytochrome b5. The CYP superfamily is responsible for the oxidation, peroxidation and/or reduction of vitamins, steroids, cholesterol, xenobiotics and the majority of cardiovascular drugs in an oxygen and NADPH-dependent manner. Although the vast majority of CYP are found in hepatic tissues, other CYP have been shown in recent years to be expressed predominantly in extra-hepatic tissues such as the heart, blood vessels, gut, kidney and lung. The role of CYP in cardiovascular disease is poorly understood, increasing evidence suggests that these enzymes play a role in the pathogenesis of a number of cardiovascular diseases. Previously, we discovered that the rat equivalent of CYP 2C9 makes a significant contribution to superoxide generation and cell death associated with I/R injury. Recently, we demonstrated that the CYP 2C inhibitor sulfaphenazole (SP) increases endothelium dependent vasodilation and decreases vascular superoxide production following ischemia and reperfusion.
4.2 Aim

CYP 2C enzymes contribute to post-ischemic vascular dysfunction and cell death. As ischemic injury is thought to contribute to CAV, we hypothesized that CYP 2C will contribute to the development of CAV. The aim of this study was to investigate whether CYP 2C inhibition during the peri-transplant period would reduce post-transplant oxidative damage and vascular remodelling associated with chronic cardiac rejection.

4.3 Materials and Methods

4.3.1 Heterotopic heart transplantation

All protocols were designed in accordance with the guidelines, and approved by, the animal care committee of the University of British Columbia. A copy of the animal care certificate for heterotopic heart transplantation is provided in Appendix I. Minor histocompatibility antigen-mismatched rat heterotopic heart transplants were performed between male Lewis donor (RT1\textsuperscript{1}, 260-330 g) and Fisher 344 recipient (RT1\textsuperscript{iv}, 230-280 g) rats. All rats were purchased from Charles River Laboratories (Wilmington, MA) and cardiac transplantation was performed as previously described.\textsuperscript{1} Donors and recipients were treated with 5 mg/kg SP intraperitoneally (IP, Clinalpha, Laufelfingen, Switzerland), or vehicle control 1 hr prior to surgery. Donors were anaesthetized with xylazine (10 mg/kg)/ketamine (120 mg/kg), IP. The inferior vena cavae (IVC) were isolated, slowly perfused with heparinized saline and clamped distally. The right and left superior vena cavae (SVC) were then ligated. The ascending aortas were cut below the brachiocephalic artery and the main
pulmonary arteries were cut proximal to their bifurcations. They were flushed with heparinized saline. Pulmonary veins were ligated together and the donor hearts were gently detached and placed in ice-cold heparinized saline. Recipients were anaesthetized with isofluorane (4% induction, 2% maintenance) Anastomoses were performed between the ascending aortas of donor hearts and the abdominal aortas and between the pulmonary arteries and the inferior vena cavae of the recipient animals. Buprenorphine was administered subcutaneously at 0.01 mg/kg immediately following surgery. A copy of the standard operating procedure outlining the detailed protocol for this operation is provided in Appendix II.

4.3.2 Tissue collection

At 4, 7 and 30 days post-transplantation the animals received heparin (50 U/kg, IP) and were anaesthetized with a combination of ketamine hydrochloride (120 mg/kg) and xylazine hydrochloride (10 mg/kg). Thoracic and abdominal cavities were opened and transplanted hearts were assessed for heartbeat. The circulatory system was flushed by injecting 25 ml of Ringer’s buffer at 80 mmHg into the right ventricle and cutting a small incision in the right atria to allow fluids to drain. Rats were then perfusion fixed by replacing Ringer’s with 4% formalin 80 mmHg and allowing it to circulate as above. The native and transplanted hearts were then removed rapidly, and transverse sections immersion fixed in 10% formalin for 24 h before being embedded in paraffin.
4.3.3 Histological staining and immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and Movat’s pentachrome stain using standard methods. IHC was performed on formalin-fixed, paraffin embedded ventricular transverse sections. Briefly, sections were deparaffinized by baking in a 60°C oven for 1 h followed by serial rehydration by immersion in 100% xylene (3X 5 min), 100% ethanol (2X 5 min), 90% ethanol (3 min), 70% ethanol (3 min) and Tris-buffered saline (TBS, pH 7.4, 2X 5 min). Antigen retrieval was performed by boiling sections in citrate-citric acid buffer (pH 6.0) for 15 min, allowing sections to reach room temperature and washing 2X with TBS. Exogenous phosphates were quenched by incubation in 10% H₂O₂ in TBS for 10 min and washing 2x with TBS. Sections were blocked by incubation of sections with blocking buffer (10% normal serum of the species the secondary antibody was raised in) for 30 min. Sections were incubated in primary antibodies overnight in blocking buffer at 4°C. Primary antibodies utilized were: 1:50 monoclonal α-rat Ki-67 clone M1B-5 and 1:100 polyclonal α-human CD3 (Dako Canada, Missisagua, ON), 1:100 monoclonal α-rat CD8 MRC OX-8 (Genetex, San Antonio, TX), and 1:800 polyclonal α-Von Willebrand Factor (Abcam Inc, Cambridge, MA). Sections were then washed 3X in TBS + 0.01% Tween 20 (TBST). Secondary detection was performed for 1 hr at room temperature with 1:350 biotinylated anti-secondary antibodies (Vector Laboratories, Burlingame, CA) in blocking buffer supplemented with 3% normal rat serum. The addition of 3% normal rat serum was required to reduce cross reactivity with rat IgG. Staining was visualized using the ABC kit (Vector Laboratories) followed by detection with the chromagens Nova Red or diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories,
Burlingame, CA), and nuclei were counterstained with hematoxylin. Slides were coverslipped using Aqua-mount® aqueous mountant (Lerner Laboratories, Pittsburgh, PA).

4.3.4 Histological assessment and quantification

Four micrometer sections were stained with H&E or Movat’s pentachrome stain. H&E-stained sections of 30 day post-transplant sections were scored on a 0–5 scale (five rats per group, n=7 arteries/rat) for general, focal, sub-epicardial, sub-endocardial, and perivascular immune infiltration. Luminal narrowing in all visible medium to large size coronary arteries (30 days post-transplantation, n = 5 rats per group, 3 sections per rat, 7 arteries per section) was evaluated on Movat’s pentachrome stained sections. Briefly, *Image-Pro Plus™* (MediaCybernetics, Silver Spring, MD) was used to quantify intimal and luminal areas, and percent luminal narrowing was calculated as the area of the lumen as a percentage of the combined area of the lumen and the intima. For assessments of immune infiltration the vascular wall area was defined as the region from the lumen to the outside of the medial smooth muscle layer and the perivascular space (PVS) was defined as the region between the medial smooth muscle layer and the myocardium. These regions were traced and quantified using *Image-Pro Plus™*.

4.3.5 Luminex analysis

Blood was collected in accordance with the guidelines, and approved by, the animal care committee of the University of British Columbia. A copy of the animal care certificate
for blood collection is provided in Appendix I. Tail vein blood was collected into heparinized tubes 1 day prior to transplant and at days 1, 3, 5 and 7 post-transplant. Serum was isolated by allowing blood to clot for 30 min and centrifuging for 15 min at 1000 g. Samples were analyzed using the rat cytokine/chemokine premixed LINCOplex 14-plex premix bead kit as per manufacturer’s recommendations (LINCO Research, St. Charles, MO). Briefly, serum samples were diluted 1:5 in LINCO Serum Matrix™ and standards were diluted in four-times serial dilutions to 1:4096. The assay filter plate was blocked using LINCO Assay buffer for 10 min at room temperature and was fluid was removed by gentle vacuum filtration. Diluted samples, standards and controls were incubated with premixed cytokine/chemokine detection beads overnight with agitation at 4°C at which time samples were drained by gentle vacuum filtration and washed two times with LINCO washing buffer. Plates were developed by the addition of the detection antibody cocktail and the streptavidin-phycocerythrin detection solution. Upon gentle vacuum filtration, plates were washed two times with wash buffer and bead-antibody complexes were solubilized in sheath fluid for analysis. Plates were analyzed on the Luminex FlowMetrix System (Qiagen, Mississauga, ON). Sample parameters required a minimum of 50 events per bead. Samples were run in duplicate and compared to an 8-point standard curve developed using a 5-parameter logistic fit graph.

4.3.6 8-Isoprostane measurements

Free 8-isoprostane measurements were performed using the 8-isoprostane EIA Kit on left ventricular blood samples collected at sacrifice on day 4 and 7 post transplantation as per manufacturer’s recommendations (Cayman Chemical Company, Ann Arbor, MI). Briefly,
samples were collected into tubes containing EDTA and 0.005% BHT (butylated hydroxytoluene) was added to prevent further production of 8-isoprostanes in the samples. Samples were stored at -80°C until time of analysis. Samples were then purified using 8-isoprostane affinity sorbent purification kit (Cayman). An equal volume of 15% KOH was added and samples were incubated at 40°C for 60 min. Four volumes of ethanol containing 0.01% BHT was added and samples were vortexed, incubated for 5 min on ice and centrifuged for 10 min at 1500 g. Supernatants were collected and ethanol was evaporated under nitrogen to less than 10% vol/vol. Purification required neutralizing the samples to pH 7.4 by addition of 2 volumes of 1 M KH₂PO₄ and 1 volume of eicosanoid affinity column buffer. Samples were then added to pre-equilibrated 8-isoprostane affinity sorbent and allowed to bind for 60 min at room temperature, centrifuged briefly at 1500 g and supernatant was discarded. Sorbent was then washed twice with ultrapure water and 8-isoprostanes were retrieved by incubating sorbent in ethanol Elution Solution with vortexing. Samples were dehydrated using a speed vacuum system and resuspended in EIA buffer for ELISA. Purified samples and 8-isoprostane standards were then incubated with EIA Buffer, AChE tracer and 8-isoprostane antiserum on the EIA plate for 18 h at room temperature. EIA plates were developed by the addition of Ellman’s reagent and 8-isoprostane tracer followed by incubation for 90 min at room temperature. Samples were measured via absorbance readings at 412 nm on the Tecan GENios Rainbow absorbance plate reader (Tecan, San Jose, CA).
4.3.7 Statistical analysis

Repeated measures general linear model analysis was performed for Luminex results using SPSS Statistical Software (Chicago, IL). Wilks’ Lambda multivariate tests were utilized to assess significance. For other assays, statistical differences between two groups were determined using a Student's t-test. For both tests, a p-value (alpha error) of 0.05 or less was considered significant.

4.4 Results

The heterotopic heart transplant model was utilized to assess the contribution of CYP 2C to peri-transplant I/R injury and the development CAV. Donor hearts were transplanted from Lewis donor rats into the abdominal cavities of Fisher 344 recipients by suturing the ascending aorta of donor hearts and the recipients abdominal aorta and the donor’s pulmonary artery to the recipients inferior vena cava. This represents a minor histocompatibility mismatch allowing us to assess vascular changes associated with chronic rejection.

4.4.1 Post-surgical morbidity and mortality

Donor and recipient rats were treated with 5 mg/kg of SP or saline control 1 h prior to surgery in order to assess the contribution of CYP 2C to cardiac rejection. SP is a specific inhibitor of CYP 2C9 in humans and is a potent inhibitor of CYP 2C6, a putative homologue
of human CYP 2C9, in rats. Surgical and post-surgical morbidity and mortality rates were similar and very low (i.e. <5%) in both SP and control groups. Post-surgical recovery was also similar in both groups with recipients regaining their pre-surgical weigh by 9.2 ± 2.9 days in the SP group versus 10.3 ± 4.0 days in the control group (Figure 4.1). Transplant recipients were euthanized and organs were harvested at days 4, 7 and 30 post-transplant. At harvesting, all transplanted hearts had palpable heart beats.

4.4.2 Assessment of CYP 2C6 expression in rat heart cross-sections

Initially, immunohistochemical studies were performed to confirm expression of CYP 2C6 in the transplanted hearts. Results demonstrate positive staining for CYP 2C6 in both the myocardium and vasculature of transplanted hearts (Figure 4.2).

4.4.3 CYP 2C contributes to luminal narrowing in rat heterotopic heart transplants

Transplanted hearts harvested at day 30 were then utilized to assess the development of CAV by measuring the degree of luminal narrowing in the large coronary blood vessels (Figure 4.3). Pre-treatment with SP at the time of transplantation resulted in a dramatic decrease in luminal narrowing by day 30 (12.1 ± 4.1% vs. 66.2 ± 13.6% for control, p=0.0002; Figure 4.4).

4.4.4 Assessment of general immune infiltration

Thirty day post-transplant hearts were then assessed for characteristics of general immune rejection. H&E-stained sectioned were scored for diffuse, focal, sub-epicardial, sub-endocardial infiltration (Figures 4.5 and 4.6). SP pre-treatment did not result in a statistically
Figure 4.1 SP treatment does not reduce post-transplant weight gain.
Rats receiving heterotopic heart transplants were weighed prior to transplantation and daily for 30-days post-transplant. Both SP and control groups had characteristic weight loss immediately following surgery followed by gradual weight gain. Values expressed as mean ± SD (n=5). There was no difference between treatment groups (p>0.1).
Figure 4.2 CYP 2C6 is expressed in transplanted rat heart blood vessels and myocardium. Paraffin-embedded transplanted hearts from Lewis rats sacrificed 30 days post-surgery were immunohistochemically stained for the presence of CYP 2C6 using Vector NovaRED as a substrate. Primary antibody-absent negative staining controls and CYP 2C6 antibody-positive staining result are shown for transplanted hearts. Scale bar equals 100 μm.
Figure 4.3 SP administration at time of surgery attenuates allograft luminal narrowing. Representative coronary blood vessels in Movat’s pentachrome stained coronary cross sections from rat heterotopic heart transplants 30 days post-transplantation.
Figure 4.4 SP administration at time of surgery attenuates allograft luminal narrowing. Percent luminal narrowing in rat heterotopic heart transplants were performed between Lewis donor and Fisher recipient rats 30 days post-transplantation. Luminal narrowing was measured using Image-Pro Plus as the percent of the area of the lumen divided by the area of the internal elastic lamina. Data are expressed as mean ± SD (n= 5 hearts, 3 sections/heart, 7 vessels/section, * p<0.005).
Figure 4.5 Histological features of diffuse and focal myocardial infiltration. H&E stained myocardial sections from 30-day post surgical rat heterotopic heart transplants. Representative images showing (A) normal myocardium, (B) diffuse myocardial infiltration and (C) focal immune myocardial infiltration.
Figure 4.6 Histological features of epicardial and endocardial immune infiltration. Representative H&E-stained epicardial and endocardial sections from native and 30-day post surgical heterotopic transplanted hearts. Transplanted hearts demonstrate subepicardial and subendocardial immune infiltration.
significant decrease in any of these parameters (Figure 4.7). A trend was observed towards a
decrease in both the number of blood vessels with perivascular infiltration and the severity of
the infiltration but, again, these findings were not significant \( (p>0.1; \text{ Figure 4.8}) \). Perivascular infiltration was not associated with luminal narrowing with some vessels
demonstrating severe perivascular infiltration with minimal luminal narrowing and others
demonstrating luminal narrowing with minimal infiltration (Figure 4.9).

4.4.5 CYP 2C does not contribute to T-lymphocyte infiltration

T-lymphocyte infiltration was further assessed by IHC staining and quantification in
both the vascular wall and the perivascular space (PVS) for general CD3\(^+\) T-lymphocytes
(Figure 4.10) and for CD8\(^+\)-positive cytotoxic T-lymphocytes (Figure 4.11). SP pre-
treatment did not alter the degree of CD3\(^+\) or CD8\(^+\) lymphocyte infiltration in either the
vascular wall (CD3\(^+\): 3.4 ± 3.6 vs. 2.0 ± 1.2 cells/100 \( \mu \text{m}^2 \) for control, \( p=0.49 \); CD8\(^+\): 2.5 ±
1.8 vs. 1.3 ± 1.0 cells/100 \( \mu \text{m}^2 \) for control, \( p=0.23 \)) or the PVS (CD3\(^+\): 8.4 ± 3.4 vs. 6.5 ±
2.9 cells/100 \( \mu \text{m}^2 \) for control, \( p=0.42 \); CD8\(^+\): 6.6 ± 1.4 vs. 6.4 ± 2.2 cells/100 \( \mu \text{m}^2 \) for control,
\( p=0.92 \)).

4.4.6 CYP 2C does not significantly alter post-transplant apoptosis

Early post-transplant apoptotic events were assessed by measuring TUNEL positivity in the
myocardium, endothelium and smooth muscle layers of transplant sections isolated 4 days
post-transplantation. TUNEL positivity was lower in SP treated rats in all regions assayed
however these differences were not statistically significant (Figure 4.12). Endothelia were
stained with Von Willebrand Factor and no loss of endothelial cells was detected (Figure
4.13).
Figure 4.7 CYP 2C does not contribute to general myocardial immune infiltration. Immune infiltration was scored from absent to severe in a blinded fashion in the cardiac muscle. Data are represented as mean ± SD. There was no significant difference between SP and control groups in any immune infiltration category.
Figure 4.8 CYP 2C does not contribute to perivascular immune infiltration.
Perivascular immune infiltration was assessed in rat heterotopic heart transplants. (A) Immune infiltration was scored from absent to severe in a blinded fashion in the cardiac muscle. (B) The percent of blood vessels with immune infiltration is shown. Percent luminal narrowing expressed as mean ± SD (n= 5 hearts, 7 vessels/heart) p>0.1.
Figure 4.9 Perivascular immune infiltration in the absence of luminal narrowing. H&E-stained, paraffin-embedded sections from 30-day post-transplant rats showing coronary blood vessels. (A) Representative image of native blood vessel. (B) Representative image of CAV associated luminal narrowing in control groups. This blood vessel shows significant luminal narrowing with only moderate immune infiltration. (C) Representative image of blood vessel showing severe perivascular immune infiltration in the absence of luminal narrowing. Scale bar equals 100 μm.
Figure 4.10 SP treatment does not alter CD3+ lymphocyte infiltration in the vasculature. Paraffin-embedded sections were immuno-stained for CD3+ lymphocytes. The number of CD3+ cells was quantified using Image-Pro Plus as the number of positive stained cells per area of the vascular wall or the PVS. PVS was defined as the area from the outside of the defined smooth muscle layer to the commencement of the myocardium. Data are represented as the mean ± SD of n=5 rats/group, n=7 vessels/rat.
Figure 4.11 SP treatment does not alter CD8$^+$ lymphocyte infiltration in the vasculature. Paraffin-embedded sections were immuno-stained for CD8$^+$ lymphocytes. The number of CD8$^+$ cells was quantified using Image-Pro Plus as the number of positive stained cells per area of the vascular wall or the PVS. PVS was defined as the area from the outside of the defined smooth muscle layer to the commencement of the myocardium. Data are represented as the mean ± SD of n=5 rats/group, n=7 vessels/rat.
Figure 4.12 CYP 2C does not significantly contribute to post-transplant apoptosis. Paraffin-embedded section from heterotopically transplanted hearts 4 days post-transplant were assessed by TUNEL staining. The percentage of positively stained nuclei relative to total nuclei relative to endothelial, smooth muscle and myocardial cells was measured using *Image-Pro Plus*. No statistically significant difference between untreated and SP treated transplanted hearts was observed. For endothelial and smooth muscle cell counts were performed on 10 blood vessels/heart, 5 hearts/group; and for myocardial measurements were performed on 10 fields of view/heart, and 5 hearts/group. Data are expressed as the mean ± SD.
Figure 4.13 CYP 2C-inhibition has an insignificant effect on EC loss at day 4 post-transplant.
Paraffin-embedded section from heterotopically transplanted hearts 4 days post-transplant were assessed by immuno-staining with the endothelial marker von Willebrand Factor and DAB staining. Endothelial loss was not observed in control hearts (A) or those treated with SP (B).
4.4.7 CYP 2C contributes to SMC proliferation following transplantation

SMC proliferation was assessed at day 4 following transplantation by counting cells with Ki-67 immunostaining. SP pre-treatment decreased the number of Ki-67 positive SMC (3.3 ± 3.3% for SP vs. 7.2 ± 2.3% for control, p=0.006). Figure 4.14 shows quantification of Ki-67 staining (A) and representative positive staining (B).

4.4.8 Peripheral cytokine and chemokine levels following transplantation

Serum which was isolated at 1 day prior to transplantation and at days 1, 3, 5 and 7 post-transplantation was utilized to analyze cytokine levels in the systemic circulation via the Luminex multiplex assays. Serum levels of granulocyte macrophage colony-stimulating factor (GM-CSF), growth-related oncogene (GRO/KC), interferon gamma (IFN-γ), monocyte chemotactic protein 1 (MCP-1), and interleukin (IL-) 1α, 1β, 2, 10, and 18 were assessed for alterations with time and with time by group. We observed a high level of variability between samples. Quantified cytokine levels are shown in Table 4.1 and a summary table to statistical analyses is shown in Table 4.2. GRO/KC and IL-1α, 1β, 2, 10 and 18 did not show any significant alterations with time or by group (p>0.1). MCP-1 levels showed a non-significant increase following transplantation (p=0.064) which was similar in both treated and non-treated groups (p=0.755). GM-CSF showed an effect with time (p=0.004), peaking at day 3 post-transplant but there was no significant difference with SP treatment (p=0.239). INF-γ levels (Figure 4.15) increased following transplantation in the control group (p=0.023) however this trend was significantly reduced in the SP treated rats (p=0.028).
Figure 4.14 CYP 2C contributes to SMC proliferation.
Paraffin-embedded section from heterotopically transplanted hearts 4 days post-transplant were assessed by immuno-staining with the proliferation marker Ki-67 and DAB staining. (A) Quantification was performed by calculating the number of Ki-67 positive nuclei of the total number of nuclei in the defined smooth muscle cell layer. Data are represented as mean ± SD of n=5 rats/group, 7 vessels/rat. * = p<0.05. (B) A representative image of positive Ki-67 staining (arrows). Scale bar represent 100 μm.
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Table 4.1 Peripheral cytokine and chemokine levels following heterotopic heart transplantation in SP treated and control rats.
Serum was isolated from tail blood vein collected 1 day before transplantation and on days 1, 3, 5 and 7 post-transplant. Samples were assessed for cytokine and chemokine levels using the Luminex assay. Data are shown as mean ± SD in pg/ml for n=5 rats per group.
Table 4.2 Repeated measures analysis of peripheral cytokine and chemokine levels following heterotopic heart transplantation in SP treated and control rats.
Serum was isolated from tail blood vein collected 1 day before transplantation and on days 1, 3, 5 and 7 post-transplant. Samples were assessed for cytokine and chemokine levels using the Luminex assay. Data were analyzed for significant differences utilizing repeated measures multivariate analysis. P-values were calculated using the Wilks’ Lambda test. (*=p< 0.05)
Figure 4.15 CYP 2C contributes to peripheral IFN-γ levels post-transplantation. Serum was isolated from tail blood vein collected 1 day before transplantation and on days 1, 3, 5 and 7 post-transplant. Samples were assessed for IFN-γ levels (pg/ml) using the Luminex assay. Data were analyzed for significant differences utilizing the repeated measures multivariate analysis. P-values were calculated using the Wilks’ Lambda test. IFN-γ levels increase with time (p=0.023) and are significantly reduced in the SP treatment group compared to control (p=0.028).
4.4.9 CYP 2C contributes to serum 8-isoprostane levels

Serum isolated at sacrifice (day 4 or 7) was analyzed by EIA to assess oxidative stress via free 8-isoprostane levels. Results, shown in Figure 4.16, demonstrated a peak in 8-isoprostane levels at day 4 in the control group compared to the SP treatment group (2746 ± 367 pg/ml for control vs. 1040 ± 181 pg/ml for SP, mean ± SEM, p=0.026). These levels were reduced by day 7 and were similar in both groups (466 ± 62 for control vs. 543 ± 197 pg/ml for SP, mean ± SEM, p=0.389).

4.5 Discussion

The findings of this study demonstrate, for the first time, that CYP 2C contributes to peri-transplant ischemic injury. We have found that inhibition of CYP 2C by SP reduces early signs of ischemic injury including oxidative stress and a trend towards a decrease in apoptosis. This finding is consistent with our earlier studies that showed a decrease in vascular and myocardial superoxide production following I/R injury following pre-treatment with SP. CYPs have been previously been shown to significantly contribute to the cellular production of ROS such as $O_2^{-}$, hydrogen peroxide and hydroxyl radicals during the CYP reaction cycle when electrons for the reduction of the central heme iron are transferred to the activated bound oxygen molecule.

We also found a reduction in early (day 4) smooth muscle cell proliferation, as indicated by Ki-67 staining, in rats pre-treated with SP. This corresponds to a significant
Figure 4.16 CYP 2C contributes to post-transplant serum free 8-isoprostane levels. Serum was isolated from left ventricular blood at days 4 and 7 post-transplantation. Serum was analyzed for free 8-isoprostane by EIA. Data represent mean ± SD for n=5 rats/group, samples were run in triplicate.
decrease in intimal thickening and luminal narrowing by day 30 post-transplantation. It was previously hypothesized that SP increases the bioavailability of nitric oxide (NO) by reducing its reaction with superoxide. In support of this hypothesis, SP is able to increase NO-mediated vasodilation following ischemia/reperfusion injury. Studies by Fleming’s group demonstrated that SP enhances endothelium-dependent vasodilator responses in patients with manifest coronary artery disease. The observed effect was suggested to be due to the increased bioavailability of NO as a consequence of reduced CYP 2C9-mediated superoxide generation and ONOO formation in endothelial cells. Both reduced NO and elevated ONOO have important implications in the pathogenesis of CAV and smooth muscle cell proliferation. Endothelial NO is known to inhibit smooth muscle cell proliferation. We also observed an increase in IFN-γ levels following transplantation in the control group and that these levels were significantly reduced in the SP treatment group. IFN-γ has been shown to induce smooth muscle cell proliferation in a phosphoinositol 3-kinase dependent manner.

In summary, CYP 2C inhibition during the peri-transplant period prevented development of CAV by inhibiting early SMC proliferation and intimal hyperplasia. This affect could be a result of reduced post-ischemic oxidative damage, contributing to increased NO bioavailability and/or prevention of IFN-γ production. As described above, both NO- and IFN-γ have previously been shown to contribute to SMC proliferation. Future studies will be geared towards further elucidating the mechanism of CYP 2C-mediated post-ischemic endothelial dysfunction.
4.6 Bibliography


Chapter 5: Cytochrome p450 2C9 in Vascular Cell Death and Oxidative Stress

5.1 Introduction

CYPs are membrane-bound heme-containing terminal oxidases that exist in a multi-enzyme system that includes a FAD/FMN-containing NADPH cytochrome p450 reductase and cytochrome b$_5$. The CYP superfamily is responsible for the oxidation, peroxidation and/or reduction of vitamins, steroids, cholesterol, xenobiotics and the majority of cardiovascular drugs in an oxygen and NADPH-dependent manner. CYP 2C9 is a monoxygenase that is strongly expressed in the liver and small intestine but is also expressed in the heart and the vasculature. CYP 2C9 is an epoxygenase and is involved in the metabolism of arachidonic acid (AA) into epoxyecosotrienoic acids (EETs). Specifically, CYP 2C9 produces 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET which are involved in NO-independent vasodilation.

During ischemia increased intracellular calcium levels induces the activation of phospholipase A2 (PLA$_2$) and the subsequent hydrolysis of AA from membrane phospholipids. AA metabolism is further increased during reperfusion. AA is metabolized by three major pathways; the cyclooxygenase (COX) pathway, the lipoxygenase (LOX) pathway, and the CYP epoxygenase pathway, described in detail in section 1.5. AA metabolism during I/R has been implicated as a key contributor in the progression of ischemic injury as inhibition of PLA$_2$ or inducible PLA$_2$ (iPLA$_2$) protects against I/R.

4 A version of this chapter is in preparation for publication. Arwen L. Hunter, Paul Hiebert, David J. Granville. Cytochrome p450 2C9 expression increases oxidative stress and cell death following hypoxia and reoxygenation in human umbilical venous endothelial cells.
However, several AA metabolites, including cyclooxygenase-2 (COX-2)-derived prostacyclin, are known to be cardioprotective. CYP 2C are the primary epoxygenases involved in AA metabolism by the third pathway. As previously described, CYP 2C also make a significant contribution to the cellular production of ROS such as $O_2^-$, hydrogen peroxide and hydroxyl radicals during AA metabolism.

Previous studies by our group and others have shown that CYP 2C9, or the rodent or rabbit equivalent, plays a role in vascular function in coronary flow following I/R injury, vasomotion in patients with coronary artery disease and post-ischemic vascular function (see Chapter 3). In recent experiments, described in Chapter 4, we found that CYP 2Cs also contribute to post-transplant oxidative stress, smooth muscle proliferation and CAV development. As AA can be metabolized by one of three possible mechanisms, it is logical that if one of these pathways were blocked, that this may result in a shift towards the other 2 pathways and increased activity of these pathways.

5.2 Aim

We hypothesized that CYP 2C9 expression would increase EC death and dysfunction following hypoxia and re-oxygenation (H/R). Further, that CYP 2C9 inhibition would reduce these effects whereas COX inhibition would exacerbate them. The aim of this research is to examine the effect of CYP 2C9 in cultured human endothelial and smooth muscle cells in response to H/R. Specifically, we examined the contribution of CYP 2C9 to cell death in
endothelial and smooth muscle cell lines. We also examined the effect of COX-2 inhibition on CYP 2C9-mediated oxidative stress following H/R.

5.3 Materials and Methods

5.3.1 Cell culture

Pooled HUVECs were obtained from Cambrex (Baltimore, MD). HUVECs were cultured in complete endothelial growth medium (EGM: endothelial basal medium supplemented with 0.4% bovine brain extract, 0.1% human endothelial growth factor (hEGF), 0.1% hydrocortisone and 0.1% gentomycin-amphotericin B (GA-1000); Cambrex) plus 5% foetal bovine serum (FBS, Invitrogen). HCASMCs were cultured in complete smooth muscle growth medium (SmGM: smooth muscle basal medium supplemented with 0.1% insulin, 0.5% human foetal growth factor B, 0.1% GA-1000 and 0.1% hEGF; Cambrex) plus 5% FBS.

5.3.2 Cell lysis and Western blotting for CYP 2C9

Cells were washed twice with ice cold PBS and lysed in CellLytic M lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich, Oakville, ON). Protein concentrations were measured using the Bio-Rad protein assay, which is a modified Bradford protein assay (Bio-Rad, Hercules, CA). This assay measures the change in absorbance of Coomassie Brilliant Blue G-250 to 595nm upon binding to basic and aromatic amino acids in proteins. Equal amounts of protein were separated by sodium dodecyl sulphate – polyacrylamide gel
electrophoresis and then transferred to nitrocellulose membranes. Equal volume amounts of purified human liver microsome (HLM) diluted 1:20 in lysis buffer was used as a control for CYP 2C9 expression. After blocking with 2% skim milk, the membranes were incubated with primary antibodies (1:500 anti-human CYP 2C9 (BD Gentest, San Jose, CA) or 1:1000 monoclonal anti-β-actin (AC-74) antibody (Sigma-Aldrich, St. Louis, MO)) overnight at 4°C, followed by incubation for 1 h with 1:4000 IRDye800- or 1:2000 IRDye700–conjugated secondary antibodies (Rockland Inc. Gilbertsville, PA). Protein expression was detected by using the Odyssey Infrared Imaging System from LI-COR Biosciences (Lincoln, NE).

5.3.3 Adenoviral expression of CYP 2C9 in HUVEC

An adenoviral vector expressing CYP 2C9 sense and an adenoviral vector expressing CYP 2C9 antisense, control, were kindly provided by Dr. Ingrid Fleming. Adenoviral infections were carried out by removal of media and addition of a 10:1 virus to cell ratio in low volume media. Cells were incubated with intermittent gentle rocking for 2 h, media levels were restored and cells were incubated overnight to allow for protein expression.

5.3.4 Optimization of hypoxic conditions

Hypoxic conditions were generated in a Coy hypoxic glove box (Coy Laboratories, Grass Lake, MI). This glove box permits control of temperature, humidity, CO₂ and O₂. Conditions were optimized to generate a PO₂ between 10 and 20 mmHg, as observed during cardiac ischemia.\textsuperscript{16-20} The PO₂ of the air and of culture media were assessed under
humidified conditions at 37°C and 5% CO₂. Media was bubbled with chamber air for 20 s prior to measurements. PO₂ was measured using Oxylab PO₂ probes and a tissue oxygenation monitor (Oxford optronix, Oxford, UK).

5.3.5 Cell viability in response to H/R

HUVECs transduced with Ad-CYP 2C9 sense or antisense and HCASMCs were seeded in 96-well plates, grown to 90% confluency. Cells were pre-treated with 10 µM sulfaphenazole (SP, Clinalpha, Laufelfingen, Switzerland) 1 h prior to induction of H/R. Cells were transferred to the hypoxic chamber, media was removed and cells were washed twice in PBS. Basal media bubbled in low oxygen conditions was then added to cells and cells were exposed to 24 h hypoxia in followed by 4 h of re-oxygenation in normoxic conditions, complete media containing 5% FBS. Viability was assessed using the CellTiter96™ AQueous Assay (MTS) (Promega, Madison, WI). MTS is described in section 2.2.6. MTS was protected from light and was added at a 1:5 ratio of MTS: media and the reaction was allowed to proceed for 1 h at 37°C. Samples were measured in triplicate for absorbance at 490 nm on the Tecan GENios Rainbow absorbance plate reader (Tecan, San Jose, CA). Data are shown as the mean ± SD and represent 3 samples per experiment for 4 experiments measured in triplicate.

5.3.6 Measurements of 8-isoprostanate

Free 8-isoprostanate measurements were performed on conditioned medium from cell culture experiments, as per manufacturer’s recommendations (Cayman Chemical Company, Ann Arbor, MI). Cells were treated as described in section 5.3.5 with the sole modification
that 6-well plates were utilized in order to collect sufficient conditioned media for analysis. Also additional treatment groups including treatment with 0.9 μM valdecoxib, and 0.75 mM aspirin were included (Cayman). Upon collection of conditioned media, 0.005% BHT (Butylated hydroxytoluene) was added to prevent further production of 8-isoprostanes in the samples. Samples were stored at -80°C until time of analysis.

For free 8-isoprostane measurements, samples were measured using the EIA kit (Cayman Chemical Company, Ann Arbor, MI) as described in section 4.3.6. Briefly, samples and 8-isoprostane standards were incubated with EIA Buffer, AChE tracer and 8-isoprostane antiserum on the EIA plate for 18 h at room temperature. EIA plates were developed by the addition of Ellman’s reagent and 8-isoprostane tracer followed by incubation for 90 min at room temperature. Samples were measured via absorbance readings at 412 nm on the Tecan GENios Rainbow absorbance plate reader (Tecan, San Jose, CA).

5.4 Results

5.4.1 Native, adenoviral, and H/R-induced expression of CYP 2C9 in HUVECs.

Hypoxic conditions were optimized by measuring the PO2 at varying oxygen concentrations in both the chamber air and bubbled media. Results, Figure 5.1, showed that we were able to obtain a PO2 of approximately 10% by presetting our oxygen concentration to 1%. Measured PO2 levels were comparable in the chamber air and in bubbled media indicating that our method of bubbling media was sufficient to control solution PO2 levels. It should be noted that these measurements were repeated while both increasing and decreasing
Figure 5.1 Relationship between hypoxic chamber oxygen concentration and measured PO\textsubscript{2}.
Oxygen concentrations were controlled using a hypoxic glove box. The PO\textsubscript{2} of the air and of bubbled culture media were assessed under humidified conditions at 37°C and 5% CO\textsubscript{2}. PO\textsubscript{2} was measured using Oxylab PO\textsubscript{2} probes.
the chamber oxygen concentration and with a second PO2 probe. We did not detect any variability in these measurements in our range of interest (PO2 5-15).

Cultured HUVECs were analyzed for native expression of CYP 2C9 by Western blot. HUVECs did not express detectable levels of CYP 2C9. HUVECs transduced with Ad-CYP 2C9 sense but not CYP 2C9 antisense expressed CYP 2C9, Figure 5.2(A). Hypoxia for 24 h followed by 4 h (H24/R4) of re-oxygenation did not induce CYP 2C9 expression in HUVECs, Figure 5.2 (B).

5.4.2 CYP 2C9 expression contributes to post H/R cell death in HUVEC

HUVECs were transduced with Ad-CYP 2C9 sense or Ad-CYP 2C9 antisense were exposed to H24/R4 or normoxic conditions (control). Cell viability was measured using the MTS assay, shown in Figure 5.3. Cells transduced with antisense demonstrated a decrease in viability following H24/R4 to 71.3 ± 0.3% of control. These results are comparable to those we routinely observe in non-transduced cells. Cells transduced with sense CYP 2C9 demonstrated a significantly greater drop in viability to 56.0 ± 1.9% of control (p<0.005 compared to antisense cells). Pre-treatment with SP did not significantly increase viability post-H24/R4 treatment in antisense transduced cells (83.0 ± 13.9% SP treated compared to 71.3 ± 0.3% untreated, p>0.1). Pre-treatment with SP did significantly increase viability post-H24/R4 treatment in sense transduced cells (77.0 ± 18.7% SP treated vs. 56.0 ± 1.9% untreated, p<0.05).
Figure 5.2 CYP 2C9 expression in HUVECs following adenoviral transduction and H/R. (A) Immunoblot for CYP 2C9 in HUVECs transduced with Ad-CYP 2C9 antisense and sense. Human liver microsomes (HLM) are used as a positive control for CYP 2C9 expression. (B) Non-transduced and CYP 2C9 sense transduced cells were exposed to 24 h hypoxia followed by 4 h reperfusion and normoxic time controls. CYP 2C9 expression was analyzed by Western blot.
Figure 5.3 CYP 2C9 expression in HUVECs reduces cell viability following H/R.
Percent viability of HUVECs compared to control of cells exposed to 24 h hypoxia followed by 4 h reperfusion and normoxic time controls. Cells were either untreated or treated with 10 \( \mu \)M SP 1 h prior to the induction of hypoxia. Values are expressed as mean ± SE (n=6, *p<0.05).
5.4.3 SP treatment does not alter SMC viability following H/R.

HCASMCs were pretreated with SP and exposed to H24/R4; the results are shown in Figure 5.4. H/R reduced cell viability in both untreated (86.4 ± 3.3% for H24/R4 vs. 100.0 ± 2.0% normoxic, p<0.005) and SP treated (82.3 ± 3.7% for H24/R4 vs. 94.8 ± 2.4% normoxic, p<0.005) cells. Unlike what was seen in HUVECs, SP treatment caused a slight decrease in viability in both normoxic (5.2%) and H/R exposed (4.2%) cells (p<0.05).

5.4.4 Effect of SP and COX-inhibition on 8-isoprostane production following H/R in CYP 2C9 expressing HUVECs

Levels of 8-isoprostane were measured as an indicator of reactive oxidant production. Conditioned media was collected from Ad-CYP 2C9 sense and antisense transduced cells. Cells were pre-treated with 10 μM SP, a reversible inhibitor of CYP 2C9 inhibitor; 0.9 μM valdecoxib, IC50 (half maximal inhibitory concentration) of the reversible COX-2 inhibitor; and 0.75 mM aspirin, IC50 for irreversible COX-1 inhibition. Cells were then exposed to H24/R4. 8-isoprostane levels were measured using the 8-isoprostane EIA, Figure 5.5. In the absence or presence of each inhibitor Ad-CYP 2C9 sense cells had higher levels of 8-isoprostanes in the condition media than Ad-CYP 2C9 antisense cells. For example, in the absence of inhibitor 8-isoprostane levels were 33.1 ± 15.3 pg/ml for antisense cells and 86.1 ± 9.5 pg/ml for sense cells. CYP 2C9 inhibition with SP was able to reduce 8-isoprostane levels in CYP 2C9 sense transduced cells from 86.1 ± 9.5 pg/ml to 55.4 ± 4.3 pg/ml. Treatment with valdecoxib has the opposing effect with 8-isoprostane levels increasing to 112.3 ± 8.4 pg/ml.
Figure 5.4 SP treatment in HCASMCs does not alter proliferation or cell viability following H/R.

Percent viability of HCASMCs exposed to 24 h hypoxia followed by 4 h reperfusion and normoxic time controls. Cells were either untreated or treated with 10 µM SP 1 h prior to the induction of hypoxia. Values are expressed as mean ± SE (n=6, * p<0.05).
Figure 5.5 CYP 2C increases 8-isoprostane levels.
HUVECs transduced with Ad-CYP 2C9 sense or antisense and HCASMCs were pre-treated with SP, valdecoxib or aspirin 1 h prior to induction of H/R. Cells were exposed to 24 h hypoxia in followed by 4 h of re-oxygenation in normoxic conditions. Free 8-isoprostane measurements were performed on conditioned medium by EIA. Data represents mean ± SD, n=3, * p<0.05).
Aspirin treatment showed little effect on 8-isoprostane levels in CYP 2C9 expressing HUVECs.

5.5 Discussion

Our previous studies have shown an important role for CYP 2C in post-ischemic vascular function and CAV development. In both of these studies it is hypothesized that CYP 2C induced its deleterious effects via oxidative stress on the endothelium. However, this hypothesis had not been directly tested. Thus, we examined the role of CYP 2C9 in cultured endothelial and smooth muscle cells following exposure to H/R, as an in vitro model of I/R.

CYP 2C9 is expressed in human endothelial cells. However, upon culturing, CYP 2C9 levels in endothelial cells rapidly decrease both at the mRNA and protein level. Therefore, our finding that CYP 2C9 is not expressed in cultured HUVECs was not unexpected. In order to examine the role to CYP 2C9 in these cells we employed adenoviral vectors expressing sense and anti-sense (control) CYP 2C9. Previous studies have shown that CYP 2C mRNA and protein levels increase following hypoxia. We examined whether exposing the cells to H/R altered CYP 2C9 levels. We were unable to detect any induction. As there are many members of the CYP 2C family it is likely that family members other than 2C9 are responsible for this induction.

We have previously shown that the CYP 2C9 inhibitor SP protects against endothelium-dependent vascular dysfunction following I/R in rats. Therefore, we wanted to examine whether CYP 2C9 would also contribute to endothelial cell death and dysfunction in
cultured endothelial cells. Our results indicate that CYP 2C9 expression results in increased cell death following exposure to H/R. Pre-treatment with SP is able to protect against H/R in HUVECs expressing CYP 2C9 sense but not anti-sense. We also found that CYP 2C9 sense expressing cells produce increased oxidant radicals, as indicated by free 8-isoprostane levels in the conditioned media, following H/R and that SP was able to reduce 8-isoprostane levels in these cells.

We have previously found that SP treatment during I/R reduces subsequent SMC proliferation following heterotopic heart transplantation in rats, see Figure 4.13. Although it is likely that this resulted from decreased bioavailability of endothelium derived NO and not from the single dose of SP given prior to transplantation, we examined the possibility that SP was directly affecting SMCs. We detected only a slight decrease in the number of viable SMCs following SP treatment. H/R cause a decrease in cell viability in both untreated and SP treated groups and exposure to H/R did not increase or decrease this effect. This result agrees with our previous finding that SMC-dependent relaxation is impaired following I/R and that SP pre-treatment is not protective against this type of vascular dysfunction.15

As described in section 1.5.4, studies related to celecoxib and paracoxib/valdecoxib have also shown an association with increased cardiovascular risks resulting in the withdrawal of paracoxib/valdecoxib from the market.24-26 Given that increased AA liberation is associated with ischemia, it is possible that under conditions of ischemia, in the presence of coxibs, that AA liberation would stimulate elevated CYP 2C activity and subsequent ROS production. This elevation in ROS production may result in vascular injury and dysfunction that could contribute to the problems associated with coxib administration in the presence of ischemia. Therefore, we were interested in whether COX inhibition could induce increased
oxidative damage by CYP 2C9. We hypothesized that decreased AA metabolism by COX-2 would lead to increased AA metabolism by CYP 2C9 and consequently increased oxidative stress. Our results show that valdecoxib treatment did increase 8-isoprostane formation in CYP 2C9 sense but not anti-sense expressing cells. However, aspirin, administered at a dose specific for COX-1 did not alter 8-isoprostane production in either CYP 2C9 expressing or control cells. Thus, it is not clear whether altered AA metabolism is involved. One factor complicating this analysis is that valdecoxib, like most COX-2 inhibitors, is largely metabolized via CYP 2C9.27 Studies have not been performed to assess whether low dose valdecoxib can induce CYP 2C9 activity although high dose valdecoxib, IC50 41 μM, acts as a moderate inhibitor of 2C9 (Bextra label information, Pfizer Inc. NY, NY).

In conclusion, we demonstrate that CYP 2C9 expression in endothelial cells leads to increased cell death and ROS production following H/R and that pre-treatment with the CYP 2C9 inhibitor SP can reduce these effects. SP did not have an effect of H/R-induced cell death in SMC. We also present intriguing data indicating that the COX-2 inhibitor valdecoxib induces increased oxidant stress in CYP 2C9 expressing endothelial cells. Further detailed experimentation will be required to elucidate the mechanism of COX-2 induced oxidant production and to determine if altered AA metabolism plays a role.
5.6 Bibliography


Chapter 6: Summary and Conclusions

6.1 Restatement of the Problem

More than 3,000 heart transplants are performed annually worldwide. Current immunosuppressive regimens are very effective in preventing acute rejection. However, chronic rejection associated with CAV remains the major hurdle to long-term graft survival of all vascularized organ transplants. CAV is an accelerated and diffuse form of arteriosclerosis\(^1\) that can be detected in up to 75% of heart transplant recipients following the first year of transplantation.\(^2\) Although immunological mechanisms clearly play an important role in the pathogenesis of CAV, non-immunological mechanisms, such as peri-transplant I/R injury, also contribute via direct damage or indirectly through cross-talk with immune responses associated with this type of vasculopathy.\(^3,4\)

There are relatively few treatments for established CAV and re-transplantation is often the only option. Given the short supply of donor organs and the added stress of an additional major surgery, even re-transplantation is not a viable option for many patients. Treatments to control risk factors, such as CMV infection\(^5\) and hyperlipidemia\(^6\), have shown the most promising results at preventing CAV development. Given the association between vascular I/R injury and CAV development our primary aim was to examine whether inhibition of peri-transplant I/R injury could reduce or prevent CAV treatment. We selected two targets that are known to protect against and contribute to oxidant injury, ARC and the
CYP 2C enzymes, respectively, to examine this link based on results shown in myocardial infarction.7,8

### 6.2 Summary of Findings

Initially, we examined the ability to ARC to protect against oxidant stress in vascular cell types (Chapter 2). We demonstrated, for the first time, that ARC is expressed in both cultured endothelial and smooth muscle cells by RT-PCR and immunoblotting. We then increased ARC levels utilizing TAT-fusion protein transduction and examined ARC’s ability to protect against oxidant-mediated cell death induced by treatment with H2O2. TAT-ARC did not confer increased protection against H2O2 treatment than did treatment with our control protein TAT-β-gal. These results differed from results obtained in H9c2 rat embryonic cardiomyocytes.8

During our control experiments analyzing the action of ARC in H9c2 cells, we observed that ARC overexpressing cells did not undergo differentiation induced by serum withdrawal. We examined this observation further and demonstrated that ARC expression levels increase and stabilize upon differentiation in non-transduced H9c2 cells. ARC-overexpression in pre-differentiated H9c2-cells suppressed differentiation; indicated by increased myotube formation, nuclear fusion and expression of the differentiation markers myogenin and troponin-T. ARC-overexpression inhibited myoblast differentiation associated caspase-3 activation, suggesting ARC inhibits myogenic differentiation through caspase inhibition. Thus, we have demonstrated a novel role for ARC in the regulation of muscle differentiation.
Chapter 6

As we were unable to obtain sufficient preliminary data indicating a role for ARC in protection against oxidative damage in the vasculature, we turned our attention to the CYP 2C family of enzymes. Specifically, we examined the role of the CYP 2C9-like enzyme in rodents. Our initial examinations involved assessment of vascular function following ischemia and reperfusion (Chapter 3). Previous studies have shown that vascular function is impaired following ischemic injury\textsuperscript{9-14} and that CYP inhibitors provide protection against myocardial infarction\textsuperscript{7} and vascular dysfunction in patients with manifest coronary artery disease.\textsuperscript{15} Therefore, we hypothesized that SP, an inhibitor of CYP 2C9, would also attenuate post-ischemic endothelial dysfunction. We utilized the Langendorff model of I/R in rats and analyzed vascular function in septal coronary resistance arteries by pressure myography.\textsuperscript{16} I/R caused impairment in both endothelium-dependent and independent vasodilation. Pre-treatment with SP restored endothelial sensitivity to ACh but did not restore sensitivity to endothelium-independent vasodilators. I/R-induced superoxide production was assessed by dihydroethidium staining of flash frozen hearts. SP treatment significantly reduced superoxide production in arterial walls following I/R injury. Therefore, we concluded that CYP 2C contributes to impaired post-ischemic endothelium-dependent, NO-mediated vasodilation by increasing superoxide production.

Given the protective role of the CYP 2C inhibitor SP in protection against vascular dysfunction following I/R and the link between peri-transplant I/R injury, post-transplant vascular dysfunction and CAV, we explored whether CYP 2C may also contribute to the onset of CAV (Chapter 4). Lewis-to-Fisher rat heterotopic heart transplants were performed. Donors and recipients were treated with 5 mg/kg SP or vehicle control 1 h prior to surgery. We were able to demonstrate that SP did not affect post-transplant morbidity, mortality or
weight gain. Assessment of coronary blood vessels from rats 30 days post-transplant indicated that treatment with SP significantly reduced luminal narrowing. However, SP did not reduce diffuse, focal, epicardial, endocardial or perivascular immune infiltration nor did it alter infiltration by lymphocytes as measured by CD3+ and CD8+ staining. SP also did not significantly alter TUNEL positivity in myocardial, endothelial or SMC populations. We did not observe endothelial loss in either the SP-treated or control groups. Analysis of rats 4 days post-transplant demonstrated a decrease in SMC proliferation in the SP-treated rats compared to controls. In addition, the SP-treatment group had decreased levels of serum IFN-γ and 8-isoprostane post-transplantation.

Our final set of experiments examined the effects of CYP 2C9 in cultured vascular cells in response to H/R. We demonstrated that HUVECs do not express CYP 2C9 following culture and that H/R does not induce expression. We successfully induced expression of CYP 2C9 through the use of adenoviral constructs and demonstrated that CYP 2C9 contributes to cell death and oxidant stress following H/R in these cells. CYP 2C9 inhibition, by SP, reduced these effects. In contrast, SP treatment had no effect on SMC viability following H/R. In addition, we examined the potential for COX inhibitors to alter CYP 2C9 production of ROS. Our results indicated that the COX-2 inhibitor, valdecoxb, but not aspirin given at a dose specific for COX-1, caused increased ROS production in CYP 2C9 expressing cells. It is unclear if valdecoxb is exerting this effect through direct induction of CYP 2C9, altered AA metabolism or an alternative pathway.
6.3 Relevance of Findings

The relationship between peri-transplant ischemic injury and CAV has been previously described. However, effective strategies for CAV by preventing ROS production during peri-transplant I/R have not been reported.

Some previous studies have linked peri-operative ROS scavenging treatments using antioxidants which reduced CAV development. Murata et al. demonstrated that peri-operative treatment with the SOD mimetic m40401 reduced CAV development. More recently, Iwanaga et al. demonstrated that peri-operative treatment with the antioxidant riboflavin reduces both acute rejection and CAV development in mice. Complementary studies have been described in renal transplantation where the use of peri-operative antioxidants have reduced both acute and chronic obliterative arteriosclerosis.

Studies targeting the sources of ROS have been examined in the field of myocardial I/R related to infarction. As described in section 1.2.2, several candidate pathways have been proposed to produce ROS during I/R. These include mitochondria, NADPH oxidases, xanthine oxidase and eNOS. However, attempts to target these systems in I/R injury have not met great success. NADPH oxidase inhibition, by use of p47-null mice, revealed no significant difference in infarct size. Xanthine oxidase inhibitors have also failed to protect against I/R and may be contraindicated in patients with ischemic disease while eNOS may play a protective role. Thus, the discovery that CYP 2C9 inhibition significantly reduced myocardial infarction was exciting.

Our studies, targeting ROS production by CYP 2C9, are the first, to our knowledge, to significantly reduce both peri-transplant vascular dysfunction and CAV. Our method of
administering SP, to inhibit CYP 2C9, only during the peri-operative period demonstrated a clear link between peri-operative I/R and the development of vascular dysfunction and CAV.

6.4 Future Directions

Throughout the course of this thesis we have made several interesting and novel findings. Each of these findings warrants further examination.

With respect to our findings related to ARC in the vasculature, we were not able to show protection against oxidative injury induce by H₂O₂. However, as ARC is expressed in the vasculature, it is logical to assume that it is serving a function in these vascular beds and ARC has been found to be a multifactorial anti-apoptotic protein.²⁹ Thus there are many potential targets for ARC in these cells. Further experimentation examining alternative inducers of apoptosis and necrosis, both oxidative and non-oxidative may uncover a role for ARC in these cells. Our findings related to ARC’s inhibition of myocyte differentiation indicate that the mechanism likely involves inhibition of caspase-3 activity. However, further experimentation would also be required to fully elucidate the detailed mechanism of action.

Our studies of CYP 2C, related to vascular cell death and dysfunction and CAV, also create many interesting questions. If our hypothesis is correct and SP treatment protects against vascular injury by increasing post-ischemic NO• bioavailability, then it is not clear why direct addition of NO•, by the NO• donor SNP, did not have a similar effect. This effect was also described by Fichtlsherer et al..¹⁵ It is possible that this difference reflects the mechanism of NO• transfer between endothelial and SMCs. It is also possible that SP’s
restoration of endothelium-dependent vasodilation involves other factors outside of maintenance of NO bioavailability, likely related to decreased oxidative damage to the endothelium. Direct examination of the role of AA release and examination of alternative CYP 2C substrates would undoubtedly provide mechanistic insight into CYP 2C role in I/R injury. Experiments related to detailed examination of alterations in AA metabolism have commenced in our laboratory. These experiments would not only provide insight into CYP 2C deleterious effects during I/R but may also provide insight into why COX-2 inhibitors have been associated with increased risk of cardiovascular events. Our finding that CYP 2C inhibition during the peri-transplant period could have clinical importance. In order to translate this research into a clinical setting there are several questions that should be addressed. One intriguing possibility is that addition of CYP 2C inhibitors to cardioplegic solutions could be sufficient to confer protection. This situation would be advantageous as it would not require pre-treatment of donors and would likely reduce unwanted drug-drug interactions with cardiovascular drugs necessary for recipient treatment. If donor and recipient treatment are required then defining the time-line for inhibition would be required. Also, dosing considerations for all therapeutic drugs that are metabolised by the CYP 2C family would also have to be considered.

6.5 Concluding Remarks

Our overarching goal of these studies was to examine methods of preventing or reducing vascular I/R injury and subsequent development of CAV. Although we did not obtain
positive results in our preliminary data related to ARC in vascular oxidative damage we did uncover a serendipitous role for ARC in myogenic differentiation. In our studies related to CYP 2C9, we were able to reduced post-ischemic oxidative stress, reduce endothelium-dependent vascular dysfunction and significantly reduce CAV development.
6.6 Bibliography


Appendix I: Animal Care Certificate for Transplantation

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A05-0019

Investigator or Course Director: David J. Granville

Department: Pathology & Laboratory Medicine

Animals:
- Rats F344 112
- Rats Lewis 205

Start Date: January 2, 2005 Approval Date: March 22, 2006

Funding Sources:
- Grant Agency: Canadian Institutes of Health Research
  Grant Title: The role of CYP2C9 in peri-transplant ischemic injury and transplant vascular disease
- Grant Agency: Dean of Medicine
  Grant Title: Start Up Funding
- Grant Agency: Heart and Stroke Foundation of B.C. & Yukon
  Grant Title: The role of CYP2C9 in peri-transplant ischemic injury and transplant vascular disease
- Grant Agency: Heart & Stroke Foundation of Canada
  Grant Title: Role of CYP2C9 in Transplant Vascular Disease
- Grant Agency: Canadian Institutes of Health Research
  Grant Title: Role of CYP2C9 in Cardiac Ischemia and Reperfusion Injury
- Grant Agency: St. Paul's Hospital
  Grant Title: Cytochrome p450 monooxygenases: Role in endothelial and smooth muscle cell death and cardiac transplant vascular disease
Appendix I

Grant Agency: Michael Smith Foundation for Health Research
Grant Title: Transplantation training program

Unfunded title: CYP2C9 contributes to cardiac ischemia and reperfusion injury

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
Appendix II: Rat Heterotopic Heart Transplantation
SOP

SOP: The technique for heterotopic cardiac transplantation in rats
Submitted by: Alexandra Kerjner
Date: 05/05/2006

The technique for heterotopic cardiac transplantation in rats

Purpose:
To describe a technique for performing heterotopic cardiac grafting in a rat model.

Policy:
The personnel conducting the surgery must have received formal training by a senior staff member proficient in the technique or have equivalent previous experience.

Responsibility:
Veterinarian, Technical Personnel, Investigator

Materials:

Micro Instruments
- 1 curved microneedle driver
- 1 straight microneedle driver
- 1 straight microscissor
- 1 45° angle microscissor
- 3-4 Jeweller’s forceps
- 1 angled Jeweller’s forceps

Macro Instruments
- 4 haemostats (1 large, 2 medium, 1 small)
- 1 small curved Steven scissor
- 1 suture scissor
- 1 large scissor
- 2 large forceps
Appendix II

- 1 large needle driver
- 1 Allis forceps
- 1 large curved paediatric clamp

The procedure is carried out using an operating microscope at a magnification between 4 to 25X.

Procedure:
In this model the donor ascending aorta is sutured end-to-side to the recipient abdominal aorta and the donor pulmonary artery is anastomosed to the recipient inferior vena cava (IVC). Hearts transplanted heterotopically behave functionally as aorto-caval fistulae. Blood enters the donor, ascending aorta from the recipient abdominal aorta and is diverted into the coronary arteries by the closed aortic valve. After the myocardium is perfused, venous blood drains into the right atrium through the coronary sinus and is pumped back into the recipient IVC by the right ventricle.

Recipient (abdominal aorta)

Donor ascending aorta

Donor pulmonary artery

Recipient IVC

Drawing of the anastomoses in the heterotopic cardiac grafts. The donor ascending aorta is sutured end-to-side to the recipient abdominal aorta and the donor pulmonary artery is anastomosed to the recipient inferior vena cava (IVC).

Donor operation:
The rat is anaesthetized with Xylazine (10 mg/kg)/Ketamine (120 mg/kg), IP. The rat is shaved, prepped with alcohol and betadine and moved to the surgery table. Sterile drapes are placed; aseptic technique being used throughout. The donor’s abdominal cavity is opened with a large longitudinal incision long the abdomen, at the bottom of the ribs. The diaphragm and the lateral aspects of the rib cage are cut and the abdominal contexts are moved to the left side, exposing the IVC. The IVC is isolated above the liver and a loose tie is placed proximally, the IVC is clamped distally. Using a 3ml syringe and a 25 gauge needle, the IVC is cannulated and slowly perfused with heparinized saline, making sure that no air gets into...
the vessel. The right and left superior vena cava (SVC) are isolated and two ligatures are placed leaving space between then. The SVC is cut between the two ligatures. The ascending aorta is cut below the brachiocephalic artery and the main pulmonary artery is cut proximal to its bifurcation, they are flushed with heparinized saline. The connective tissue between the ascending aorta and pulmonary artery is cut away at this stage. The pulmonary veins are ligated together with a single 6-0 silk tie. The donor heart is gently detached and flushed with heparinized saline. At this point it might be necessary to tie off a small vessel very close to the aorta and pulmonary artery. The heart is then weighed and placed into a heparinized saline bowl on ice.

Donor preparation. Superior and inferior vena cava, azygos vein and pulmonary vein were ligated. Ascending and pulmonary artery were cut in this order.

Recipient preparation:

The recipient is prepared for grafting prior to procedure of the donor organ in order to reduce the ischemic times. The animal is weighed, anaesthetized with isoflurane inhalation anaesthesia in an induction chamber set at 4% anaesthesia and prepped in the same manner as the donor. Ophthalmic ointment (Lacri-lube) is put in the eyes to prevent drying and the animal is ear marked. A well insulated heating pad is positioned under the rat so that the...
body temperature is maintained at 38°C. A midline incision is made down to the pubic region. The bowel is brought out and wrapped in warm moist gauze. The abdominal wall is retracted on either side using a needle and silk to get good exposure of the aorta, inferior vena cava, and left-kidney vessels. The IVC and abdominal aorta are cleaned and appropriate sites for anastomoses are located. Haemostat is applied to both vessels below the level of the kidneys. The donor heart is then removed from the ice and placed in the right flank of the rat. After ensuring that the orientation of the donor PA is correct, end-to-side anastomosis between donor PA and recipient IVC is performed using continuous 9-0 nylon sutures. The posterior wall is sutured within the vessel lumen without repositioning the graft. The anterior wall is then closed externally using the same suture. Once the venous anastomosis is completed, the vein is gently stretched before tying the sutures to avoid narrowing at the anastomotic site.

Arterial anastomosis: The arterial anastomosis between the donor aortic cuff and recipient aorta is performed in the fashion as the venous anastomosis. A small quantity of microfibrillar collagen (Avitene) is placed around the arterial anastomosis before releasing the clamp. Gentle pressure is applied to the anastomotic site with a dry cotton swab for 1-2 minutes after the clamp is removed. Sites of possible leaks are checked. The bowel is returned to the abdominal cavity. The abdomen and skin are closed in a two layer closure, muscle then skin. The rat receives 10 cc of saline once the incision is closed. The rat is placed in the paediatric incubator for a few hours with supplemental oxygen. Buprenorphine is administered subcutaneously at 0.01 mg/kg immediately following surgery. If necessary additional buprenorphine will be administered twice every 8-12 hours given signs of postoperative pain. Signs of postoperative pain include: decreased activity or a reluctance to move, abnormal posture or gait (i.e., arched back or lameness), rough, greasy-looking coat (due to lack of normal grooming), dark red porphyrin staining around the eyes and nose in rats (Chromodacryorrhoea), unusual aggressiveness when handled.
**Scoring System for Heart Transplant Animals**

This system is designed to give an overview of the health status of experimental mice and rats involved in heart transplant projects. These animals undergo the very strenuous and stressful surgical procedure of having a heart transplanted into their abdomen. Each animal is monitored very carefully and scored daily using this system. The animal unit’s staff is always available to give assistance and advice. Please do not hesitate to contact us if you have any questions or concerns.

Animals are evaluated on a number of criteria and given a score from 0 to 4.

Score from: 0=normal
1= minimal/mild but noticeable
4= moderate to severe

If the score is 4 for any criteria, consult with the animal unit group and/or designated experiment representative to have a second scoring done by that person. If the score is still 4, appropriate steps for treatment or euthanasia may need to be taken.

If the combined score for all of the criteria is 10 or higher, consult with the animal unit group and/or designated experiment representative regarding the appropriate action to be taken (i.e. euthanasia or treatment). A score of 10 or higher indicates a significant problem and will require intervention.

**NOTE:** If treatment or intervention of any kind will render the experiment unusable please inform animal unit staff at the onset of the experiment. If this is the case, animals which display illness or other problems will be euthanized.

**Attitude:**

0= BAR (bright, alert/active and responsive)
1= Burrowing or hiding, quiet but rouses when touched
4= No cage exploration when lid is off, burrows/hides, head presses, may or may not vocalize or be unusually aggressive when touched, no nesting, may seem confused/irritated or hyper responsive.

**Appearance:**
0= normal  
1= mild piloerection, mild to moderate dehydration, soft stool  
4= severe piloerection, moderate to severe dehydration (obvious at first glance), sunken/wasted appearance, diarrhoea (moderate to severe can be smelled easily and seen on light coat colors easily), laboured breathing, yellowing or whitish looking mucus membranes (skin) colour, animal is hot or cold to the tough.

**Gait/Posture:**
0= normal  
1= mild in coordination when stimulated, slight hunched posture, slight limp  
4= obvious ataxia or head tilt, severe hunching, tippy-toeing, favouring of limb/noticeable limp or paralysis of limb(s)

**Weight: (post surgical or post experiment)**
0= none or up to 10% weight loss  
1= 11-15% weight loss  
4= 16-20% weight loss

**Appetite:**
0= normal, eats dry food, evidence of urine and feces on cage bottom, food missing from hopper or floor, Jell-O or supplements gone after 8 hours  
1= no evidence of eating dry food but likes Jell-O or supplements  
4= no interest in food or supplements
In addition to the categories which are given a score, here is a list of things which must also be evaluated on a regular basis. If any of these are noted, please consult with animal unit staff or designated experiments representative for advice on appropriate action to be taken.

- Suture dehisce (incision comes open)
- Check incision/experimental site daily
- Skin lesions/sores appear
- Porphyrin staining in rats can be none to mild staining around eyes or nostrils (face) but if heavy or noted on pays may indicate a problem.
- Fighting/ scabbing noted or excessive barbering with sores. (If barbering without sores is seen, note on cage card but you do not need to inform animal unit staff)
- Weigh experimental animal daily
- Check own animals (minimum) once daily or as often as required depending on experiment and reactions. Have others check your experimental animals occasionally to minimize bias.

*** If any treatments are indicated they must be approved beforehand by the PI in order to assure the treatment will not interfere in any way with the experiment.

Heart Transplant Specific Items:
There are daily observations needed specific to heart transplant project and must be made each time the animal is evaluated. Animals are evaluated daily beginning 2 days prior to transplant surgery and continuing for the duration of the animal’s participation in the experiment.

- Transplant heart palpation
  a) Palpate and score the quality of the heart beat. Record with observations. Heartbeat is graded as A (strong heartbeat), B (weak heartbeat), or C (no heartbeat felt).
  b) If the heart is no longer beating before the animal reached day 7, the data obtained will not be considered useful and the animal should be euthanized. Contact Dr. Kerjner as tissues may be needed. If Dr. Kerjner is unavailable, euthanize the animal and collect the native and transplanted hearts and placed in separate containers of formalin. Notify Dr. Kerjner when she is available

- Hind limp paralysis
  a) If one leg is paralyzed, record observations daily and euthanize if paralysis persists longer than 1 week.
  b) If both legs are paralyzed, euthanize animals immediately. Notify Dr. Kerjner and collect native and transplanted hearts in formalin

- Weigh animals at each evaluation and record with observations.
• Animals are kept for 4, 7 or 30 days post surgery and treated daily with immunosuppressive drugs for 14 to 30 days. Make sure to record injections and any treatments or manipulations in daily record sheets.

This scoring/evaluation system was taken and adapted from the Animal Care and Use Guidelines of the University of Florida.
Appendix III: List of Publications, Abstract, Oral Presentation and Awards

Published Refereed Papers


Book Chapters

Published Abstracts


Oral Presentations

Invited presentations
1. “Cytochrome p450 2C Contributes to Ischemia and Reperfusion Injury and Cardiac Allograft Vasculopathy” (2007). Center for Cardiovascular Biology and Regenerative Medicine, University of Washington Medicine, Department of Pathology, Seattle, WA.
2. “Cytochrome p450 2C Contributes to Post-Ischemic Vascular Dysfunction and Cardiac Allograft Vasculopathy” (2006), Heart Transplant Research Group at the Alberta Stollery Children's Hospital, Edmonton, AB.

Podium presentations

Awards
1. Heart and Stroke Foundation of Canada, Doctoral Research Award, $62,000, 06/06-06/09
2. Michael Smith Foundation (MSFHR), Junior Trainee Award, $45,000, 09/04-09/06
3. MSFHR, Senior Trainee Award, $45,000 (partially declined), 06/04-08/08
4. Canadian Society for Atherosclerosis, Thrombosis and Vascular Biology (CSATVB), 2005 Top Oral Presentation Award, $500
5. Canadian Cardiovascular Society, 'Have a Heart Bursary', approx $2,500, 10/05
6. CSATVB, Travel Award, $1,500, 05/05
7. Canadian Society of Transplantation, Travel Award, $1,500, 05/04
8. Centre for Blood Research/IMPACT, Best Oral Presentation Award, $150, 2006
9. UBC, Department of Pathology, 1st Place Oral Award, $300,06/05
10. ICAPTURE Centre, Rookie of the Year Award, $100, 2003
11. BC Transplant Society, 1st Place Oral Award, $500, 2003