MECHANISMS OF AIDS AND COCAINE RELATED CARDIOVASCULAR DISEASE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

Alysia Anne Chaves, B.A.

* * * *

The Ohio State University
2003

Dissertation Committee:

Associate Professor, John A. Bauer, Advisor
Professor, Lane J. Wallace
Assistant Professor, Cynthia A. Carnes
Associate Professor, Dale G. Hoyt

Approved by

------------------------------------
Advisor
College of Pharmacy
ABSTRACT

My primary thesis focus deals with understanding the unique pathology behind retroviral (HIV/AIDS) and cocaine related cardiovascular complications (Section I and II), in an attempt to provide novel mechanistic insight into these complications so that specialized therapy can be developed to treat these complications. Murine models of cardiovascular disease are also becoming increasingly important in the mechanistic study of this grave disease, and my thesis also includes a section dealing with the appropriate use of murine models in cardiac disease (Section III). Recent developments in food science may offer new and rational approaches for prevention and/or management of cardiovascular conditions. Thus, I also evaluated the value of grapes in improving or protecting endothelial function in humans (Section IV).

HIV-related cardiovascular disease is a unique form of cardiovascular disease, and traditional cardiovascular therapy may not be optimal for this population. HIV infection and cardiac complications in humans are often complicated by illicit drug abuse and covariate infections. These co-variates clearly impart adverse effects on disease progression, but the mechanisms involved are very poorly understood. LPBM5 infection, a well-established experimental model for the immunologic complications of HIV-infection, caused early cardiac contractility deficits and, later, more severe
cardiovascular impairment in mice, with a time course that preceded the development of overt immunodeficiency. The “murine AIDS” model appears appropriate for the mechanistic study of RTV-induced cardiac complications. Although the infectious agent was not HIV itself, the murine AIDS model recapitulates many of the important features of AIDS-related cardiac disease in humans, suggesting that the systemic and/or cardiovascular response to RTV infection may mediate many of these changes, rather than direct effects of the virus itself. We observed dramatic changes in immune cell levels and trafficking patterns. We also describe first-time evidence that cardiac RNS formation and oxidative injury may play a significant role in the development of cardiac complications in both the murine AIDS model and in a relevant population of AIDS-related cardiomyopathy patients.

A multi-pathogen setting is commonly observed in HIV related cardiomyopathy cases, but few studies have addressed important interactions with respect to retroviral pathogenesis vs. organ dysfunction. We have demonstrated that a modest exposure of LPS (e.g. at doses that did not yield significant effects alone in vivo) amplified abnormalities in cardiac structure and function observed in a murine AIDS model. The observed cardiac dysfunction was associated with selective increases in non-focal infiltration of CD68+ cells; these cells were found to be NOS2 positive and correlated to extent of cardiac dysfunction. This amplification interaction was not associated with alterations in retroviral progression or cardiac retroviral content, but important increase in TLR4 was observed in the combination treatment group
Evidence of cardiac myocyte induction of TLR4 was also observed in human tissues from AIDS related cardiomyopathy.

Despite the high incidence and medical costs, the mechanisms responsible for cocaine-related cardiovascular disease are not well defined. Furthermore traditional therapies commonly employed for acute coronary syndrome have not been demonstrated effective, and at this time there is no established therapeutic strategy proven safe and effective for this setting. Given the facts that nearly 25 million people in the U.S. have used cocaine at least once and more than 5 million people abuse the drug on a regular basis, optimized and cost-effective strategies for cardiac complications are warranted.

We observed that a single dose of cocaine caused acute electrical abnormalities in mice, analogous to clinical phenomenon observed in humans. Cocaine administration also resulted in protracted cardiac dysfunction (slower conduction capabilities and depressed cardiac output and stroke volume) as measured by electrocardiography and echocardiography. Our murine model has apparent value in investigating cocaine related cardiac toxicity as the mouse exhibits cardiac insufficiencies similar to human cocaine abusers. Furthermore, the mouse has definable cardiotoxic endpoints that allow for the evaluation of novel therapeutics. In addition, we observed extensive dysregulation of NO as evidenced by increased cardiac prevalence of NOS2, reactive nitrogen species and protein-3NT. These initial findings, in mice, suggest that novel therapeutics to control excessive prevalence of
cardiac NOS2 and reactive nitrogen species might be useful in managing cocaine related cardiac dysfunction.

The mouse seems to be an appropriate model to study cocaine related vascular dysfunction especially since a 30mg/kg dose of cocaine causes protracted endothelial dysfunction analogous to the human condition. Cocaine related endothelial toxicities involve concentration- and time-dependent production of oxidants. Antioxidants may have value ameliorating endothelial dysfunction due to cocaine induced vascular toxicity, and preliminary evidence suggests that cocaine may mediate oxidant production through sigma receptors. Cocaine induced endothelial dysfunction and toxicity in vitro involves both apoptotic and necrotic events that may explain protracted endothelial dysfunction with cocaine use and abuse.

Transgenic animal techniques have greatly expanded studies of cardiovascular disease mechanisms and pharmacological research. However, important considerations regarding anesthetic selection exist for physiologically relevant and statistically reliable performance evaluations. We found that significant advantages exist for inhalation anesthesia with halothane (or other similar agents) relative to injectable agents (ketamine/xylazine). These include increased convenience and throughput, maintenance of physiologically relevant hemodynamics, and decreased measurement variability leading to fewer animals required for detection of differences among groups.
We also found that six-lead measurements provided high quality ECG recordings in lightly anesthetized mice and that the choice of anesthetic played an important role in the observed electrophysiological parameters and in the intra-group variability of these parameter estimates. This effect on variability had an important impact on the statistical power and greatly influenced the numbers of animals required per group to detect differences. Inhalation anesthetics were found to be advantageous in that they provided rapid and convenient onset and offset and an appropriately stable depth of anesthesia for data collections. We also detected significant age effects both with respect to baseline ECG parameters and with respect to optimal approaches for QT interval corrections. These observations demonstrate that noninvasive and high fidelity measurements of ECG are possible in mice but that careful selection of experimental conditions and appropriate control groups may play a critical role in parameter identification, and detection of differences among groups.

Brachial artery ultrasound technology has become an important tool in assessing endothelial health in major cardiovascular risk assessment studies. Our study used this technology to evaluate endothelial function, in healthy male subjects, in response to a single dose of standardized grape product and chronic consumption of grape product. We have established a reproducible method for assessing endothelium dependent responses in humans using brachial artery ultrasound. We observed that an acute administration of a standardized grape product significantly increased shear-stress induced
vasorelaxation responses in healthy normal males, and this effect was further enhanced during chronic administration. The standardized grape product completely inhibited the acute endothelial dysfunction induced by a single high fat meal, by a mechanism that was independent of circulating triglyceride levels. These data suggest that standardized grape product may have beneficial effects on endothelial health and function in subjects with no evidence of cardiovascular disease, that the beneficial effects of the grape product were not ethanol-dependent, and that this standardized grape product may have substantial protective effects against the vascular toxicities associated with a high fat diet.
ACKNOWLEDGMENTS

My graduate life here at the division of pharmacology would not have been the same without the support and encouragement from several individuals that helped me on my journey through graduate school.

My advisor, Dr. John Bauer has been very important to my growth as a scientist and even more importantly to my growth as an individual as his guidance and encouragement helped me overcome my introversion to become the scientist I am today. I am greatly appreciative of the efforts of Drs. Uretsky, Wallace, Young, Hoyt, Rahwan, and Patil in the enrichment of my training. Financial support from The Ohio State University Graduate School, and NHLBI is appreciated.

I would like to thank my parents Jacqui and Chelston Chaves for their steadfast love, support, encouragement, and through their example shown me that value of honest and hard-work to achieve any goal that has helped through my years at graduate school. I would also like thank my sister Chrysa Chaves unwavering support and encouragement whenever I have need her especially through all the rough spots of school. I would also like to thank Spencer for not only sharing in the good times, but also giving me a shoulder to lean on during the shaky times.
The encouragement and training from several Post-doctoral fellows (Dr. Dave Weinstein, Dr. Cynthia Liu, Dr. Liang Jing, Dr. Suvara Wattanapityakul, and Dr. Michael Mihm) in our lab who have helped me through several experiments and rough spots during my years in graduate school is also greatly appreciated. I am also thankful to the many friends (Reshma, Jane, Hong, Mandar, Shawn, and Ben) in the Department of Pharmacology who have provided moral support and a lot of fun during my time as a student at Ohio State.

I am also greatly appreciative of the research insight and technical support provided by the several collaborators (Dr. Cynthia Carnes, Dr. Robert Hamlin, Dr. Tomohiro Nayakama, Dr. Tsonwin Hai, Dr. Yoshi Okamoto, Dr. Valerie Bergdall, Dr. Leona Ayers, and Dr. Freddie Robertson) that I have had the good fortune to work with. I am also very grateful for the research support I have received from technical (Brandon Schanbacher, Christen Coyle, Debbie Amann, and Angela Cook) and undergraduate assistants (Matthew Hallenburg and Anupam Basuray) in our lab.
VITA

September 27th, 1975.............................Born—Bombay, India

1997......................................................B. A. Chemistry and Zoology

Ohio Wesleyan University, Delaware, Ohio

1997-1999.................................Graduate Teaching Assistant, The

Ohio State University, Columbus, Ohio

1999-present.............................Graduate Research Associate, The

Ohio State University, Columbus, Ohio

PUBLICATIONS


FIELDS OF STUDY

Major Field: Pharmacy
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>viii</td>
</tr>
<tr>
<td>Vita</td>
<td>x</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xvi</td>
</tr>
<tr>
<td>Chapters:</td>
<td></td>
</tr>
<tr>
<td>1. Introduction and Thesis Objectives</td>
<td>1</td>
</tr>
<tr>
<td>SECTION I: HIV/AIDS Related Cardiovascular Disease</td>
<td></td>
</tr>
<tr>
<td>3. Cardiomyopathy in a murine model of AIDS: evidence of reactive</td>
<td>64</td>
</tr>
<tr>
<td>nitrogen species and corroboration in human HIV/AIDS cardiac</td>
<td></td>
</tr>
<tr>
<td>tissues</td>
<td></td>
</tr>
<tr>
<td>4. Bacterial Lipopolysaccharide enhances retroviral cardiomyopathy</td>
<td>100</td>
</tr>
<tr>
<td>during murine AIDS: Roles of macrophage infiltration and myocyte</td>
<td></td>
</tr>
<tr>
<td>expression</td>
<td></td>
</tr>
<tr>
<td>SECTION II: Cocaine related cardiovascular disease</td>
<td></td>
</tr>
<tr>
<td>5. Cocaine induced electrophysiological and contractile abnormalities</td>
<td>138</td>
</tr>
<tr>
<td>in mice; evidence of reactive nitrogen species and nitric oxide</td>
<td></td>
</tr>
<tr>
<td>synthase 2</td>
<td></td>
</tr>
</tbody>
</table>
6. Cocaine induced endothelial dysfunction in mice and oxidant production in murine endothelial cells ......................................................... 165

SECTION III: Murine Models in Cardiovascular Disease

7. Non Invasive echocardiographic studies in mice: influence of anesthetic regime .................................................................................. 194

8. Age and anesthetic effects on murine electrocardiography ............. 217

SECTION IV: Vasoprotective effects of a standardized grape product (Use of brachial artery ultrasound to evaluate endothelial dysfunction in humans)

9. Vascular endothelial effects of a standardized grape product in humans .................................................................................................. 247

10. Conclusions .............................................................................................................................. 283

Bibliography ........................................................................................................................................ 293

Appendices: Work completed through collaborative investigations

A. Reactive nitrogen species formation and cardiac oxidative injury in human HIV-related cardiomyopathy .................................................. 329

B. Transgenic mice with cardiac-specific expression of activating transcription factor 3, a stress-inducible gene, have conduction abnormalities and contractile dysfunction ........................................ 364
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Time-line of events that outline HIV/AIDS related</td>
</tr>
<tr>
<td>2.2</td>
<td>Retrospective studies of HIV/AIDS related cardiac dysfunction</td>
</tr>
<tr>
<td>2.3</td>
<td>Prospective studies of HIV/AIDS related cardiac dysfunction</td>
</tr>
<tr>
<td>2.4</td>
<td>Cardiotoxicity of drugs used for HIV and HIV related conditions</td>
</tr>
<tr>
<td>7.1</td>
<td>Variability of cardiovascular parameter measurement as a function of anesthesia</td>
</tr>
<tr>
<td>9.1</td>
<td>Averaged electrocardiographic, hemodynamic, blood lipid and total antioxidant capacity values at baseline and post test product (sugar control and grape product)</td>
</tr>
<tr>
<td>9.2</td>
<td>Average change in brachial artery blood flow post release of the occluder at baseline and post test product (sugar control or grape product)</td>
</tr>
<tr>
<td>9.3</td>
<td>Averaged electrocardiographic, hemodynamic, blood lipid, and Δ flow values before and after 21 days (2X) of grape product consumption</td>
</tr>
</tbody>
</table>
9.4 Averaged electrocardiographic, hemodynamic, blood lipid and total antioxidant capacity values at baseline and post test product (High fat meal alone and in conjunction with grape product)………………………………………………………………………………281

9.5 Average changes in brachial artery blood flow at baseline and post test product (high fat meal alone and in conjunction with grape product)…………………………………………………………………………282

A.1 General patient characteristics………………………………………………………356

A.2 Heart weight and heart/brain ratios…………………………………………………357

B.1 Morphometric analysis of Mice……………………………………………………392

B.2 Electrophysiological analysis of mice…………………………………………….393
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>93</td>
</tr>
<tr>
<td>3.2</td>
<td>94</td>
</tr>
<tr>
<td>3.3</td>
<td>95</td>
</tr>
<tr>
<td>3.4</td>
<td>96</td>
</tr>
<tr>
<td>3.5</td>
<td>97</td>
</tr>
<tr>
<td>3.6</td>
<td>98</td>
</tr>
<tr>
<td>3.7</td>
<td>99</td>
</tr>
<tr>
<td>4.1</td>
<td>130</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>4.2</td>
<td>Cardiac alterations during murine AIDS, and RTV infection in combination with LPS.</td>
</tr>
<tr>
<td>4.3</td>
<td>Splenomegaly and increased viral load in cardiac and splenic tissue during the progression of murine AIDS.</td>
</tr>
<tr>
<td>4.4</td>
<td>Circulating monocyte levels are significantly diminished during murine AIDS while circulating eosinophils are significantly enhanced with RTV infection in combination with LPS.</td>
</tr>
<tr>
<td>4.5</td>
<td>Increased cardiac presence of CD68+ infiltrates during murine AIDS.</td>
</tr>
<tr>
<td>4.6</td>
<td>Increased cardiac TLR4 expression and protein prevalence during RTV infection in combination with LPS.</td>
</tr>
<tr>
<td>4.7</td>
<td>Increased cardiac TLR4 prevalence in HIV dilated cardiomyopathy.</td>
</tr>
<tr>
<td>5.1</td>
<td>A representative signal averaged Lead I recording from an untreated C57BL/6 mouse.</td>
</tr>
<tr>
<td>5.2</td>
<td>Acute electrocardiographic abnormalities post a single intra-peritoneal injection of 30mg/kg cocaine.</td>
</tr>
<tr>
<td>5.3</td>
<td>Protracted electrocardiographic dysfunction in mice 5 days post cocaine (30mg/kg, ip.) administration.</td>
</tr>
<tr>
<td>5.4</td>
<td>Protracted echocardiographic dysfunction in mice 5 days post cocaine (30mg/kg, ip.) administration.</td>
</tr>
</tbody>
</table>
5.5 Increased cardiac RNS formation and attendant protein nitration five days post cocaine (30mg/kg, ip)……………………………………163

5.6 Increased cardiac presence of NOS2 five days post cocaine (30mg/kg, ip.)……………………………………………………………164

6.1 Cocaine (30 mg/kg, ip.) caused acute increases in heart rate and cardiac output………………………………………………………188

6.2 Cocaine caused protracted (5 days) endothelial dysfunction in mice - evidence of selective reduction in endothelial NO production or availability………………………………………………...189

6.3 In vitro cocaine exposure caused an increase in murine endothelial cell oxidant production (as measured by DCF fluorescence)……………………………………………………...190

6.4 In vitro cocaine exposure caused a time-dependent increase in intracellular oxidant production in murine endothelial cells…….191

6.5 Cell death (apoptosis/necrosis) due to an in vitro exposure (24hr) of cocaine (10, 100, 1000 µM) (as measured by annexin V and propidium iodide labeling……………………………………………….192

7.1 Cardiac function assessment via echocardiography………………...213

7.2 Correlation analyses of hemodynamic variables. ......................214

7.2 Power analysis to determine sample size……………………………215

8.1 A representative signal averaged Lead I recording from an 8-week old C57BL/6 mouse under sevofluorane anesthesia………240

8.2 Cardiac function evaluated via electrocardiography………………241
8.3 Age- and Anesthetic-Dependent Effects on the Electrocardiograms of C57BL/6 mice………………………………….242
8.4 Heart Rate Dependence of Electrocardiographic Intervals………………243
8.5 QT interval correction with Bazett’s correction and Fridericia correction at both 8 and 48 weeks of age……………………………...244
8.6 Number of Animals Required to Detect Significant Differences in Electrocardiographic Variables: Effect of Anesthetic Regimen and Age………………………………………………………………………….245
9.1 Representative images of brachial artery diameter and flow in response to hyperemia…………………………………………………..272
9.2 Average responses in brachial artery flow and cross-sectional (xs) area post release of occluder………………………………………273
9.3 DCF fluorescence to measure total plasma antioxidant capacity……274
9.4 A single dose of grape product significantly enhanced endothelial function at 45min and 90min post administration…………………...275
9.5 Responses to a 21day (2X) consumption of grape product………….276
9.6 Blunting of high-fat induced endothelial dysfunction by a standardized grape product………………………………………….......277
A.1 A tissue microarray approach for human AIDS cardiac autopsy tissues…………………………………………………………………358
A.2 Cardiac interstitial fibrosis measures…………………………………..359
A.3 Cardiac immune cell infiltrates…………………………………………360
A.4 Protein-3NT immunohistochemistry..............................................361
A.5 TNF-α immunohistochemistry.........................................................362
A.6 NOS 2 immunohistochemistry.........................................................364
B.1 ATF3 is induced by myocardial ischemia-reperfusion.......................394
B.2 MyHC-ATF3 transgenic mice express the transgene in the heart......395
B.3 TG1 transgenic mice have enlarged atria and dilated right
ventricles. .........................................................................................396
B.4 MyHC-ATF3 transgenic mice have increased gene expression
indicative of hypertrophy.................................................................397
B.5 Histological analyses indicate myocyte disarray and fibrosis in
TG1 hearts. .........................................................................................398
B.6 Ultrastructural analyses indicate myocyte degeneration in TG1
hearts. ..............................................................................................399
B.7 Cardiomyocytes derived from MyHC-ATF3 transgenic ventricles
have decreased contractile function Impairment in left ventricular
diastolic performance precedes .......................................................400
B.8 TG2 transgenic mice have time-dependent cardiac performance
deficits..........................................................................................401
CHAPTER 1

INTRODUCTION AND THESIS OBJECTIVES
As a graduate student in Dr. John Bauer’s lab, I was fortunate enough to have the unique opportunity to be involved in several projects involving various aspects of cardiovascular disease, which is the leading cause of death in the United States. Traditional cardiovascular therapy, often very expensive, has often failed to ameliorate the cardiovascular complications due to some unique pathological contributors. Therefore, novel therapies to treat expensive and unique pathologies of heart disease are warranted. My primary thesis focus deals with understanding the unique pathology behind retroviral (HIV/AIDS) and cocaine related cardiovascular complications (Section I and II), in an attempt to provide novel mechanistic insight into these complications so that specialized therapy can be developed to treat these complications. Murine Models of cardiovascular disease are also becoming increasingly important in the mechanistic study of this grave disease and my thesis also includes a section dealing with the appropriate use of murine models in cardiac disease (Section III). Recent developments in food science may offer new and rational approaches for prevention and/or management of cardiovascular conditions (1). Thus, we also evaluated the value of grapes in improving or protecting endothelial function in humans (Section IV).

Section I: HIV related cardiovascular disease

Human immunodeficiency virus (HIV) and the resultant development of Acquired Immunodeficiency Syndrome (AIDS) may represent the most pressing medical challenge of the last 20 years. With the worldwide incidence
of HIV infection approaching an estimated 5,000,000 cases annually, the crisis of HIV/AIDS is not likely to soon subside, despite substantial research and clinical efforts to manage infectivity and disease progression (2). These efforts have resulted in significant advances in addressing the primary symptomology of HIV/AIDS—immunodeficiency and high susceptibility to opportunistic infections—such that the turn of the 21st century may mark an important shift in the disease presentation, morbidity and mortality of HIV/AIDS (3, 4). This shift transpired as a result of the dramatic success of Highly Active Anti Retroviral Therapy (HAART) regimens that were introduced in 1993 (4). HAART, a cocktail of several anti-retroviral drugs that interrupt various stages of the replication cycle of HIV, has significantly improved overall survival and increased time of AIDS-free living for patients infected with HIV (4). However, this same prolongation of life expectancy and increased quality of life has evinced a variety of HIV-related complications that are apparently not directly related to immunodeficiency or opportunistic infections (4, 5). For example, HIV-related cardiovascular complications represent increasingly important contributors to the overall morbidity and mortality of HIV/AIDS patients (6, 7). The prevalence of cardiac complications in HIV patients has been estimated to be as high as 80%, with an estimated 5-20% progressing to severe dilated cardiomyopathy and cardiac failure (4, 8). As the worldwide incidence continues to increase, HIV/AIDS-related cardiovascular disease might soon become a significant etiology of cardiac failure. In Chapter 2 we review the epidemiology and scope of this unique form of cardiovascular disease. The
mechanism by which HIV-related cardiovascular disease is initiated and progresses are still incompletely understood, but Chapter 2 will attempt to outline several mechanisms that have been suggested to contribute (CHAPTER 2).

Unfortunately, the study of relationships between human HIV infection and cardiac alterations is often complicated by the use of various drug therapies, illicit drugs known to be cardiotoxic, limited tissue availability and variable disease progression (9). Demonstration of relevant animal models for the investigation of retrovirus-related cardiac dysfunction and pathologies could provide opportunities for further mechanistic insight and therapeutic intervention. In Chapter 3, we employed a well-established murine model of retroviral infection (LPBM5 virus) and defined the time-dependencies of retroviral progression and cardiac dysfunction, in an attempt to extend its relevance as a disease model. The LPBM5 model of retroviral infection is commonly called the "murine AIDS" model, due to its high similarity to many of the immune-related complications seen during human HIV infection, including aberrant cytokine release, changes in T-cell populations, and increased susceptibility to opportunistic infections. An additional goal of Chapter 3 was to define the contributions of nitric oxide (NO)-related biochemistry during murine retrovirus related cardiac dysfunction (CHAPTER 3). To corroborate our experimental findings in the murine AIDS model, we explored the relevance of our experimental results in a sample of cardiac autopsy tissues from HIV/AIDS patients with dilated cardiomyopathy and relevant controls
In Appendix A, (a collaborative effort) we have further investigated the mechanisms involved in retroviral related cardiovascular disease using a well-controlled library of human cardiac autopsy tissues.

Although the concept of concurrent pathogens driving retroviral complications have been suggested, no previous studies have tested this hypothesis directly in a well-controlled set of experimental conditions. We have recently established that a well-defined murine model of retroviral disease (the LPBM5 model of “murine AIDS”) recapitulates the major cardiovascular features already documented in HIV/AIDS patients, with initially detectable contractility deficits prior to overt immune compromise followed by more severe cardiomyopathy (CHAPTER 3) (10). Given our prior observations in this model and the potential importance of combined pathogen effects with respect to retrovirus related cardiovascular alterations, we investigated potential interactions among retroviral infection and a modest exposure to a bacterium related pathogen in Chapter 4. In the studies described in Chapter 4, we tested the hypothesis that exposure to LPS (at doses that did not induce cardiac toxicities alone) can modulate retrovirus-related cardiac deficiencies in this well-established model of the immunologic and cardiac complications of retroviral infection (10, 11). We used LPS as the immune activator rather than live bacteria to avoid confounding differences in pathogen growth in immunocompromised hosts. Our investigative focus was on aspects of retroviral progression, leukocyte populations and trafficking to cardiac tissues; by using a specific pathogen-free mouse colony we had an unique opportunity
to evaluate interactions between these two immune system challenges and limit influences of other potential covariates commonly found in humans (CHAPTER 4). An additional component of our study was to address mechanisms by which such multi-pathogen interactions might develop. As key participants of the innate immune system, toll-like receptors (TLR) are a newly identified class of receptors involved in the recognition and transmission of pathogenic stimuli (12). In Chapter 4 we tested the hypotheses that the TLR4 receptor system may be involved in retrovirus related cardiac dysfunction, in both the murine AIDS model and in a small set of human tissues from HIV related cardiomyopathy autopsy cases.

SECTION II – Cocaine related cardiovascular disease

Cocaine abuse remains an epidemic in the U.S. and is associated with high social and medical costs. Estimates of the annual total costs for drug abuse has been estimated to be $98 billion dollars, and a majority of these costs are related to cocaine emergency cardiac care as these incidents account for nearly 30% all drug related emergency room (ER) visits, with an annual expenditure of nearly $83 million (13, 14). For example, acute episodes of angina pectoris, cardiac arrhythmias and myocardial infarction have all been commonly reported in cocaine abusers, and occasionally occur in first-time users. Separate from these acute events, chronic conditions including myocarditis, vasculopathy, cardiomyopathy, congestive heart failure have also been demonstrated in frequent cocaine users (15-18). Despite the
high incidence and medical costs, the mechanisms responsible for cocaine-related cardiovascular disease are not well defined. Furthermore traditional therapies commonly employed for acute coronary syndromes have not been demonstrated effective and at this time there is no established therapeutic strategy proven safe and effective for this setting (19-22). Given the facts that nearly, 25 million people in the U.S. have used cocaine at least once and more than 5 million people abuse the drug on a regular basis, optimized and cost-effective strategies for cardiovascular complications are warranted (13).

Over the last three decades, much of the in vivo investigations of cocaine’s cardiovascular pharmacology and toxicology have been conducted in a wide array of animal models, including sheep, dogs, rabbits, and rats. In addition, other investigators have employed murine models for investigation of cocaine related hepatotoxicity. In Chapter 5 our goal was to define the acute and protracted cardiovascular effects in a murine model, using our established expertise measuring contractile and electrophysiological parameters in this small species. An additional component of these investigations was to evaluate potential contributions of altered nitric oxide (NO) control and reactive nitrogen species during cocaine induced cardiac dysfunction (CHAPTER 5).

One critical component of vascular integrity and function is endothelial monolayer, and important component of normal endothelial cell function is the production of nitric oxide (NO) via metabolism of arginine. NO is now recognized as a critical component to a healthy endothelium, providing local modulation of vascular tone and inhibiting blood cell adhesion (23, 24). An
impaired capacity of the endothelium dependent and NO mediated relaxant response has been observed in a wide variety of patients with vascular risk factors, including diabetes, smoking history, elevated cholesterol, and familial history (25). Cocaine has been shown to cause chronic endothelial dysfunction in humans as measured by forearm plethysmography (26). Cocaine induced impaired endothelial function has been theorized to be related to increased levels of endothelin-1 mediated by direct influence of cocaine on sigma receptors (27). However, the exact mechanisms involved in cocaine induced vascular dysfunction are not completely understood. In Chapter 6 we employed a murine model to evaluate cocaine induced vascular endothelial dysfunction. In parallel studies, in Chapter 6, we investigated mechanistic aspects using isolated murine endothelial cells to evaluate direct cellular effects, testing the hypotheses that sigma receptor activation, oxidants and/or apoptosis are affected by cellular exposures to cocaine.

SECTION III – Murine models in cardiovascular disease

Transgenic animal techniques have greatly expanded studies of cardiovascular disease mechanisms and pharmacological research. These animal models, primarily developed in mice, include selective gene deletion ("knock-out"), tissue-specific gene promotion, and selective gene induction strategies (28-30). While these strategies have enhanced research potential in the identification of molecular and genetic factors contributing to disease,
they have also created significant challenges for reliable physiological parameter measurements in these small rodents.

Given the limited availability and high cost associated with transgenic animal models, optimal conditions for valid echocardiographic measures are important. While other reports have demonstrated the potential value of these methods, conditions required for reliable measurements have not been defined. This aspect is particularly important for long-term serial study designs and drug trials, as well as the rapid screening of large numbers of animals.

In evaluating the optimal conditions for murine echocardiography, investigators must consider not only animal and instrumentation conditions but also which cardiac functional parameters are most reliable in detecting differences among groups. In Chapter 7 we have compared the utility of an injectable mixture of ketamine/xylazine, widely used for small animal research and previously used for echocardiographic measures (31-33), to that of a halothane inhalation approach. In addition, we considered the potential impact of anesthetic choice on statistical power, contrasting several traditional parameters of cardiac performance (CHAPTER 7). The goals of our investigations in Chapter 7 were to identify conditions and parameters that provide reliable, convenient, and relevant echocardiographic measurements in mice.

We, and others, have recently demonstrated the potential value of electrophysiological recordings in mice to characterize transgenic models of cardiac disease or dysfunction (34-36). Thus far, most published reports using
such methods have focused on qualitative evidence of conduction disturbances, and in many cases the abnormalities are overt and beyond changes typically observed in patients (owing to the severe cardiac pathologies often reported in transgenic models) (37-39). In addition, the quantitative ECG measures, when reported, often vary widely in a given treatment group. For example, the reported QT interval (an index of ventricular conduction) for a “wild type” mouse strain has varied roughly three-fold in prior publications; this discordance still remains in the published literature even if this parameter is heart rate corrected (QTc, rate corrected QT interval) (40, 41). This parameter is now recognized as an important toxicological endpoint for assessing risk of cardiac arrhythmias in vivo (42-44), suggesting that reliable measurement in rodent models may enhance drug safety evaluations.

Given the limited availability and high cost associated with transgenic animal models, optimal conditions for valid electrophysiological measures are important. This aspect is particularly important for long-term serial study designs and drug trials, as well as the rapid screening of large numbers of animals. The ability to detect relatively small changes in ECG parameters is also important for toxicity screening; for example, a less than 20% prolongation of QT interval is considered clinically relevant (45).

In Chapter 8 we tested the hypothesis that anesthesia selection has an influence on the measured ECG parameters and potentially influences study conclusions. We systematically compared the utility of an injectable mixture of ketamine/xylazine, (a combination widely used for small animal research and
previously used for many cardiovascular studies in mice), to that of two inhalation anesthetics (halothane and sevoflurane) (CHAPTER 8). We focused on several clinically relevant ECG parameters in vivo and an additional component of our study, in Chapter 8, was to assess the influence of animal age on these effects. We also considered the potential impact of anesthetic choice and age on statistical power to detect clinically relevant ECG changes (CHAPTER 8).

SECTION IV – Vasoprotective effects of a standardized grape product (Use of Brachial Artery Ultrasound to evaluate endothelial function in humans)

Coronary artery disease (CAD) currently afflicts more than 12 million Americans, and results in over 500,000 deaths annually, making CAD the leading cause of death in America. The mechanisms by which CAD is initiated and progress are multi-factorial, involving a number of different cell types (vascular smooth muscle cells, immune cells, vascular endothelial cells) and co-variates (diet, exercise, genetic, etc). Injury to the vascular endothelium and resultant endothelial dysfunction has been recently demonstrated to be a precipitating event in the development of CAD (46). An important component of normal endothelial cell function is the production of nitric oxide (NO) via metabolism of arginine. NO is now recognized as a critical component to a healthy endothelium, providing local modulation of vascular tone and inhibiting blood cell adhesion (23, 24). An impaired capacity of the endothelium
dependent and NO mediated relaxant response has been observed in a wide variety of patients with vascular risk factors, including diabetes, smoking history, elevated cholesterol, and familial history (25). Thus it appears that the phenomenon of early endothelial cell dysfunction may be a common and unifying feature of vascular disease development (47).

Several studies suggest that grape products may have important actions on vascular endothelial performance—this action may help to explain epidemiological data regarding the reduction in cardiovascular disease risk associated with grape products (48-52). However, the vascular effects of chronic consumption of grape products in healthy normal subjects, in the absence of ethanol, have not been evaluated. An intake of a high-fat meal has been shown to cause acute and time-dependent impairment of endothelial function (53). Thus, an improvement in endothelial performance in vivo, or a reduction of impairment following high-fat meals, may provide an opportunity to delay or prevent deleterious cardiovascular effects. Thus, the primary goal of the study in Chapter 9 was to test the hypothesis that a standardized product from fresh grapes (acute and chronic consumption), in the absence of ethanol, improves endothelial performance in normal subjects, alone or in combination with a standardized high-fat meal.

Finally, a general overview of my dissertation research is provided in Chapter 10.
REFERENCES


immunodeficiency in the mouse: MAIDS as a model for AIDS [editorial]. 


SECTION I

HIV/AIDS RELATED CARDIOVASCULAR DISEASE
CHAPTER 2

A REVIEW OF HIV/AIDS RELATED CARDIOVASCULAR DISEASE
**Introduction and Outline**

Human immunodeficiency virus (HIV) and the resultant development of Acquired Immunodeficiency Syndrome (AIDS) may represent the most pressing medical challenge of the last 20 years. With the worldwide incidence of HIV infection approaching an estimated 5,000,000 cases annually, the crisis of HIV/AIDS is not likely to soon subside, despite substantial research and clinical efforts to manage infectivity and disease progression (1). These efforts have resulted in significant advances in addressing the primary symptomology of HIV/AIDS—immunodeficiency and high susceptibility to opportunistic infections—such that the turn of the 21st century may mark an important shift in the disease presentation, morbidity and mortality of HIV/AIDS (2, 3). This shift transpired as a result of the dramatic success of Highly Active Anti Retroviral Therapy (HAART) regimens that were introduced in 1993 (3). HAART, a cocktail of several anti-retroviral drugs that interrupt various stages of the replication cycle of HIV, has significantly improved overall survival and increased time of AIDS-free living for patients infected with HIV (3). However, this same prolongation of life expectancy and increased quality of life has evinced a variety of HIV-related complications that are apparently not directly related to immunodeficiency or opportunistic infections (3, 4). For example, HIV-related cardiovascular complications represent increasingly important contributors to the overall morbidity and mortality of HIV/AIDS patients (5, 6). The prevalence of cardiac complications in HIV patients has been estimated to be as high as 80%, with an estimated 5-20% progressing to severe dilated cardiomyopathy and cardiac failure (3, 7). As the
worldwide incidence continues to increase, HIV/AIDS-related cardiovascular disease might soon become a significant etiology of cardiac failure.

Here, we review the epidemiology and scope of this unique form of cardiovascular disease. The mechanism by which HIV-related cardiovascular disease is initiated and progresses are still incompletely understood, but this review will attempt to outline several mechanisms that have been suggested to contribute. The mechanistic study of this disease in humans is further complicated by the existence of a wide variety of confounding co-variates, including co-existent pathogens, concurrent therapies used to treat HIV that may have cardiac toxicities in their own right, and illicit drug use. Subsequently, no specialized therapeutic approaches currently exist for this unique, complex, costly, and increasingly prevalent patient population.

The problem:

In the early 80’s, the United States was faced with a mysterious medical condition that predominated in the gay community (8). The cause of this disease was unknown, and it was initially labeled as Gay Related Immunodeficiency Disease (GRID). As it spread outside this community, the disease was renamed Acquired Immunodeficiency Syndrome (AIDS). A primarily T cell infecting retrovirus was soon found to be cause of AIDS (9). Anti-retroviral drugs were quickly developed against Human Immunodeficiency Virus (HIV) to inhibit the rapid progression to AIDS in these patients. In 1987, the first of the nucleoside reverse transcriptase inhibitors, Zidovidune or AZT, was introduced, which was
joined by other classes of drugs (non-nucleoside reverse transcriptase, protease, and integrase inhibitors), directed at targets of HIV’s replication cycle. When administered as single agents, HIV rapidly developed tolerance to these drugs—a combination strategy consisting of a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor, and a protease inhibitor was employed to avoid this problem. This regimen became known as High Active Antiretroviral Retroviral Therapy (HAART), and was adopted as the standard therapy to combat AIDS beginning in the early 1990’s. The advent of HAART revolutionized therapy and vastly improved survival and time of AIDS free living among HIV-infected individuals.

Along with improved survival came a dramatic reduction in common HIV-related opportunistic infections like cytomegalovirus (CMV) and *Pneumocystis carinii* pneumonia (PCP) (10, 11). Similarly, the incidence of Kaposi Sarcoma (KS), a cancer commonly seen in AIDS patients, was drastically reduced from a 4% prevalence in the early eighties to approximately 0.03% post-HAART (12). However, as survival improved, the opportunity to observe a variety of HIV-related complications that were apparently not directly related to immunodeficiency or opportunistic infections. HIV-related pulmonary, neurological, and cardiovascular complications represent important contributors to the overall morbidity and mortality in HIV/AIDS patients (3, 4). These complications are often present as accelerated conditions of “aging-related” disorders, and are resulting in much poorer outcomes and higher medical costs for an increasingly large number of people living with HIV. For example, HIV-
related cardiovascular disease, even using the lowest estimated incidence of this condition, will result in approximately four million people with cardiac insufficiencies. Currently in the United States, approximately 72,000 people have some form cardiovascular disease due to HIV/AIDS (1). As the worldwide incidence of AIDS approaches an estimated 5,000,000 new cases annually, HIV-related cardiovascular disease could soon become a significant etiology of cardiac failure (1).

The etiology of cardiovascular disease in an HIV infected population

Cardiovascular complications were first reported in several post mortem analyses of HIV infected patients. Shown in Table 1 is a timeline of the events that shaped the current understanding of HIV-related cardiovascular complications. Outlined in Table 2 is a collection of retrospective studies describing the presence of cardiac insufficiencies in the early stages of the AIDS. The prevalence of cardiac complications (myocarditis, endocarditis and pericardial effusion) in HIV patients has been estimated to be as high as 50%, with an estimated 5-40% progressing to dilated cardiomyopathy and congestive heart failure (Table 2). Early cardiac autopsy studies were the initial indicators that cardiac insufficiencies might be significant complication of HIV infection, as a number of AIDS patients with no prior documented history of cardiovascular disease often presented with “inflammatory” cardiac pathologies (e.g. myocarditis, pericarditis, chamber remodeling) at autopsy (13-15). Subsequent prospective cardiac evaluations in HIV+ positive patients showed that cardiac
dysfunction may be involved in more than just end-stage AIDS patients (Table 3). Even pre-symptomatic HIV patients consistently demonstrate cardiac abnormalities, ranging from slight but significant systolic deficiencies, to relaxation deficits, to conduction abnormalities (16-19). The incidence and severity of these complications increase as patients progress to the development of AIDS, and the existence of cardiac complications to AIDS generally results in poorer patient outcomes (17, 20). AIDS patients with cardiovascular involvement have a significantly reduced (5-fold) life expectancy at an equivalent stage of AIDS progression (by CD4+ count), and AIDS-related cardiovascular disease has extremely poor outcomes relative to more traditional etiologies of cardiac failure (AIDS-related cardiomyopathy has an adjusted hazard ratio of 5.86 relative to the more common etiology of idiopathic cardiomyopathy) (5, 6). Additionally, the prevalence and intensity of these complications are not limited to the adult population as nearly 50% of pediatric AIDS patients (vertical transmission) develop chronic cardiovascular disease by age 2, and cardiac failure is a primary cause of mortality in this patient population (21).

Despite the high incidence of cardiovascular abnormalities and the high costs associated with patient care, the mechanisms involved remain largely undefined. One of the most prominent and controversial theories is related to a direct cardiotoxic effect of HIV (or its protein constituents) on myocytes—such studies have been the focus of a preponderance of animal studies of HIV-related disease. Several other studies are based on the hypotheses that HIV alone does not solely lead to the cardiac damage, but works in concert with the host’s
immune system to stimulate undesirable cardiac effects (cardiac immune cell infiltration). Human HIV infection and cardiac alterations are often complicated by the use of various drug therapies (HAART and therapy directed towards various other HIV-related complications), illicit drugs with known cardiotoxicities (cocaine), and covariate infections (other viruses and bacteria) that might modulate the cardiac condition in HIV. New opportunities for therapy in this unique form of cardiac disease will eventually come from investigations in relevant animal models of retrovirus-related cardiac dysfunction in the light of what is known from pathological studies in the human population.

**Cardiac myocyte effects of HIV infection**

*Can HIV infect a myocyte?*

The most prominent theory regarding HIV-related cardiovascular dysfunction is based on a highly controversial position that HIV can directly infect cardiac myocytes. In 1987, Calabrese and his colleagues provided some early evidence that HIV could be isolated and cultured from a cardiac biopsy sample (22). Human studies in a pediatric HIV infected population detected the presence of HIV-1 RNA in cardiac tissue homogenates via PCR based methods (23). Several later studies using either immunohistochemical (in situ hybridization to localize HIV) and/or PCR based techniques also corroborated the presence of HIV in myocardial tissue and myocytes (24-26). The techniques, employed in these studies, were sensitive but they lacked the ability to provide
conclusive evidence as to which cells were harboring HIV. Rebolledo’s investigations in human fetal cardiomyocytes argued against HIV infection of cardiomyocytes, because HIV-1 isolate could not infect the myocytes (27). Cellular infection by HIV-1 requires the presence of CD4 and chemokine (CXCR4 and CCR5) receptors on the surface of cells (28). Chemokine receptors have been recently identified on the surface of myocytes isolated from non-infected dilated cardiomyopathy patients (29). However, there has been no indication that myocytes have CD4 receptors, which leads to an unanswered question of how HIV infects a myocyte. Twu and her colleagues, using an in vitro model of neonatal rat ventricular myocytes (NRVM), shed some light on this question. They found that HIV-1 could infect NRVMs by binding to ganglioside GM1 and entry through macropinocytosis (30). This mechanism of infection had been previously proposed to occur in brain and coronary endothelial cells too and was inhibited by dimethylamiloride (Na/K channel inhibitor), cyclodextrin (cholesterol extracting agent), and the polyanion heparin (30). Even though HIV-1 could infect NRVMs, it was not able to replicate in vitro. On the other hand, studies done in Simian models of retroviral infection indicate that even though SIV can be found in the myocardium, it is colocalized with cells bearing a CD4 epitope. In the absence of inflammation, SIV resides in cardiac dendrites and in infiltrating macrophages when inflammatory infiltrates are present (31). In light of these studies it is possible that even if the retrovirus (HIV or SIV) does not directly infect the myocyte it may be contributing to the pathology of retroviral related cardiac dysfunction through the host’s immune system.
Cardiac toxicity of the components of HIV

A role for the direct cardio toxic effect of HIV on a myocyte has also been examined through studying the effects of recombinant proteins of HIV on myocyte function and integrity. HIV’s glycoprotein, glycoprotein120 (gp120) has been documented to be involved in the production of nitric oxide (NO) via an induction of inducible nitric oxide synthase (NOSII) in isolated neonatal rat ventricular myocyte (NRVM) cultures (32). This induction of NOSII was also found to be dependent on p38 MAP kinase pathways involving NFkB activation. However, gp120 did not elicit these effects alone, it was only in conjunction with interleukin-1β (IL-1β) that promoted the release of NO through NOSII (32). Twu’s studies in NRVMs also looked at the toxic effect of gp120. They found that gp120 could elicit apoptotic events in a myocyte that were controlled through mitochondrial pathways (30). This study also suggested that cardiac myocyte apoptosis is receptor mediated via chemokine receptors, specifically CCR3 on NRVMs. To date CCR4 has been the only documented chemokine receptor on human cardiac myocyte (29). HIV’s glycoprotein gp120 has also been shown to inhibit cardiac myocyte contraction in isolated rabbit ventricular myocytes. Gp120 inhibition of L-type Ca^{2+} channels was found to be responsible for the impaired myocyte contractility (33). Even though, these in vitro studies are mechanistically insightful, they still represent only a small piece of HIV related cardiovascular disease.
Cardiac immune cell infiltration

A pathogenic role for the immune system in acute cardiac injury as well as progressive cardiac and vascular disease is becoming increasingly apparent (34, 35). The interactions between immune cells and cardiac myocytes may have particular relevance in the setting of retroviral- and HIV/AIDS-related cardiac disease. Monocytes/macrophages are carriers for HIV, SIV, and LPBM5, and recent studies in humans have demonstrated that trafficking of HIV-infected CD68+ cells to the heart occurs in patients with AIDS-related cardiomyopathy (36, 37). Investigations in our laboratory on human autopsy samples revealed a 2-3 fold increase in cardiac presence of CD68+ cells (monocytes/macrophages) in HIV+/CVD+ hearts, compared to their respective controls for cardiac disease (HIV-/CVD+) and viral infection (HIV+/CVD-) (38). Interestingly, our model of murine AIDS recapitulated the human phenomenon in spite of being a different retrovirus (LP-BM5) (39). The simian models of SIV related cardiac dysfunction might also involve the increased presence of cardiac CD68+ cells (31).

Macrophages are high capacity production sites for reactive oxygen and reactive nitrogen species and may contribute to cardiac oxidative damage in this setting (40). It is possible that these immune cells have the potential to modulate cardiac function in all types of retroviral infection, implying there is the unifying feature of a cardiac selective infiltration of immune cells in retroviral related cardiac dysfunction. Interestingly, it is seemingly evident that it may not be solely a direct cardiotoxic effect by HIV, but a dysregulated immune response to the retrovirus that might be involved in the cardiac dysfunction. The factors involved
in the recruitment and/or activation of these immune cells to the heart still remain unknown. Further studies defining the molecular interactions and consequences involved are warranted.

Oxidative pathways - Cardiac Protein 3-NT and NOS II induction

Nitric oxide (NO) is a key mediator of both immune function and cardiovascular homeostasis and may be an important pathophysiologic link between immune and cardiovascular system control (41). The reactivities of NO in vivo are highly dependent upon its interactions with other oxidants and its capacity to participate in the production of reactive nitrogen species. Reactive nitrogen species (RNS) are a family of biologically relevant oxidants derived from the interaction of nitrogen-based intermediates (most notably NO) with reactive oxygen species (e.g. superoxide anion, hydroxyl radical, hydrogen peroxide) (42). RNS can have profound cellular effects and toxicities due to the distinct reactivities of RNS relative to their reactive oxygen precursors (43). These reactivities include the avid capacity to cause nitration of tyrosine residues, both protein bound and free, resulting in the stable formation of 3-nitrotyrosine residues (44). Protein-3NT formation has been demonstrated to be a potent structural and functional post-translational protein modification, has deleterious effects on cardiac contractility, and has been observed in a panoply of cardiovascular disease states (45-50). We have recently shown that a mouse model of retroviral infection displays time-dependent increases in cardiac RNS formation during retroviral progression and that the extent of cardiac myocyte
protein-3NT formation was inversely correlated to cardiac performance (39). Our human studies recapitulated this phenomenon as cardiac prevalence of protein-3NT was drastically increased in HIV+/CVD+ patients as compared to HIV+/CVD- and HIV-/CVD+ (38). These changes occurred in the absence of cardiac NOS II induction. These murine and human findings suggest that RNS formation may participate in HIV/AIDS related cardiac pathology rather than NOS II induction. These results are in opposition to recent results published by Barbaro et al, which showed that increased cardiac NOSII staining may be a consistent phenomenon in AIDS patients with cardiac disease (51, 52). However, these data are consistent with the bimolecular reaction kinetics of RNS formation and support the under appreciated concept that NOS II induction is not obligatory for promotion of RNS formation and the sustained presence of protein-3NT \textit{in vivo}.

\textbf{Illicit drug abuse - Cocaine}

HIV/AIDS-related cardiovascular disease is often complicated by illicit drugs, most notably cocaine. In fact, since the beginning of the epidemic, intravenous drug use (IDU) directly and indirectly (enhanced at-risk sexual behavior) has accounted for nearly 36% of all AIDS cases in the United States (1). Unfortunately, the trend appears to be continuing and is not limited to intravenous drug use as “crack” smoking individuals were found to be three times more likely to be infected with HIV than non smokers (1, 53). The combination of cocaine abuse and HIV infection represents a unique challenge to the
optimization of therapy for HIV-related cardiovascular disease. Cardiac complications of cocaine abuse include chest pain, myocardial ischemia and infarction, myocarditis, dilated cardiomyopathy, congestive heart failure, cardiac arrhythmias, and sudden cardiac death. Vascular complications of cocaine abuse include coronary spasms, aortic dissection, atherosclerosis and vasculitis (54, 55). The exact mechanisms of cocaine induced cardiovascular toxicity alone and in combination with HIV infection are incompletely defined. Studies in animals and in in vitro cultures have shown that cocaine produces a wide range of effects on the immune system including alterations in lymphocyte populations, decreased immune reactivity and reduced macrophage cytotoxicity (56-58). Additionally cocaine has been shown to increase retroviral replication in cultured cells, apparently through the depletion of available glutathione stores and enhanced oxidative stress (59). Cocaine abuse has been associated with an increased susceptibility to neuroinvasion by its ability to modulate endothelial cell permeability (60, 61). Cocaine has been shown to increase mast cell recruitment to vascular endothelial cells and might also be able to modulate cardiac immune cell infiltration that has been shown to promote retrovirus related cardiovascular dysfunction (62-64). Cocaine’s ability to modulate immune responses and retroviral infection along with a lack of optimal therapy to block cocaine induced cardiovascular toxicities are serious concerns, as they may exacerbate cardiovascular influences of HIV infection. In summary, the combination of cocaine abuse and HIV infection is a reality that has to be considered when developing treatment strategies for HIV-related cardiovascular complications.
Co-infectious agents – bacterial and viral infections

Although HIV is now well established as the causative pathogen of AIDS, other covariate pathogens have been suspected as contributors to viral progression and complications (65-67). Several viral pathogens, such as the Coxsackie virus, Cytomegalovirus and Hepatitis C virus have been linked to AIDS related cardiovascular pathology (68-70). Coxsackievirus (CVB3) and cytomegalovirus (CMV) alone or in combination have been shown to aggravate retroviral-induced myocarditis in murine AIDS (LP-BM5 infection) (71, 72). Thus far, retroviral related cardiac problems have been linked to concurrent viral infections, but bacterial infections, with chlamydia pneumonia, helicobacter pylori, and mycobacterium tuberculosis, are emerging as important risk factors for cardiovascular disease in non HIV-infected populations (73-75). Furthermore, concurrent bacterial infections with mycobacterium avium complex, mycobacterium tuberculosis, haemophilus influenza, staphylococcus aureus, and escherichia coli are becoming increasingly important modulators of the severity and morbidity of AIDS (76-81). LPS, an integral cell wall component of gram-negative bacteria, is especially important because it has an important role in enhancing HIV’s ability to defeat the potent initial immune response against the retrovirus (66, 67, 82). On the other hand, LPS can also prevent macrophage infection by HIV when administered prior to HIV infection (83, 84). Concomitant bacterial pathogens may be critical modulators of cardiovascular complications, but its relationship to progression of cardiac complications in HIV
infection are not fully understood. A recent study from our laboratory in a murine model of retroviral infection has documented that a low dose of LPS could aggravate retroviral related cardiac pathology via cardiac innate immune regulation and immune cell trafficking (85, 86). Interestingly, the aggravated cardiac condition was not related to an enhanced retroviral progression, implying that bacterial (LPS mediated) modulation of retroviral pathogenesis might not be the sole mechanism involved in the co-infectious pathology of retroviral related cardiac dysfunction. Apparently, host immune response may play a role in these effects too.

**Drug modulation of HIV/AIDS related cardiovascular toxicity**

Given the dramatic need for agents that could interrupt the rapid progression to AIDS in HIV patients, a number of anti-retrovirals were developed and “fast-tracked” for use in humans. While HAART therapy is the most significant advance in HIV therapy, a number of unexpected toxicities are becoming more apparent with the use of these agents, both alone and in their combination. Most prominent among these toxicities have been cardiovascular effects, which may contribute, at least in part, to the cardiovascular complications seen in these patients. Drugs that are currently in use to treat HIV infection and its related complications that have been documented to have undesirable cardiovascular effects in both human and animal studies are shown in Table 4;
examples of these toxicities that have been described in the literature are reviewed below:

*Non-nucleoside Reverse Transcriptase Inhibitors (AZT)*

Zidovidune (AZT) was one of the first drugs introduced to combat HIV infection and now is currently used in HAART. Murine models of retroviral infection treated with AZT alone and in combination with other HAART drugs develop cardiac dysfunction along with cardiac mitochondrial damage (Lamividune & Indinavir) (87, 88). AZT is an important drug used to prevent prenatal transmission of HIV, and fortunately a prospective study following children treated with AZT *in utero* revealed that there was no long-term (~5 years) evidence of cardiac dysfunction (89, 90).

*HAART and protease inhibitors*

Other HAART drugs, most notably the protease inhibitors, have recently overshadowed AZT’s cardiotoxic potential. Initial case reports followed by extensive studies have documented that protease inhibitors may be linked to hypertriglyceridemia (due to abnormal lipid handling) and insulin resistance, both of which are risk factors for cardiovascular disease. Several studies have linked the use of these drugs to an increased evidence of coronary calcium and atherosclerosis in HIV infected patients. HIV infected individuals on protease inhibitors had approximately a 3-fold higher risk for coronary-events and myocardial infarction (91). However, a recent study in VA hospitals revealed
that HAART therapy might not increase the risk of cardiovascular events in the HIV population (92). The VA study may not be complete as it followed the medical records of only male patients. Protease inhibitors are also known to cause endothelial dysfunction, an initiating factor in atherosclerosis, in both humans and animal models (93, 94). Protease inhibitors, most notably ritonavir, have the ability to inhibit several metabolizing enzymes of cytochrome P450 pathway 3A4 (CYP3A4). Several lipid lowering drugs, statins, follow the same pathway, which might lead to increase levels of the statin drugs exacerbating their toxicity (rhabdomyolysis and myopathy). Therefore, this unique population of patients must be carefully evaluated before employing lipid-lowering therapy.

Cardiotoxic potential of drugs used to treat the HIV-related conditions and complications

Opportunistic infections are a common occurrence with HIV infection. Erythromycin, an anti-infective drug, has the potential of extending the QT interval, which predisposes an individual to Torsade de Pointes, ventricular arrhythmia (95). Similar QT interval prolongation has been observed with clarithromycin use (treatment of mycobacterial disease) (96). The fluorquinolones have also been associated with electrocardiographic alterations (97). Anti-fungal agents like amphotericin B andazole antifungals, widely used in HIV+ patients, have also been associated with long QT diseases and arrhythmias (98). Pentamidine (Pneumocystis carinii pneumonia treatment and prophylaxis) has also been shown to have QT interval toxicities (99). A high percentage
(~50% women and & 70% men) of HIV+ patients suffer from some sort of psychiatric disorder including mild anxiety, depression and psychosis (100). QT prolongation has been observed with the use of antidepressants like fluoxetine and doxepine (101). Several case studies have suggested antipsychotic drugs have cardiotoxic potential (102). Allergic reactions are fairly prevalent in the HIV+ population, and astemizole can prolong the QT interval. Cardiac dysfunction has been well documented with use of anti cancer drugs like doxorubicin (103). Interferon-α, used in the early stages of Kaposi Sarcoma and in chronic hepatitis C infection, has been shown to precipitate acute cardiovascular events (arrhythmias & acute coronary events) (104). IL-2, used to stimulate natural killer cells and induce differentiation of T-cells, has been documented to potential dose dependent cardio toxicity (105). Several of the drugs listed above have dose-limiting cardiotoxic effects that could potentially be aggravated when used in combination with protease inhibitors, many by virtue of their pharmacokinetics and metabolism. Clearly, the HIV patient needs to be carefully monitored with respect to drug treatment and considered with a broad appreciation for the multi-organ pathophysiology of HIV/AIDS.

Management of HIV-related cardiovascular disease (Early detection of cardiac disease, monitoring of concurrent drug therapy and novel therapeutic options)

There is currently little information regarding the management of cardiac disease in HIV/AIDS patients, despite substantial evidence that the presentation
and several mechanistic aspects of this form of cardiac disease may be unique to these patients. The following paragraphs outline some general suggestions and identify some underutilized therapeutic options that the current literature suggests might be beneficial to patient outcomes and care.

*Early detection using echocardiography and electrocardiography in the HIV+ population*

Most prospective studies evaluating cardiac studies in the HIV+ population primarily evaluated performance in the late stages of HIV infection. However, other studies document cardiac dysfunction in the early stage of the disease as well (16-19). Fortunately, non-invasive measures of cardiac function (echocardiography and electrocardiography) might provide early diagnosis in this population before they develop end stage heart failure. Even though cardiac echocardiography and electrocardiography could aide in early detection of cardiac anomalies, these measures might be expensive initially. However, the cost-effectiveness of early detection leading to optimization of therapy may help reduce expenses associated with HIV-related cardiac disease. Detection paradigms might be particularly useful in monitoring the complicating drug regimes that accompany HIV+ infection (Table 4). Electrocardiographic monitoring would enable physicians to titrate or modify therapy for HIV related conditions that are arrhythmogenic (Table 4). HAART is extremely important to overall survival in an HIV+ individual, but its potential cardiovascular toxicities have recently been overshadowing its therapeutic benefit. Therefore, serial
monitoring (echocardiography) prior to HAART along with follow up measures might aide in better management of cardiac complications. Endothelial dysfunction, an early event in coronary heart disease, can be non-invasively measured through flow mediated dilation studies and can also be used as an adjunct to cardiac evaluation. Fortunately, the wide spread documentation of cardiac disease, even in asymptomatic HIV+ patients, has prompted an increase in diagnostic cardiovascular measures in the HIV+ pediatric population, but could be improved upon in the adult population (106-110).

Prevention of direct HIV toxicity to the myocyte – Cardiac immune cell infiltration as a target for therapy

Unlike other most other forms of cardiovascular disease HIV-related cardiovascular disease has a single known “toxicant” – the retrovirus executing several pathways detrimental to cardiac myocytes. If HIV is directly infecting the myocyte, then one must develop drugs to block HIV’s entry into the myocyte. Such drugs have not been developed because there is still controversy as to what receptors are allowing HIV entry into myocytes. Chemokine receptors have been suggested as a likely suspect, but known HIV-1 chemokine receptors are yet to be found on a myocyte. Furthermore, myocytes have not been shown to bear a CD4 epitope, a primary receptor for HIV-1 entry into a cell. Even if HIV does not directly infect a myocyte, it has still been found in cardiac tissue, which is not to rule out HIV-1 eliciting cardiotoxic effects through its carriers, most notably the macrophages. We have found that the prevalence of cardiac CD68+
cells (monocytes and macrophages) strongly correlated to cardiac dysfunction in a murine model of retroviral infection (85). Corroborative studies in human autopsy samples revealed a 2-3 fold increase in cardiac presence of CD68+ cells (monocytes/macrophages) in HIV+/CVD+ hearts, compared to their respective controls for cardiac disease (HIV-/CVD+) and viral infection (HIV+/CVD-) (38). Cardiac infiltration was distinctly elevated in only the HIV+/CVD+ group, and may be a mechanism by which HIV drives cardiac toxicity in this patient population. While the specific factors involved in the recruitment and/or activation of these immune cells to the heart in this setting remain unknown, much is known generally about organ recruitment of immune cells, and many potential candidates could be proposed (selectins, immunoglobulin adhesion molecules, etc). Further studies defining the molecular interactions and consequences involved are warranted to help design and develop therapy targeted against cardiac immune cell pathology.

Cardiac Protein 3-NT prevention?

We have found that peroxynitrite (ONOO-') and related reactive nitrogen species (RNS) may play an important role in retroviral related cardiac dysfunction. Peroxynitrite, an aggressive free radical is formed by the diffusion rate-limited reaction of nitric oxide and superoxide anion and is known to oxidize cellular lipids, proteins, DNA, and promote cardiac cell death via both necrosis and/or apoptosis (41, 111). Peroxynitrite has the avid capacity to cause nitration of tyrosine residues, both protein bound and free, resulting in the stable formation
of 3-nitrotyrosine residues (44). Protein-3NT formation has been demonstrated to be a potent structural and functional post-translational protein modification, and has deleterious effects on cardiac contractility (45, 46, 49, 50, 112). Cardiac prevalence of protein-3NT was drastically increased in HIV+/CVD+ patients as compared to HIV+/CVD- (seropositive controls) and HIV-/CVD+ (cardiac disease controls), indicating that RNS mediated cardiac protein 3-NT formation might be a good therapeutic target (38). Antioxidants have already shown to be useful in controlling HIV/AIDS, but their utility in HIV-related cardiac pathology is yet to be determined (113-117). Specific peroxynitrite scavengers like ascorbate may be especially useful in managing HIV/AIDS related cardiac pathology as it has been recently documented to be an effective peroxynitrite decomposition catalyst in a biological setting (118). We have also recently shown that a mouse model of retroviral infection displays time-dependent increases in cardiac RNS formation during retroviral progression, and that the extent of cardiac myocyte protein-3NT formation was inversely correlated to cardiac performance (39). Thus, this murine model might be a good paradigm to test the therapeutic potential of peroxynitrite scavengers in retroviral related cardiac pathology.

Prevention of premature coronary heart disease

HAART has dramatically increased survival among HIV+ patients, but the drug cocktail, specifically protease inhibitors, has been recently blamed for hypertriglyceridemia, insulin resistance and abnormal fat distribution. Hypertriglyceridemia and insulin resistance are both risk factors for myocardial
infarction and coronary artery disease. Lipid lowering therapy should be used with caution in an HIV+ infected population on protease inhibitors. Protease inhibitors may aggravate the side effects (skeletal muscle toxicity) of effective lipid lowering drugs (atorvastatin), as they interfere with the CYP3A enzymes. Pravastatin is better choice because it has a different clearance route as compared to such drugs as atrovastatin. Diet and exercise are recommended alternatives to controlling lipids, but somehow this option seems drastic and impossible in a set of patients “wasting” away from AIDS. However, this option has been shown to have some benefit in HIV+ patients (119). Fibric acid analogues (gemfibrozil and fenofibrate) and niacin are also useful in lowering serum triglycerides in uninfected populations, but its usefulness in HIV+ patients is unclear. HIV+ patients are a unique population of cardiac patients because regular cardiovascular therapy has to be used with caution to reduce complications. Furthermore, even though the protease inhibitors may aggravate and/or promote HIV-related cardiac pathology, they are too important to HIV management not to be used.

Controlling co-infectious pathology in an effort to prevent worsened cardiac pathology

Concurrent pathogen exposure is not uncommon in HIV/AIDS. Bacterial infections, specifically gram-negative infections have been shown to modulate HIV pathogenesis via lipopolysaccharide (LPS). We have recently documented that low dose LPS augments retroviral related cardiac pathology in a murine
model of AIDS, through an upregulation of cardiac innate immune pathology. Under normal physiologic settings, Toll like receptors (TLR), as gatekeepers of the innate immune system, are involved in recognition and transmission of pathogenic stimuli. Most notable are the TLR4 receptors that sense the presence of bacterial LPS by eliciting inflammatory and oxidative pathways in immune cells (120). Interestingly, these receptors have been detected on cardiac myocytes, and the upregulation of TLR4 has been noted in several cardiac disease settings (121). Our finding in the mouse might suggest that cardiac TLR4 receptors could be a potential target for HIV-related cardiac pathology, especially if concurrent bacterial infections are involved (122). However, specific inhibitors of cardiac TLR4 are yet to be developed, but several groups have been working on developing a therapeutic TLR4 inhibitor for the treatment of sepsis related conditions.

Overall conclusions

HIV infection is a devastating disease that affects 900,000 people in the United States and 42 million people worldwide (1). Cardiovascular disease is an important complication of HIV infection, affecting a large proportion of these patients. Severe cardiac complications, like congestive heart failure, are primarily seen in AIDS patients, but asymptomatic patients also have detectable and significant signs of LV dysfunction—the connections between these conditions, (i.e. Does early pathology mediate more severe latent events? Can early
intervention delay or prevent AIDS-related cardiomyopathy?), remain to be defined. Prognoses for HIV patients with these events are often significantly worsened. Despite the general recognition that cardiovascular abnormalities develop in these patients and the high costs associated with patient care, the mechanisms involved remain largely undefined. HIV-related cardiovascular disease is a unique form of cardiovascular disease and traditional cardiovascular therapy may not be optimal for this population. HIV infection and cardiac complications in humans are often complicated by illicit drug abuse and covariate infections—these co-variates clearly impart adverse effects on disease progression, but the mechanisms involved are very poorly understood. While the number of patients affected by this complication of HIV/AIDS is likely to continue to grow, a number of promising experimental approaches have been developed to address this problem, including more relevant animal models, a larger appreciation for the broad scope of HIV/AIDS related effects, and the development of larger and better controlled patient populations for clinical and histopathological study. These advances, combined with a greater appreciation of the importance of this condition to the pathology of HIV/AIDS, will hopefully result in the development of therapeutic approaches that are specific to and effective for this unique and growing patient population.
REFERENCES


<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1981</td>
<td>1st reported case of “unknown” case of PCP in LA, that was later associated with AIDS</td>
<td>(8)</td>
</tr>
<tr>
<td>1983-1984</td>
<td>Isolation of AIDS-associated virus</td>
<td>(123, 124)</td>
</tr>
<tr>
<td>1983</td>
<td>Documentation of cardiac involvement in AIDS-myocardial Kaposi Sarcoma</td>
<td>(125)</td>
</tr>
<tr>
<td>1984</td>
<td>HIV (HTLV-III) identified as the causative agent in AIDS</td>
<td>(9)</td>
</tr>
<tr>
<td>1984</td>
<td>Echocardiographic detection of cardiac abnormalities in AIDS</td>
<td>(126)</td>
</tr>
<tr>
<td>1985</td>
<td>Autopsy on AIDS patients &gt; cardiac lesions might be related to morbidity &amp; mortality</td>
<td>(15)</td>
</tr>
<tr>
<td>1986</td>
<td>1st report of dilated cardiomyopathy in HIV+ patients</td>
<td>(127)</td>
</tr>
<tr>
<td>1987</td>
<td>Isolated &amp; cultured HIV-1 from cardiac biopsy</td>
<td>(22)</td>
</tr>
<tr>
<td>1990</td>
<td>Initial PCR detection of HIV-1 RNA in cardiac tissue from a patient infected with HIV related cardiovascular abnormalities</td>
<td>(23)</td>
</tr>
<tr>
<td>1990</td>
<td>Suggestion of HIV-1 presence in human myocyte</td>
<td>(25)</td>
</tr>
<tr>
<td>1993</td>
<td>Cardiac dysfunction a serious concern in children with HIV infection</td>
<td>(21)</td>
</tr>
<tr>
<td>1996</td>
<td>Highly Active Anti-Retroviral Therapy (HAART) introduced &gt; HIV+ patient survival improving – emergence of complications</td>
<td>(128)</td>
</tr>
<tr>
<td>1998</td>
<td>Asymptomatic HIV patients with left ventricular (LV) dysfunction</td>
<td>(129)</td>
</tr>
<tr>
<td>1998</td>
<td>peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors</td>
<td>(130)</td>
</tr>
<tr>
<td>1998</td>
<td>Pediatric Pulmonary and Cardiac Complications of Vertically Transmitted HIV Infection (P2C2 HIV) Study initiated – detection of LV dysfunction in pediatric population too</td>
<td>(109)</td>
</tr>
<tr>
<td>2000</td>
<td>Poor prognosis with cardiomyopathy due to HIV infection</td>
<td>(6)</td>
</tr>
<tr>
<td>2000</td>
<td>Endothelial Dysfunction noted in HIV+ patients</td>
<td>(131)</td>
</tr>
</tbody>
</table>

**Table 2.1:** Time-line of events that outline HIV/AIDS related cardiovascular disease.
### Incidence of Cardiac Complications in an HIV+ patient population

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Patient #</th>
<th>Dilated Cardiomyopathy</th>
<th>Myocarditis</th>
<th>Endocaditis</th>
<th>Pericardial effusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>(126)</td>
<td>15</td>
<td>20%</td>
<td></td>
<td>7%</td>
<td>53%</td>
</tr>
<tr>
<td>(15)</td>
<td>41</td>
<td></td>
<td>2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(132)</td>
<td>54</td>
<td>31%</td>
<td>55%</td>
<td></td>
<td>9%</td>
</tr>
<tr>
<td>(133)</td>
<td>26</td>
<td></td>
<td>35%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(134)</td>
<td>187</td>
<td>9%</td>
<td></td>
<td></td>
<td>18%</td>
</tr>
<tr>
<td>(13)</td>
<td>71</td>
<td>10%</td>
<td>52%</td>
<td></td>
<td>21%</td>
</tr>
<tr>
<td>(131)</td>
<td>86</td>
<td>15%</td>
<td></td>
<td></td>
<td>21%</td>
</tr>
<tr>
<td>(135)</td>
<td>58</td>
<td></td>
<td>45%</td>
<td></td>
<td>19%</td>
</tr>
<tr>
<td>(136)</td>
<td>115</td>
<td>3%</td>
<td></td>
<td></td>
<td>32%</td>
</tr>
<tr>
<td>(14)</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(137)</td>
<td>60</td>
<td>38%</td>
<td>42%</td>
<td></td>
<td>21%</td>
</tr>
<tr>
<td>(138)</td>
<td>440</td>
<td>3%</td>
<td>7%</td>
<td>6%</td>
<td>12%</td>
</tr>
</tbody>
</table>

**Table 2.2:** Retrospective studies of HIV/AIDS related cardiac dysfunction.
<table>
<thead>
<tr>
<th>Ref.</th>
<th>Patient #</th>
<th>Durat. (mths)</th>
<th>Incidence of Cardiac complications in an HIV+ patient population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DCM</td>
</tr>
<tr>
<td>(126)</td>
<td>15</td>
<td></td>
<td>20%</td>
</tr>
<tr>
<td>(127)</td>
<td>71</td>
<td></td>
<td>10%</td>
</tr>
<tr>
<td>(139)</td>
<td>102</td>
<td></td>
<td>41%</td>
</tr>
<tr>
<td>(140)</td>
<td>38</td>
<td>24</td>
<td>11%</td>
</tr>
<tr>
<td>(18)</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(141)</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(142)</td>
<td>15</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>(143)</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(144)</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(145)</td>
<td>70</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td>(146)</td>
<td>28</td>
<td></td>
<td>21%</td>
</tr>
<tr>
<td>(147)</td>
<td>70</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>(148)</td>
<td>173</td>
<td>18</td>
<td>8%</td>
</tr>
<tr>
<td>(17)</td>
<td>114</td>
<td>45</td>
<td>17%</td>
</tr>
<tr>
<td>(149)</td>
<td>69</td>
<td>11</td>
<td>6%</td>
</tr>
<tr>
<td>(150)</td>
<td>124</td>
<td>18</td>
<td>12%</td>
</tr>
<tr>
<td>(5)</td>
<td>296</td>
<td>48</td>
<td>4%</td>
</tr>
<tr>
<td>(17)</td>
<td>136</td>
<td>54</td>
<td>5%</td>
</tr>
<tr>
<td>(151)</td>
<td>174</td>
<td>54</td>
<td>22%</td>
</tr>
<tr>
<td>(16)</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(152)</td>
<td>157</td>
<td>12</td>
<td>9%</td>
</tr>
<tr>
<td>(20)</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(147)</td>
<td>68</td>
<td>32</td>
<td>15%</td>
</tr>
<tr>
<td>(138)</td>
<td>952</td>
<td>60</td>
<td>8%</td>
</tr>
<tr>
<td>(129)</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(153)</td>
<td>181</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>(154)</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(19)</td>
<td>61</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3: Prospective studies of HIV/AIDS related cardiac dysfunction.

[(DCM: Dilated Cardiomyopathy, MyoC: Myocarditis, EndoC: Endocarditis)
*p<0.05 vs. healthy non-infected controls]
<table>
<thead>
<tr>
<th>Reference</th>
<th>Drugs</th>
<th>Therapeutic use in HIV+ infected populations</th>
<th>Cardiotoxic potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>(156, 157)</td>
<td>Nucleoside transcriptase inhibitors (NRTIs): Zidovudine, Didanosine, Zalcitabine, Stavudine, Lamivudine, Abacavir</td>
<td>Control HIV infection</td>
<td>Cardiomyopathy?</td>
</tr>
<tr>
<td>(158)</td>
<td>Non-nucleoside transcriptase inhibitors (NNRTIs): Nevirapine, Delavirdine, &amp; Efavirenz</td>
<td>Control HIV infection</td>
<td>Increased risk for cardiovascular disease?</td>
</tr>
<tr>
<td>(130, 159)</td>
<td>Protease Inhibitors: Saquinavir, Ritonavir, Indinavir, Nelfinavir, Amprenavir, Lopinavir</td>
<td>Control HIV infection</td>
<td>Increased risk for Coronary Artery Disease &amp; cardiovascular risk?</td>
</tr>
<tr>
<td>(96, 160)</td>
<td>Anti-infective drugs; Ethromycin, Clarithromycin, Trimethoprim/Sulfamethoxazole, Pentamidine, Fluoroquinolones, Amphotericin B, Azole antifungals</td>
<td>Opportunistic infections therapy</td>
<td>QT Interval prolongation</td>
</tr>
<tr>
<td>(102)</td>
<td>Antipsychotics: Thioridazine, Chlorpromazine, Pimozide, Sertindole, Haloperidol</td>
<td>Psychotic Disorders</td>
<td>QT interval prolongation</td>
</tr>
<tr>
<td>(101, 161)</td>
<td>Antidepressants; Amitryptiline, Doxepine, Desipramine, Imipramine, Clomipramine</td>
<td>Depression</td>
<td>QT interval prolongation</td>
</tr>
<tr>
<td>(95)</td>
<td>Antihistamines: Astemizole, Terfenadine</td>
<td>Allergic reactions neoplasms</td>
<td>QT interval prolongation</td>
</tr>
<tr>
<td>(103)</td>
<td>Anti-cancer agents: Anthracylines (Doxorubicin)</td>
<td></td>
<td>Cardiomyopathy</td>
</tr>
<tr>
<td>(104)</td>
<td>Interferons: IFNα</td>
<td>Kaposi Sarcoma &amp; Hepatitis C infection</td>
<td>Cardiomyopathy</td>
</tr>
<tr>
<td>(162)</td>
<td>Interleukin2 (IL-2)</td>
<td>Immunotherapy</td>
<td>Cardiomyopathy</td>
</tr>
</tbody>
</table>

**Table 2.4:** Cardiotoxicity of drugs used for HIV and HIV related conditions.
(modified from Fantoni et al, 2000)(155)
CHAPTER 3

CARDIOMYOPATHY IN A MURINE MODEL OF AIDS: EVIDENCE OF REACTIVE NITROGEN SPECIES AND CORROBORATION IN HUMAN HIV/AIDS CARDIAC TISSUES

This chapter has been presented in the format of the journal (Cardiovascular Research) that will publish this article in their October 2003 Issue.
ABSTRACT

Objective: Cardiomyopathy and other vascular complications are now recognized as significant components of HIV/AIDS pathogenesis. Although the mechanisms involved in cardiomyopathy are poorly defined, a role for direct retroviral action and/or focal infiltration of activated immune cells have been postulated. Here we investigated mechanisms in retrovirus associated cardiomyopathy using a well-defined mouse model of acquired immunodeficiency. Methods: Mice were dosed with LPBM5 retrovirus; cardiac performance was assessed by echocardiography followed by tissue collection at 5 and 10 weeks post-infection. Results: Contractile deficits were observed at 5 and 10 weeks post retrovirus infection and preceded the development of overt immunodeficiency. Selective and widespread cardiac infiltration of CD68+ cells, but not neutrophils, mast cells, or eosinophils was also observed at both 5 and 10 weeks. LPBM5 retrovirus was readily detectable in cardiac samples by RT-PCR. Time dependent increases in cardiac protein nitration (biomarker of reactive nitrogen species) were observed and were correlated to extent of cardiac dysfunction whereas no changes in NOSII occurred at 5 and 10 weeks. We corroborated the mouse findings using cardiac tissues and clinical findings from human HIV/AIDS autopsies. Conclusions: These studies demonstrated that cardiac myocyte protein nitration in AIDS related cardiomyopathies, rather than focal immune
cell lesions characterize retrovirus associated cardiomyopathies and differentiate them from non retroviral cardiomyopathies.

**Keywords:** experimental & clinical, heart, organism & organ, pathophysiology nitric oxide, reactive nitrogen species, HIV, AIDS, cardiomyopathy, retrovirus
INTRODUCTION

Following the advent of highly active antiretroviral therapy (HAART) regimens, AIDS-free living and overall survival have significantly improved for patients infected with human immunodeficiency virus (HIV) [1]. However, a variety of HIV-related complications have become increasingly evident in this population. For example, HIV-related pulmonary, neurological, and cardiovascular complications represent important contributors to the overall morbidity and mortality of HIV/AIDS patients [1,2]. HIV-related cardiovascular disease was first recognized in the early 1980’s, primarily via autopsy cases in which myocarditis and cardiac inflammatory lesions were observed in hearts from AIDS patients with no documented evidence of cardiovascular disease [3,4]. Later studies demonstrated that HIV/AIDS patients develop an array of cardiovascular pathologies, ranging from left ventricular deficits in pre-symptomatic seropositive individuals to pulmonary hypertension, dilated cardiomyopathy and congestive heart failure [5,6]. The prevalence of cardiac complications in HIV patients has been estimated to be as high as 80%, with an estimated 5-20% progressing to dilated cardiomyopathy and failure [1,7]. As the worldwide incidence of AIDS approaches an estimated 5,000,000 new cases annually, HIV-related cardiovascular disease may become a significant etiology of cardiac failure [8,9]. The mechanisms by which this unique form of cardiovascular disease develops and its relation to more traditional settings (non-retroviral) of cardiomyopathy remains incompletely defined. As a result,
no specialized therapeutic approaches for this patient population currently exist.

Unfortunately, the study of relationships between human HIV infection and cardiac alterations is often complicated by the use of various drug therapies, illicit drugs known to be cardiotoxic, limited tissue availability and variable disease progression [10]. Demonstration of relevant animal models for the investigation of retrovirus-related cardiac dysfunction and pathologies could provide opportunities for further mechanistic insight and therapeutic intervention. In this study, we employed a well-established murine model of retroviral infection (LPBM5 virus) and defined the time-dependencies of retroviral progression and cardiac dysfunction, in an attempt to extend its relevance as a disease model. The LPBM5 model of retroviral infection is commonly called the "murine AIDS" model, due to its high similarity to many of the immune-related complications seen during human HIV infection, including aberrant cytokine release, changes in T-cell populations, and increased susceptibility to opportunistic infections (for a detailed review see Watson 1989).

An additional goal was to define the contributions of nitric oxide (NO)-related biochemistry during murine retrovirus related cardiac dysfunction. Under normal physiological conditions, NO is known to modulate cardiac myocyte contractility and blood flow distribution [11]. In contrast, high levels of NO production (via inducible nitric oxide synthase, NOSII) are associated with several forms of cardiac disease, and have recently been implicated in human
HIV-related cardiomyopathy [12]. Once formed, NO rapidly reacts with available superoxide anion to form peroxynitrite, a reactive nitrogen species known to conduct several oxidative reactions in a cellular environment [13]. Of these, nitration of protein tyrosine residues, producing 3-nitrotyrosine, is a unique feature of reactive nitrogen species when compared to actions of reactive oxygen species (e.g., superoxide anion, hydroxyl radical, etc.) [14]. We and others have demonstrated that reactive nitrogen species formation may be an important participant in the initiation and/or progression of a wide array of cardiac pathologies and that nitration of protein tyrosine residues can inhibit cardiac enzyme function, and promote cardiac myocyte death via necrosis and/or apoptosis [15-17]. In addition, peroxynitrite mediated protein nitration has previously been demonstrated in the setting of acute viral myocarditis, but its contribution to cardiac dysfunction during retroviral pathogenesis has not been established [18]. Therefore, we tested the hypotheses that cardiac NOS II expression and/or cardiac protein nitration are associated with time dependent cardiac dysfunction during murine AIDS progression.

To corroborate our experimental findings in the murine AIDS model, we explored the relevance of our experimental results in a sample of cardiac autopsy tissues from HIV/AIDS patients with dilated cardiomyopathy and relevant controls.
METHODS

*Murine AIDS model and study design:*

All aspects of our animal use were in accordance with the guidelines of the National Institutional of Health and approved by the Institutional Animal Care and Use Committee. Active LP-BM5 virus was prepared according to the methods of Watson et al. [19]. Retrovirus-containing cell-free supernatant was collected from infected SCI/MuLV cells (AIDS Research and Reference Reagent Program, Bethesda, MD), and concentrated by centrifugation (Advanced Biotechnologies, Inc., Columbia, MA). Titers of esotropic MuLV were determined by the standard S’L plaque assay [20] and by units of reverse transcriptase activity using a commercially available kit (Boehringer Mannheim, Germany).

Pathogen-free female C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) were housed in a sterile cage rack system with HEPA filtered air circulation (approximately 50 air changes/hour, Allentown Caging Inc., Allentown, PA). Following 1-2 weeks of acclimatization LP-BM5 retrovirus was dosed via a single intraperitoneal injection (100 µl dose containing 200 reverse transcriptase units). Control animals received an identical injection of vehicle. At the time of injection all mice were six weeks old and weighed 16-18g.

Cardiac performance was assessed by echocardiography, as previously described [21], at selected times throughout 10 weeks of retroviral infection (8-12 per group). To account for time-variations, animal age and body weight, two groups of control animals were studied at 0 week and 10 weeks.
post injection \(n=6\) in each group). Immediately following echocardiography, animals were sacrificed by pentobarbital overdose. Spleen weight was measured as an index of retroviral progression, as documented by others [19,22].

**Reverse transcriptase-polymerase chain reaction (RT-PCR) for LPBM5:**

Total RNA was isolated from frozen cardiac and splenic tissue (Trizol, Gibco BRL). RNA quantity was monitored at 260nm. RNA integrity was verified by fractionating on agarose gels. Two \(\mu\)g of total RNA was reverse transcribed to cDNA (cDNA cycle kit, Invitrogen) in 20 \(\mu\)L reverse transcription reaction mix at 42°C (60 min). The reaction was stopped by heat inactivation at 95°C (2 min) and chilled on ice. Subsequently 5 \(\mu\)L of the resulting cDNA was amplified using primers specific for the p12 region in the gag gene in the LP-BM5 genome [23] and \(\beta\)-actin [24]. The RTV primers: sense primer, 5’-CCT TTATCGACACTTCCCTT-3’; antisense primer: 5’-CCGCCTCTTCTTAACTGGTC-3’. Similarly \(\beta\)-actin primers: sense primer: 5’-ATGGATGACGATATCGCT-3’; antisense primer: 5’-ATGAGGTAGTGCTGCTAGGT-3’ were used in PCR reactions under the same conditions which included an initial denaturation at 95°C (5 min), followed by a cycle of denaturation (95°C/1 min), annealing (55°C/1 min), and extension (72°C/1 min). Each sample was subjected to 30 cycles followed by a final extension (72°C/10 min). PCR products were separated and visualized on a 2% agarose ethidium bromide stained gel. Band intensity was assessed using
imaging software (UVP-Labworks analysis), normalized to β-actin expression in each tissue. Cardiac RTV expression was expressed as a percentage of splenic RTV expression.

**Blood sampling and analysis:**

Whole blood was collected at sacrifice. Liver, pancreatic and muscle enzyme activity (AST, ALT, lipase and amylase), as well as total cholesterol and triglyceride levels were measured. Complete blood chemistries using whole blood smears were used to measure eosinophils, neutrophils, monocytes and lymphocytes (Antech diagnostics).

**Histology and immunohistochemistry:**

Following functional analyses, hearts were rapidly isolated, equatorially sectioned (mitral valve), and processed for immunohistochemical studies using standard protocols [17]. Cardiac and splenic cross-sections were stained using hematoxylin/eosin and Masson's trichrome for routine morphologic and histologic assessments. Cardiac 3-nitrotyrosine (anti-3NT antibody, Upstate Biotechnology, Lake Placid, NY, 1:400 dilution) and nitric oxide synthase 2 (anti-NOS2, Transduction Labs, Lexington, KY, 1:400 dilution) were assessed in cardiac cross-sections [17]. Staining controls included antibodies preadsorbed with purified 3NT or murine NOS II; addition of antigen eliminated positive staining in each case, demonstrating antibody specificity. Histological stains for mast cells (Astra Blue stain, Sigma, St. Louis, MO) and eosinophils
(Vital Red stain, Sigma, St. Louis, MO), as well immunohistochemical probes for neutrophil (anti-myeloperoxidase (MPO) antibody, Neomarkers, 1:2000 dilution) and monocyte/macrophage presence (anti-CD68+, Neomarkers, 1:400) were employed. In additional studies, NOSII stained tissues were assessed for NOSII positive cell bodies as well. Diaminobenzidine (0.06% w/v) was used to provide visualization of immunoreactivity, with methyl green counterstaining.

**Digital image analysis:**

Digital images were acquired using a Polaroid DMC camera and Olympus microscope (model BX40) and transferred to Image Pro Plus software (Media Cybernetics, Silver Spring, MD) for both area and intensity analyses. Total cross-sectional images of spleen and heart were captured at 4x magnification and circumferential traces were used to calculate tissue areas. Images from cardiac samples assessed for specific immune infiltrates (MPO+, CD68+, mast cells, NOSII+) were captured at 400x magnification, positive cells were segmented and gated based on size, then counted and expressed per tissue area. In NOSII and 3NT studies, left ventricular images were captured at 400x, and relative intensity was determined using image threshold analysis, as we have previously described [17].
**HIV/AIDS cardiac specimens:**

Left ventricular sections (LV anterior wall) were obtained as paraffin-embedded autopsy specimens from AIDS patients and non-HIV infected controls from the National Cancer Institute AIDS Cancer and Specimen Resource. All autopsy samples studied were collected within 4 hours of death. Autopsy samples were collected between 1983 and 1998. Patient histories were reviewed to subclassify samples into 2 groups: HIV infected patients with documented evidence of dilated cardiomyopathy (HIV-DCM, n=4) and non-HIV infected patients with no evidence of cardiac disease (CTRL, n=3). HIV/AIDS patients with confounding risk factors for cardiovascular disease (smoking, diabetes, protease inhibitor therapy, etc) were omitted from analysis. Age at autopsy was not different between patient groups (CTRL: 40±2.3 yrs, HIV-DCM: 36.5±3.2yrs, p=NS), and was not a significant factor for cardiovascular disease. Average ejection fraction in HIV+ patients with dilated cardiomyopathy was 27.5±7.5%, mean CD4+ counts were 123.5±46.5 cells/mm³. These cardiac specimens were then assessed for evidence of NOS II and protein-3NT, using immunohistochemical methods identical to those described above.

**Statistical analysis:**

All data presented represent 6-12 observations per group. Statistical comparisons were made by one-way ANOVA’s with Student-Newman-Keuls
post hoc tests. Significant correlations were assessed using Spearman's non-parametric correlation analysis. A total of 30-35 data points were used for each regression analysis (CTRL, 5, 10 weeks RTV), providing statistical power $>0.95$ at $r^2=0.5$ and $\alpha=0.05$. $p<0.05$ defined statistical significance.
RESULTS

Murine AIDS studies

Two groups of control animals were studied at 0 and 10 weeks post vehicle injection to account for time-variations, animal ages and body weights. In all parameters presented below, no statistically significant differences were observed in these two control groups. Therefore, they were pooled for further comparisons to retrovirus-infected mice.

Splenomegaly and cardiac expression of LP-BM5 during murine retroviral pathogenesis

Splenomegaly is consistently observed in murine AIDS and used as a marker of retroviral progression and immune dysfunction [19]. Representative mid-line splenic cross sections from control and LPBM5-treated mice at 10 weeks post-injection are shown in Figure 1A (Upper panel), illustrating increases in organ size and disordered organ architecture (hematoxylin and eosin staining shown). Total spleen weight in control vs. retrovirus infected animals at sacrifice are shown in Figure 1A (lower panel). Spleen weight was significantly increased, indicating retroviral progression and immune dysfunction. Additional staining with Masson's trichrome did not reveal evidence of splenic fibrosis (data not shown).

LPBM5 infectivity is facilitated by the gag, pol, and env genes [25]. The p12 region in the gag gene is unique to the LPBM5 virus and important to infectivity [26]. Shown in Figure 1B (Upper panel) are representative RTV
expression bands in cardiac and splenic tissue from RTV infected mice at five and ten weeks. The 229bp RT-PCR product, specific for the p12gag region of LPBM5, was confirmed by restriction enzyme analysis with Sma I (data not shown). Viral load in cardiac tissue (expressed as percentage of splenic viral load) is depicted in Figure 1B (Lower panel). Virus was present in both cardiac and splenic tissue (homogenates) in RTV-treated animals at 5 and 10 weeks post-treatment; cardiac load was approximately 50% of splenic load (normalized to β-actin) at each time point.

**Blood leukocytes during retroviral pathogenesis**

LPBM5 infection results in an immunosuppressive profile that closely resembles human AIDS. Shown in Figure 2 are average total circulating blood monocytes, neutrophils, lymphocytes and eosinophil counts from control and retrovirus infected animals. Total levels of monocytes in whole blood were reduced at five and ten weeks post infection with RTV (Figure 2, Upper left panel). In contrast, circulating levels of neutrophils, lymphocytes and eosinophils remained unchanged.

**Cardiac morphology and histology**

Shown in Figure 3 are representative photomicrographs of equatorial cardiac cross-sections from control and RTV-treated mice at 10 weeks. Hearts from RTV-treated mice developed a hypertrophic response by 5 weeks, which persisted throughout the study (Figure 3, middle panel). LPBM5-treated
animals did not develop significant cachexia until after 10 weeks; the increase in normalized heart weight was not artifactually caused by body weight loss. A significant increase in LV cross-sectional area (at the level of the mitral valve) was observed at 5+ weeks post infection. This measure of LV mass (roughly a 25% increase from control) paralleled changes in total heart weight observed at 5 and 10 weeks.

**Time-dependent reduction in cardiac performance during murine AIDS**

Cardiac performance was assessed at 5 and 10 weeks using echocardiography (Figure 4). No difference in heart rate was detected at any time point. Murine AIDS was associated with progressive decrements in left ventricular (LV) fractional shortening, cardiac output, and stroke volumes. At 10 weeks retroviral infection, reductions in these parameters were approximately 15, 30, and 35% respectively, relative to control (Figure 4, Lower panel). Reductions in fractional shortening were predominantly related to increases in LV end systolic dimension (1.72 ± 0.04 mm vs. 1.60 ± 0.02 mm at 10 weeks, RTV vs. control).

**Cardiac immune cell prevalence during retroviral pathogenesis**

Leukocyte infiltration into cardiac tissue plays an important role in promoting and sustaining infectious cardiac diseases [27]. Shown in Figure 5 are averaged data of cardiac infiltrating immune cells as measured by histochemical techniques. Nominal presence of neutrophils and eosinophils
were detected at 10 weeks post-infection, whereas cardiac infiltration of CD68+ cells (monocytes/macrophages) was significantly increased at 10 weeks post-infection, compared to controls (Figure 5, Upper middle panel). Cardiac presence of neutrophils, eosinophils, and mast cells were not significantly elevated with retrovirus infection (p=NS). The levels of CD68+ and neutrophils (MPO+ cells) were not measured at five weeks post infection, due to technical problems. Although changes in immune cell prevalence were detected by our imaging approach, we observed limited evidence of classical focal lesion sites in any region of the cross-sectional areas studied.

**NOSII immunohistochemistry and cardiac protein nitration**

Representative photomicrographs of cardiac NOS II and protein-3NT immunoprevalence during murine AIDS are shown in Figure 6, where brown staining indicates positive immunoprevalence. NOSII content was not significantly altered in cardiac myocytes at five and ten weeks post infection, whereas sustained and extensive cardiac myocyte protein nitration was observed at 5 and 10 weeks during retroviral infection (p<0.05). Additional observation of the cross-sections revealed the presence of NOS II positive mononuclear cells, which was consistent with the general distribution of CD68+ infiltrates. Using automated cell counts by methods identical to those in Figure 5, we found that NOS II positive cell bodies are significantly elevated in hearts from MAIDS mice at 10 weeks of retroviral infection, relative to control (NOS II positive cells: 14.8±2.1 vs. 21.0±3.0 cells/mm², CTRL vs. 10
week RTV, p<0.05). The relative increases in these NOS II positive cells matches that observed for CD68+ cells over the same time course (CD68+ cells: 14.8±2.2 vs. 21.1±1.4 cells/mm²), thus confirming that these NOS II positive cells are apparently monocyte/macrophage lineage. In contrast to this focal distribution of NOS II, immunoprevalence of 3NT was widely distributed throughout myocardium and was therefore dissociated from the distribution of NOS II.

**Relationships of cardiac performance and immunohistochemical measures**

Non-parametric correlation analyses were used to test for statistical relationships among in vivo cardiac functional parameters and immunohistochemical results. A total of 60 data points were used for each regression analysis, providing a statistical power of 0.986 at $r^2=0.5$ and $\alpha=0.05$. Cardiac immunoprevalence of 3NT was statistically correlated to LV fractional shortening % ($r^2 = -0.600, p<0.01$), maximal aortic flow velocity ($r^2 = -0.559, p<0.01$), and LV cross-sectional area ($r^2 = +0.515, p<0.02$). In contrast, no statistically significant relationships were observed between NOSII immunoprevalence and any cardiac functional parameter.
**Human tissue investigations**

**Cardiac NOSII versus protein nitration**

Shown in Figure 7 are representative photomicrographs in human cardiac autopsy tissues from AIDS patients with non-ischemic dilated cardiomyopathy (HIV-DCM) and seronegative controls with no documented evidence of cardiac disease. Identical to our mouse studies, we observed striking evidence of cardiac myocyte protein-3NT formation, with no corresponding increases in NOSII presence. Similar to the murine studies described above, NOS II positive mononuclear cells were detected among the cardiac tissue cross-sections in this small population of HIV/AIDS patients and these were spatially dissociated from cardiac myocyte protein nitration.
DISCUSSION

Cardiovascular disease is a significant complication of HIV/AIDS. However, the mechanistic study of this phenomenon in humans has proven difficult. Here, we pursued the development of a relevant and convenient animal model for the mechanistic study of retrovirus-related cardiovascular dysfunction. Several recent reports have described transgenic mouse models that express some components of the HIV genome in mice; these animals develop a variety of cardiac abnormalities, especially focal lesions and production of cardiac autoimmune antibodies [28,29]. While these studies have demonstrated that HIV related proteins may be cardiotoxic, a causal role for HIV or associated proteins in cardiac muscle per se in humans remains controversial [30]. An additional consideration of the existing transgenic mouse reports is the presence of viral components and/or foreign proteins during development, as opposed to an infectious exposure following development. Since a physiological response to retroviral pathogen (rather than direct pathogen actions) may play an important role in the cardiac complications of HIV/AIDS, we chose to employ a mouse model of retroviral infection. Although the retroviral pathogen is distinct from HIV, this model recapitulates several features observed in humans, including aberrant cytokine release, changes in T-cell populations, and increased susceptibility to opportunistic infections. Here we tested the hypothesis that cardiovascular dysfunction occurs during retroviral progression and murine AIDS development.
The time course of murine AIDS development during retroviral progression has been well characterized by others (for a thorough review see Morse et al. 1992). While viral replication and increased cytokine profiles are detectable in the first few weeks post infection, overt immune deficiency occurs at approximately 10 weeks [22]. Hallmark features of retroviral activity include B-cell proliferation and splenomegaly [19]. Therefore, we used total spleen weight and viral load (by RT-PCR) as measurements of retroviral replication and disease progression. Progressive increases in spleen weight were observed throughout the study. LPBM5 presence in splenic tissue was confirmed by RT-PCR. Interestingly, LPBM5 was found to be present in cardiac tissue as well. The retroviral load in cardiac tissue was substantial (approximately 50% of the splenic retroviral load), suggesting that retrovirus is likely infecting cardiac myocytes. The presence of HIV in cardiac myocytes themselves has been highly controversial; a few recent studies have documented its presence in human myocytes in vivo [31] and its potential to weakly infect rat cardiomyocytes, but not replicate, in vitro [32]. However, studies in simian models of retroviral infection have shown that SIV does not infect myocytes [33]. Our studies in cardiac homogenates did not establish the cell types infected, and cardiac load did not increase from 5 to 10 weeks, despite significantly worsening cardiac performance in RTV-treated mice. Further studies defining the direct role of cardiac myocyte infection in RTV-related dysfunction are ongoing in our laboratory.
LV systolic performance was significantly impaired within five weeks of infection, seen measured by LV fractional shortening and stroke volume. These early performance deficits occurred at a time known to precede overt immune deficiency in this model, a phenomenon similar to observations in asymptomatic HIV patients [5]. Further declines in stroke volume and cardiac output were observed at 10 weeks—a 35-45% reduction in cardiac output is consistent with moderate to severe heart failure in mice. This biphasic decay of cardiac performance is similar to the presentation of cardiac dysfunction in HIV/AIDS patients and suggests that more than one mechanism may be involved [34-36]. The relationships between initial cardiac contractility deficits and later, more severe cardiac impairments are not well understood in this model or in humans. The identification of early markers of dysfunction, and a more complete understanding of their relevance to the development of progressive cardiac failure, may have predictive and/or preventative value in this patient population. Recent studies have also shown that the effects of relevant cardiotoxins (alcohol, cocaine, coxsackievirus B3) are aggravated by concurrent retroviral infection [37,38]. Thus, important interactions may exist in the development of RTV-induced cardiac injury in both mice and humans.

A pathogenic role for the immune system in acute cardiac injury as well as progressive cardiac and vascular disease is becoming increasingly apparent [27,39]. The interactions between immune cells and cardiac myocytes may have particular relevance in the setting of RTV- and AIDS-related cardiac disease. While LPBM5 infection was associated with a striking
reduction in circulating monocytes, we observed selective infiltration of CD68+ cells into cardiac tissue, with no corresponding increases in the cardiac residence of any other leukocyte studied (neutrophils, mast cells, eosinophils). Monocytes/macrophages are carriers for both HIV and LPBM5, and recent studies in humans have demonstrated that trafficking of HIV-infected CD68+ cells to the heart occurs in patients with AIDS-related cardiomyopathy [40,41]. Macrophages are high capacity production sites for reactive oxygen and reactive nitrogen species, and may contribute to cardiac oxidative damage in this setting [42]. The factors involved in the recruitment and/or activation of these immune cells to the heart remain unknown; further studies defining the molecular interactions and consequences involved are ongoing in our laboratory.

We observed a progressive increase in the cardiac presence of protein-3NT during the development of RTV-related cardiac dysfunction. In contrast, NOSII levels were not increased at any time point studied. In addition, significant associations between several measures of cardiac dysfunction and extent of cardiac 3NT immunoprevalence were detected, whereas no such relationships were observed for NOS II. These findings suggest that cardiac RNS formation and attendant protein nitration may participate in RTV-related cardiac pathology, rather than NOS II induction. This is consistent with the bimolecular reaction kinetics of RNS formation, and supports the under-appreciated concept that NOS II induction is not obligatory for promotion of RNS formation and the sustained presence of protein-3NT in vivo [15,17].
Staining patterns indicate that cardiac myocytes themselves carried a majority of the heart’s protein nitration burden—staining was widespread throughout the myocardium and was not confined to focal immune infiltrates. Further studies defining the putative sources for RNS and intracellular targets for protein nitration in this setting are ongoing in our laboratory.

An important consideration in these studies was our capacity to detect NOSII *in situ*—demonstrating that the lack of NOSII positive staining was not a “false negative” result. While we observed only nominal evidence of NOSII prevalence in cardiac myocytes from murine RTV hearts, we did observe intense positive staining for NOSII in infiltrative cells. We therefore quantified these cell bodies in controls and at 10 weeks of RTV infection, using methods identical to our cardiac infiltrate counts. We found that the residence of NOSII positive infiltrates was increased at 10 weeks relative to controls, and that the relative prevalence in these cells paralleled that of CD68+ cells (NOS II positive cells: 14.8±2.1 vs. 21.0±3.0 cells/mm²; CD68+ cells: 14.8±2.2 vs. 21.1±1.4 cells/mm², CTRL vs. 10 week RTV). These studies illustrate our capacity for the *in situ* detection of NOSII in cardiac tissue, and further validate the unexpected spatial dissociation between cardiac myocyte protein nitration and NOSII prevalence. In contrast to our finding of statistical association between cardiac protein nitration and LV function, we detected no such relationship between NOS II intensities or positive cell prevalence and any of the functional parameters measured, thus suggesting that reactive nitrogen species may be more directly related to dysfunction than NO production.
Further studies defining the contributions of these infiltrates to the protein oxidative events and functional impairments in this setting are warranted, and are ongoing in our laboratory.

To validate our murine findings, we conducted further histopathological studies in human cardiac autopsy tissues from HIV seropositive patients with documented evidence of AIDS-related cardiomyopathy, that were in decompensated heart failure (average ejection fraction 27%). We observed striking increases in protein-3NT presence in cardiac tissue from patients with AIDS-related cardiomyopathy compared to HIV seronegative controls, while cardiac NOSII protein levels remained unchanged. These results are in opposition to recent results published by Barbaro et al, which showed that increased cardiac NOSII staining may be a consistent phenomenon in AIDS patients with cardiac disease [12,30]. As has been shown in experimental studies, important context- and time-dependencies may exist between the induction of NOSII (and other extramitochondrial oxidases) and the formation of protein-3NT in cardiac tissue. Consistent with our experimental studies, staining for protein-3NT predominated in cardiac myocytes themselves; immune cell infiltration may incompletely explain the oxidative events (and perhaps the cardiac myocyte dysfunction) that were observed in this setting.

In summary, LPBM5 infection, a well-established experimental model for the immunologic complications of HIV-infection, caused early cardiac contractility deficits and later, more severe cardiovascular impairment in mice, with a time course that preceded the development of overt immunodeficiency;
the “murine AIDS” model appears appropriate for the mechanistic study of RTV-induced cardiac complications. Although the infectious agent was not HIV itself, the murine AIDS model recapitulates many of the important features of AIDS-related cardiac disease in humans, suggesting that the systemic and/or cardiovascular response to RTV infection may mediate many of these changes, rather than direct effects of the virus itself. Although we observed dramatic changes in immune cell levels and trafficking patterns, a number of structural and biochemical alterations occurred in the cardiac myocytes themselves in RTV-treated animals. Immune cell interactions incompletely explain the global cardiac muscle changes that occur in this setting, and the cardiac myocyte itself may represent an important site of RTV-related pathogenesis. Here, we describe first-time evidence that cardiac RNS formation and oxidative injury may play a significant role in the development of cardiac complications in both the murine AIDS model and in a relevant population of AIDS-related cardiomyopathy patients. Further studies defining the sources and putative intracellular targets of these oxidants will provide important mechanistic insights, and may reveal new therapeutic opportunities for this unique and important cardiovascular disease setting.
REFERENCES


Figure 3.1: Splenomegaly and increased viral load in cardiac and splenic tissue during the progression of murine AIDS.

Panel A: **Upper panel**- Representative Hematoxylin & Eosin mid-line splenic cross sections of a control mouse and RTV-infected mouse at 10 wks. **Lower panel**- Average data for total spleen weight in control vs. retrovirus infected animals at sacrifice. Data are expressed as mg per g of body weight (n = 6-12). *, p<0.05 as compared to control.

Panel B: **Upper panel**- Representative images of retroviral RT-PCR products from cardiac (C) and splenic (S) tissue in mice infected with LPBM5. **Lower panel**- Total cardiac viral load at 5 and 10 weeks post RTV infection (expressed as a percentage of splenic RTV expression). Data is represented as mean ± SEM (n=3-6); *, p<0.05 as compared to control.
Figure 3.2: Circulating monocyte levels are significantly diminished during murine AIDS.
Total number of monocytes, neutrophils, lymphocytes and eosinophils per µL of whole blood from control and RTV-infected mice. *, p<0.05 as compared to control.
Figure 3.3: Development of cardiac left ventricular hypertrophy in murine AIDS.

Upper panel- Representative images (magnification 25X) of equatorial cardiac cross-sections of control mouse and RTV-infected mouse at 10 wks. Middle panel- Average heart weights (normalized to body weight) at 5 and 10 weeks post-RTV infection. Lower panel- Average LV cross-sectional areas (normalized to bodyweight). *, p<0.05 as compared to control.
Figure 3.4: Bi-phasic decay in cardiac performance during the progression of murine AIDS.  

**Upper panels:** Average data for heart rate (HR), fractional shortening (FS%), cardiac output (CO), and stroke volume (SV).  

**Lower panel:** Relative comparisons of the functional parameters as percent of uninfected control values. *, p<0.05 as compared to control.
Figure 3.5: Increased cardiac presence of CD68+ infiltrates during murine AIDS. Total number of infiltrating immune cells per mm$^2$ of cardiac tissue were assessed using digital imaging approaches. *, p<0.05 as compared to control.
Figure 3.6: Increased cardiac RNS formation and attendant protein nitration during murine AIDS. **Upper Panels**- Representative images of NOS II and 3-nitrotyrosine immunoreactivity in left ventricular myocardium of control and virus-infected mice at 10 weeks (400x magnification). **Lower Panel**- Relative immunoprevalence of NOS II and 3-nitrotyrosine staining was determined by digital imaging and thresholding analyses. Average data from 5 and 10 weeks post-treatment shown. *, p<0.05 as compared to control.
Figure 3.7: Increased cardiac myocyte protein nitration, but not NOS II, in HIV dilated cardiomyopathy. Upper Panels: Representative images (400x magnification) of NOS II and 3-nitrotyrosine immunoreactivity in left ventricular myocardium of a seronegative patient with no evidence of cardiovascular disease (CTRL) and a HIV+ patient with dilated cardiomyopathy (HIV-DCM). Lower Panel: Relative immunoprevalence was determined by digital imaging and thresholding analyses. *, p<0.05 as compared to control.
CHAPTER 4

BACTERIAL LIPOPOLYSACCHARIDE ENHANCES RETROVIRAL CARDIOMYOPATHY DURING MURINE AIDS: ROLES OF MACROPHAGE INFILTRATION AND MYOCYTE TLR4 EXPRESSION
ABSTRACT

Cardiovascular disease may be an important complication of HIV infection, but the mechanism(s) involved are poorly understood. Other infectious diseases (other viruses or bacteria) may participate in HIV related pathogenesis, but few studies have investigated these interactions in a relevant animal model. We have recently demonstrated that the murine AIDS model (LPBM5 retroviral infection) mimics HIV related cardiac dysfunction and pathology in humans. Here we tested the hypothesis that low – dose non – septic lipopolysaccharide (LPS) would affect LPBM5 progression and cardiovascular dysfunction during murine AIDS development. LPS (5 mg/Kg, E coli 0111:B4) was administered at one, six and eight weeks during LPBM5 infection. Cardiac performance was evaluated at ten weeks using noninvasive echocardiography. A modest exposure of LPS amplified abnormalities in cardiac structure and function observed in a murine AIDS model. Cardiac dysfunction was associated with selective increases in non-focal infiltration of CD68+ cells; these cells were found to be NOS2 positive and correlated to extent of cardiac dysfunction. This amplification interaction was not associated with alterations in retroviral progression or cardiac retroviral content, but important increase in TLR4 was observed in the combination treatment group only. Evidence of cardiac myocyte induction of TLR4 was also observed in human tissues from AIDS related cardiomyopathy. These studies demonstrate a high degree of similarity of this reproducible animal model relative to studies
in human tissues; furthermore they provide first-time evidence of multi-pathogen enhancements to retrovirus related cardiac complications and implicate innate immunity responses in this setting.
INTRODUCTION

Following the advent of highly active antiretroviral therapy (HAART) regimens, AIDS-free living and overall survival have significantly improved for patients infected with human immunodeficiency virus (HIV) (1-4). However, a variety of chronic complications that are apparently not directly related to immunodeficiency or opportunistic infection have become evident in these patients. For example, early-onset cardiovascular disease is an increasingly significant cause of morbidity and mortality for HIV/AIDS patients, and may affect as many as 70-80% of this population (2, 5, 6). HIV/AIDS-related cardiovascular disease encompasses a wide spectrum of chronic cardiac and vascular disease states, ranging from asymptomatic left ventricular contractile deficits and ECG abnormalities in HIV+ individuals to pulmonary hypertension, dilated cardiomyopathy and congestive heart failure in AIDS patients (7-10). A variety of mechanisms have been proposed to explain these phenomena, but the mechanisms by which cardiac injury and dysfunction occurs in these patients, and the direct role for HIV or related proteins in these events, remain incompletely defined. As a result, no specialized therapeutic strategy currently exists for cardiac complications in this unique patient population.

The progression of HIV/AIDS and its secondary complications are highly variable among patients, with few valuable prognostic indicators for duration of AIDS-free living or the development of secondary cardiovascular, pulmonary, or neurological complications. While the molecular sequelae that trigger progression from pre-symptomatic HIV infection to AIDS are still being
revealed, the variability in the time course and sequence of these events suggests that this transition may be highly context-dependent (11-13). Important co-variates to HIV infection may delay or even predispose patients to alternative disease manifestations (14, 15). For example, co-existing pathogens may be a particularly important risk factor that predisposes HIV patients to more severe outcomes, and a broad spectrum of other viral and bacterial pathogens have been identified as potential contributors to HIV disease progression, including cardiovascular complications (14, 16-18). Several recent reports illustrate that gram-negative bacteria are also frequent concomitant pathogens to HIV infection, and these organisms have been implicated in promoting HIV-1 pathogenesis through bacterial lipopolysaccharide (LPS) (19). In addition to contributing to HIV related progression and complications, several of these pathogens have individually been linked to cardiovascular disease alone (20). Therefore, concomitant bacterial pathogens may be critical but under-appreciated modulators of cardiovascular disease progression, particularly in HIV/AIDS patients.

Although the concept of concurrent pathogens driving retroviral complications has been suggested, no previous studies have tested this hypothesis directly in a well-controlled set of experimental conditions. We have recently established that a well-defined murine model of retroviral disease (the LPBM5 model of “murine AIDS”) recapitulates the major cardiovascular features already documented in HIV/AIDS patients, with initially detectable contractility deficits prior to overt immune compromise followed by more
severe cardiomyopathy (21). Increased cardiac oxidative injury, and left ventricular immune cell infiltration was also observed, similar to changes observed by others in human tissues (21). We also corroborated our findings of reactive nitrogen species in this mouse model with a small set of human tissues, further supporting the relevancy of the animal preparation (21). Given our prior observations in this model and the potential importance of combined pathogen effects with respect to retrovirus related cardiovascular alterations, we investigated potential interactions among retroviral infection and a modest exposure to a bacterium related pathogen. In the studies described herein, we tested the hypothesis that exposure to LPS (at doses that did not induce cardiac toxicities alone) can modulate retrovirus-related cardiac deficiencies in this well-established model of the immunologic and cardiac complications of retroviral infection (21, 22). We used LPS as the immune activator rather than live bacteria to avoid confounding differences in pathogen growth in immunocompromised hosts. Our investigative focus was on aspects of retroviral progression, leukocyte populations and trafficking to cardiac tissues. By using a specific pathogen-free mouse colony, we had a unique opportunity to evaluate interactions between these two immune system challenges and limit influences of other potential covariates commonly found in humans.

An additional component of our study was to address mechanisms by which such multi-pathogen interactions might develop. As key participants of the innate immune system, toll-like receptors (TLR) are a newly identified class of receptors involved in the recognition and transmission of pathogenic
stimuli. Of particular importance are TLR-4 subtype receptors that bind LPS and transduce rapid response to bacterial infection, eliciting inflammatory and oxidative pathways in immune cells (23). These receptors have been detected on cardiac myocytes, and the upregulation of TLR4 has been noted in several human cardiac disease settings (24). We tested the hypotheses that the TLR4 receptor system may be involved in retrovirus related cardiac dysfunction, in both the murine AIDS model and in a small set of human tissues from HIV related cardiomyopathy autopsy cases.
METHODS

**LPBM5 retroviral infection in mice:**

Active LPBM5 virus was prepared according to the methods of Watson et al, as we have previously described. (21, 25). Retrovirus-containing cell-free supernatant was collected from infected SCI/MuLV cells (AIDS Research and Reference Reagent Program, Bethesda, MD), and concentrated by centrifugation (Advanced Biotechnologies, Inc., Columbia, MA). Titers of esototropic MuLV were determined by the standard S’L plaque assay (26) and by units of reverse transcriptase activity using a commercially available kit (Boehringer Mannheim, Germany).

Specific effort was made to procure and maintain pathogen-free female C57BL/6 mice (two specific barrier rooms at Harlan Laboratories, Indianapolis, IN), which were housed in a sterile cage rack system with HEPA filtered air circulation (approximately 50 air changes/hour, Allentown Caging Inc., Allentown, PA). Following 1-2 weeks of acclimatization, 40 mice were divided into 4 treatment groups: control (CTRL), retrovirus infected alone (RTV), LPS treated alone (LPS), or retroviral + LPS treated mice (RTV+LPS). LPBM5 retrovirus was dosed via a single intraperitoneal injection (100 µl dose containing 200 reverse transcriptase units). Control animals received an identical injection of vehicle. At the time of injection all mice were six weeks old and weighed 16-18g. At 1, 6 and 8 weeks post LP-BM5 infection, animals in the LPS and the RTV+LPS treatment groups were administered an 5mg/Kg
ip dose of LPS (E Coli 0111:B4, Sigma Chemical Co.). This dose is below the threshold required to induce a septic shock response in these mice (27).

Cardiac performance was assessed by echocardiography (see below) at 10 weeks of retroviral infection, allowing for sufficient time to clear LPS from the system (t_{1/2} LPS- 2-3 days). Immediately following echocardiography, animals were sacrificed with an overdose of pentobarbital. Whole blood was collected from the descending abdominal aorta at the time of sacrifice, and complete blood chemistries were provided (Antech Diagnostics). Total cholesterol and triglyceride levels as well as AST, ALT, lipase, amylase activities were also measured. Entire hearts were then rapidly isolated, rinsed in ice-cold physiological buffer, weighed, equatorially sectioned at the mitral valve, and fixed in 10% buffered formalin for later analysis (28). Spleen weight was also measured as an index of retroviral progression, as documented by others (22, 29).

**Murine Echocardiography:**

Mice were placed under light anesthesia with halothane inhalation (0.5–1% halothane USP in a mixture of 95% O₂ and 5% CO₂), as we have previously described (30). Two-dimensional and M-mode echocardiographic images were recorded and analyzed by a Sonos 5500 echocardiograph and a 15 MHz ultrasonic probe (Agilent Technologies). Two-dimensional transverse LV imaging was used to position the probe just distal to the mitral valve leaflets and M-mode images were then captured. Three loops of M-mode data
were captured from each animal at approximately 5-minute intervals and stored on digital disk until analysis. Each of these captured image loops provided 7-12 heart cycles, data were averaged from at least 5 cycles/loop. LV systolic (LVIDs) and diastolic (LVIDd) internal dimensions were measured according to the American Society for Echocardiography leading-edge technique by a blinded investigator (31). These parameters allowed the determination of LV fractional shortening (%FS), a measure of systolic function, by the equation: %FS = [(LVIDd – LVIDs) / LVIDd] x 100%.

Ascending aortic flow velocity was determined using the continuous Doppler wave mode. Peak aortic flow velocity and velocity-time integral (VTI) were determined for at least 5 beats/loop for each animal. At sacrifice aortic outflow tract (aortic root) was isolated and cross sectional area was measured via light microscopy with area-calibrated digital image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). Stroke volume (SV) was calculated by VTI x aortic root cross-sectional area (30). Cardiac output (CO) was calculated by SV x heart rate.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis of LPBM5 and TLR4 expression:**

Total RNA was isolated from frozen cardiac and splenic tissue (Trizol, Gibco BRL). An absorbance reading at 260nm was used to quantify the amount of RNA in each sample. The integrity of the RNA was verified by fractionating the RNA on a formaldehyde agarose gel. Two µg of total RNA
was reverse transcribed to cDNA (cDNA cycle kit, Invitrogen) in 20 µL reverse transcription reaction mix at 42°C (60 min). The reaction was stopped by heat inactivation at 95°C (2 min) and chilled on ice. Subsequently 5 µL of the resulting cDNA was amplified using primers specific for the p12 region in the gag gene in the LP-BM5 genome (32), a region spanning 317-726 bp of the murine TLR4 genome (24), and β-actin (33). The RTV primers: sense primer, 5’-CCT TTATCGACACTTCCCTT-3’; antisense primer: 5’-CCGCCTCTTTCTAATCTGGTC-3’. The TLR4 primers for the murine gene were: sense primer, 5’-GCTTACACCCACCTCTCAAACTTGAT-3’; antisense primer: 5’-ATTACCTCTTAGATCAGTTCATGG-3’. Similarly β-actin primers: sense primer: 5’-ATGGATGACGATATCGCT-3’; antisense primer: 5’-ATGAGGTAGTCTGCTAGGT-3’ were used in PCR reactions under the same conditions which included an initial denaturation at 95°C (5 min), followed by a cycle of denaturation (95°C/1 min), annealing (55°C/1 min), and extension (72°C/1 min). Each sample was subjected to 30 cycles followed by a final extension (72°C/10 min). The PCR products were separated and visualized on a 2% agarose ethidium bromide stained gel. Band intensity was assessed using imaging software (Labworks 4.0, Media Cybernetics, Silver Spring, MD). RTV expression was normalized to β-actin expression in each tissue. Cardiac RTV expression was further expressed as a percentage of splenic RTV expression.
**Histology and immunohistochemistry:**

Following formalin fixation (48 hrs) heart and spleen tissues were embedded in paraffin for histological studies (cross-sectional orientations were used). Tissue sections (5 µm) were mounted onto microscope slides and prepared for histological and immunohistochemical analyses, as we have previously described (28). Cardiac and splenic cross-sections were stained using hematoxylin/eosin and Masson's trichrome for routine morphologic and histologic assessments. Cardiac tissues from each treatment group were assessed for evidence of specific leukocyte infiltrates assessed in the complete blood chemistry profile. Histological stains for mast cells (Astra Blue stain, Sigma, St. Louis, MO) and eosinophils (Vital Red stain, Sigma, St. Louis, MO), as well immunohistochemical probes for neutrophil (anti-myeloperoxidase antibody, Neomarkers, 1:2000 dilution) and monocyte/macrophage presence (anti-CD68+, Neomarkers, 1:400) were employed. In additional studies, cardiac myocyte prevalence of TLR4 was assessed in left ventricular cross-sections (anti-TLR4, Santa Cruz Biotechnology, raised against the carboxy terminus of murine TLR4, 1:400 dilution). Isotypic and preadsorbed staining controls demonstrated antibody specificity. NOS2 stained tissues were assessed for NOS2 positive cell bodies (monocytes/macrophages) as well. Diaminobenzidine (DAB, 0.06% w/v, DAKO, Carpinteria, CA) was used to provide visualization of immunoreactivity, with methyl green counterstaining.
**Digital image analysis:**

Digital images were acquired using a Polaroid DMC camera and Olympus microscope (Model BX40) and transferred to Image Pro Plus software (Media Cybernetics) for both area and intensity analyses, as we have previously described (34). All images were captured using identical light and software settings. For morphological studies, cross-sectional images of whole spleen and heart stained with H&E were captured at 4x magnification and circumferential traces were used to calculate tissue areas. All other images were captured at 400x, and then segmented to eliminate background and nuclear counterstain from analysis. Cardiac residence, of specific immune infiltrates, were quantitated, using a digitized cell counting approach. Tissue areas were calibrated and positive cells were segmented and gated based on size, then counted and normalized as a function of left ventricular area. In parallel studies, immunoreactivity for cardiac TLR4 was determined by measuring optical density of diaminobenzidine signal in each tissue, giving a quantitative measure of relative staining intensities, as we have previously described (34). Over 200 cardiac images were analyzed, and intra-observer and inter-observer variability for these automated procedures were each <2%.
**HIV/AIDS cardiac specimens:**

Left ventricular sections (LV anterior wall) were obtained as paraffin-embedded autopsy specimens from AIDS patients and non-HIV infected controls from the National Cancer Institute AIDS Cancer and Specimen Resource. All autopsy samples studied were collected within 4 hours of death. Autopsy samples were collected between 1983 and 1998. Patient histories were reviewed to subclassify samples into 2 groups: HIV infected patients with documented evidence of dilated cardiomyopathy (HIV-DCM, n=4) and non-HIV infected patients with no evidence of cardiac disease (CTRL, n=3). HIV/AIDS patients with confounding risk factors for cardiovascular disease (smoking, diabetes, protease inhibitor therapy, etc) were omitted from analysis. Age at autopsy was not different between patient groups (CTRL: 40±2.3 yrs, HIV-DCM: 36.5±3.2yrs, p=NS), and was not a significant factor for cardiovascular disease. Average ejection fraction in HIV+ patients with dilated cardiomyopathy was 27.5±7.5%, mean CD4+ counts were 123.5±46.5 cells/mm$^3$. These cardiac specimens were then assessed for evidence of TLR4, using immunohistochemical methods identical to those described above.

**Statistical analysis:**

All data presented herein represent 6-12 observations per group. All statistical analyses were performed using Sigma Stat statistical software (Jandel Scientific, San Rafael, CA). ANOVA’s were used for statistical
comparisons among groups with Student-Newman-Keuls for post hoc analysis. Spearman's non-parametric correlation analysis was used to define significant associations. A total of 30-35 data points were used for each analysis (controls, RTV alone, LPS alone and RTV in combination with LPS), providing a statistical power of greater than 0.95 at \( r=0.5 \) and \( \alpha=0.05 \). \( p<0.05 \) was considered statistically significant.
RESULTS

Murine AIDS Studies

Cardiac structure and function following retrovirus, LPS, or their combination

Shown in Figure 1 are changes in total heart weight, and systolic and diastolic dimensions from mice treated with retrovirus, LPS, or their combination. Representative photomicrographs from control and RTV+LPS treated animals illustrate the significant LV chamber remodeling induced by combination treatment (Figure 1, upper panel). Neither retrovirus nor LPS alone significantly altered LV dimensions, but their combination caused a significant increase in lumenal LV dimensions at both systole and diastole (Figure 1, lower panels). Hearts from RTV-treated mice developed a hypertrophic response with both RTV alone and in combination with LPS (Figure 1, middle panel). Total body weights were not different among groups and no animals in the study developed cachexia during the 10 week study; thus the increase in heart weight normalized to body weight was apparently not related to changes in body mass.

In vivo echocardiographic cardiac performance indicators are shown in Figure 2. Heart rate was maintained throughout the studies at physiological rates, using light halothane anesthesia, and no differences were observed among treatment groups. Similar to our previous report, LPBM5 retrovirus alone caused significant reductions in fractional shortening, cardiac output and
stroke volume (21). LPS alone caused a slight increase in stroke volume when administered alone, but had no other demonstrable effects on cardiac performance. In contrast, LPS exacerbated the cardiac contractility deficits observed in RTV treated mice, as RTV+LPS treatment caused a significantly lower stroke volume, cardiac output, and LV shortening fraction relative to RTV treatment alone.

Influence of LPS on retroviral progression in vivo

Given the capacity of LPS to modulate RTV-induced cardiac functional and structural changes, we tested the hypothesis that LPS treatment can promote retroviral progression in spleen and cardiac tissue. Splenomegaly is consistently observed in the murine AIDS model; and is used as a marker of retroviral progression and immune dysfunction (25). LPBM5 retrovirus caused significant increases in spleen weight compared to CTL and LPS alone treatment groups (Figure 3), but this effect was not further augmented in the RTV+LPS group.

LPBM5 infectivity is facilitated by the gag, pol, and env genes (35). The p12 region in the gag gene has been shown to be unique to the LPBM5 virus and important to infectivity (36), we selected this region to develop a primer set specific to LPBM5. A representative agarose gel following separation of RT-PCR products is shown in Figure 3 (Upper Panel). The 229bp RT-PCR product, specific for the p12gag region of LPBM5, was confirmed by a restriction enzyme analysis with Sma I (data not shown). RTV treated mice
yielded a positive PCR product for LPBM5 in splenic and cardiac tissue. This signal was absent in CTL and LPS alone treatment groups, with equivalent β-actin signal. RTV+LPS treated mice had comparable levels of LPBM5 signal to RTV alone; LPS apparently did not augment retroviral load in splenic or cardiac tissue, despite dramatic alterations in cardiac functional changes.

**Circulating leukocytes following retrovirus, LPS, or their combination**

Shown in Figure 4 are circulating blood leukocyte counts for the 3 treatment groups at 10 weeks. RTV alone caused a significant reduction in circulating Monocytes, with neutrophils, total lymphocytes, and eosinophils remaining unchanged. LPS alone caused no significant changes on any measured cell type, whereas the combined treatment caused a reduction in monocytes equivalent to RTV alone, plus a marked eosinophilia with no alterations in total lymphocytes of neutrophils.

**Cardiac leukocyte infiltration**

*In situ* measures were employed to assess the cardiac residence of multiple immune cell infiltrates by immunohistochemistry (see Methods). In our preliminary examinations, inflammatory lesion sites were not observed in any of the treatment groups (e.g., no focal accumulations of cells); thus the following measures assessed the interstitial presence of immune cells throughout the myocardium. Cardiac presence of CD68+ infiltrates were significantly elevated in RTV treated mice, while cell counts for LPS alone
treated mice were not different from control. This cellular infiltration was further elevated in RTV+LPS treated mice (p<0.05 vs. RTV alone). The extent of myocardial infiltration of CD68+ cells was inversely correlated to cardiac performance parameters in these same mice—LV fractional shortening, stroke volume and cardiac output each yielded significant negative correlations to CD68+ cell densities in the same hearts. Nominal cardiac presence of neutrophils, mast cells and eosinophils were detected, with no significant increases in any treatment group studied (data not shown).

Additional immunohistochemical studies revealed the presence of NOS2 positive mononuclear cells, which were consistent with the general distribution of CD68+ infiltrates. Using automated cell counts by methods identical for the studies described above, we found that NOS2 positive infiltrates were significantly elevated in hearts from RTV mice at 10 weeks of retroviral infection, relative to control, and this was further enhanced in conjunction with RTV+LPS (NOS II positive cells: 14.8±2.1, 21.0±3.0*, 16.5±2.6, 27.0±2.8† cells/mm² -CTRL, RTV, LPS, RTV+ LPS; *, p<0.05 as compared to control. †, p<0.05 as compared to RTV alone). The relative increases and densities paralleled those observed for CD68+ cells over the same time course, consistent with these cells being of apparent monocyte/macrophage lineage.
**TLR4 expression and prevalence following retrovirus, LPS, or their combination**

Shown in Figure 6A (Upper panel) is a representative gel for TLR4 and \( \beta \)-actin expression in cardiac tissue by RT-PCR. Band intensities for the 410bp TLR4 PCR product are expressed as a percentage of \( \beta \)-actin expression, and is illustrated in Figure 6A (Lower panel). Cardiac mRNA expression of TLR4 was greatly enhanced by LPS stimulation of retroviral disease. This mRNA expression was translated into increased cardiac TLR4 protein levels as determined by immunohistochemistry (Figure 6B), such that mRNA expression was significantly correlated to protein content by immunohistochemistry (\( r = 0.5, \ p < 0.05 \)). Integrated optical density analysis for TLR4 protein was reflective of cardiac myocytes themselves, not infiltrative immune cells—LPS stimulated expression of TLR4 in cardiac myocytes during RTV disease.

**Human tissue investigations**

**Evidence of TLR4 expression in human cardiac tissues**

Shown in Figure 7 are representative photomicrographs in human cardiac autopsy tissues from AIDS patients with non-ischemic dilated cardiomyopathy (HIV-DCM) and seronegative controls with no documented evidence of cardiac disease. Identical to our mouse studies, we observed striking evidence of cardiac myocyte TLR4 formation (Figure 7, Lower panel).
DISCUSSION

Although concurrent pathogen presentation is a well-appreciated phenomenon in retrovirus-induced immunodeficiencies, few studies have investigated mechanistic consequences, especially as they relate to cardiovascular complications. Similar to our previous report, we found that LPBM5 retroviral infection alone, in pathogen free C57BL/6 mice induced detectable ventricular dysfunction (21). Interestingly, despite the fact that the LPBM5 retroviral preparation is different from HIV per se, this murine model recapitulates nearly all major complications of the HIV/AIDS condition, including pulmonary infections, neurocognitive deficits, increased tumor incidence (22). Based on our observations, cardiac dysfunction can be included in this list (21). Since the retroviral pathogen is substantially different from HIV, an immune system response to retrovirus rather than retrovirus per se may be the operable mechanism for many of these parenchymal organ abnormalities.

As expected, the low LPS dose we employed (in this mouse strain) had no significant effects on cardiovascular status alone. In contrast, LPS substantially enhanced retrovirus related cardiac structural and functional alterations. In these studies, we chose to use LPS rather than a live gram-negative pathogen to avoid confounding issues of bacterial growth differences among treatment groups. Since previous in vitro studies have shown that various pathogen exposures (including LPS) could enhance HIV replication rate and infectivity, we investigated content of retroviral RNA in spleen and
cardiac homogenates by RT-PCR (14, 17, 19). As demonstrated by others, retrovirus was readily detectable in the LPBM5 treated animals and was not significantly enhanced in the RTV+LPS group; total spleen weight was also not enhanced in the combination treatment, suggesting that the LPS dosing did not alter retroviral pathogenesis or cardiac prevalence of retrovirus in vivo. These findings are consistent with work by others suggesting that cardiac content of retrovirus per se is not closely linked to organ dysfunction in vivo (21, 37)). Isolated cellular experiments demonstrate that HIV and other retroviruses can infect cardiac myocytes, but whether this phenomenon occurs substantially in vivo and plays a causative role in commonly observed cardiomyopathies remains to be resolved (37, 38).

Our measurements of circulating leukocytes demonstrated that LPBM5 infection alone caused a reduction in circulating monocytes with no change in total lymphocytes, neutrophils, or eosinophils at 10 weeks. LPS alone caused no significant effects, but their combination caused marked increases in eosinophilia (Figure 4). While previous studies have also shown that eosinophilia is a common occurrence in HIV/AIDS patients, the significance and mechanisms of this “allergen-like” response are unclear. Eosinophilic myocarditis has been previously documented in HIV case reports, but we did not observe significant prevalence of this cell type in cardiac cross-sections, and total cell counts were not associated with any index of cardiac performance in the mouse model (see below).
In parallel to blood measurements, we investigated the prevalence of macrophages, neutrophils, mast cells, and eosinophils in cardiac cross sections using in situ techniques. LPBM5 alone caused significant enhancement of CD68+ (monocyte/macrophage) cells in cardiac cross-sections, and these were found to be widely distributed throughout cardiac tissues. Indeed, we found no significant evidence of classical focal lesions in this model in any treatment group, suggesting that this typically employed endpoint for evidence of myocarditis may not serve as an adequate marker of myocyte/immune cell interaction in this setting. While LPS alone caused no significant changes in cardiac CD68+ cells (shown in Figure 5) or other leukocytes (data not shown), the treatment combination caused further increase in CD68+ prevalence relative to retrovirus alone. We found that these cells were positive for NOS2, but surrounding myocardium was not. In addition we found that the extent of macrophage prevalence in cardiac tissue was correlated to the degree of LV dysfunction we observed in vivo (Figure 5). These findings are consistent with a cell specific monocyte/macrophage induction of inflammatory responses, and widely distributed infiltration of these activated cells may play an important role in cardiac parenchymal injury and dysfunction. Many previous reports have investigated NOS2 induction as a potential contributor to various forms of cardiac failure, and thus far the findings have been very inconsistent. Our in situ approach illustrated a strong NOS2 prevalence in the relatively low CD68+ abundance cell type, suggesting that homogenates and western blotting techniques may not offer adequate
sensitivity to detect such changes. Recent investigations have demonstrated an enhanced prevalence of CD68+ cells in brain regions from HIV dementia autopsy cases and in human HIV cardiac tissues (39, 40). Our findings in this murine model recapitulate these human findings and further argues the relevance of the animal preparation and the potential importance of immune cell interactions with cardiac myocytes in RTV related cardiac complications.

Several very recent reports have suggested that innate immunity pathways, more specifically the toll-like receptor family, may play a critical role in many forms of pathogen induced cardiovascular disease states (41). Thus far the TLR4 receptor has been shown to mediate much of the host response to gram-negative bacterial infections in vivo, and these processes are upstream from several inflammatory cascades and transcriptional controllers already implicated in cardiomyopathies (e.g., NFkB) (42). In the mouse model, neither retrovirus alone nor LPS alone had any significant impact on cardiac TLR4 expression of protein content; however, the combined treatment caused substantial increases in gene expression. This increased expression was associated with increased protein prevalence, which appeared widely dispersed and in cardiac myocytes (rather than infiltrating immune cells). In a small sample of human tissues (explanted control hearts and HIV cardiomyopathy cases), we also observed significant increases in TLR4 prevalence. These data provide first-time evidence that the activation of the cardiac innate immunity pathways may contribute to cardiac complications
during retroviral infection and may play an important role in a multi-pathogen setting commonly observed in humans.

In summary, a multi-pathogen setting is commonly observed in HIV related cardiomyopathy cases, but few studies have addressed important interactions with respect to retroviral pathogenesis vs. organ dysfunction. Here we have demonstrated that a modest exposure of LPS (e.g. at doses that did not yield significant effects alone \textit{in vivo}) amplified abnormalities in cardiac structure and function observed in a murine AIDS model. The observed cardiac dysfunction was associated with selective increases in non-focal infiltration of CD68+ cells; these cells were found to be NOS2 positive and correlated to extent of cardiac dysfunction. This amplification interaction was not associated with alterations in retroviral progression or cardiac retroviral content, but important increase in TLR4 was observed in the combination treatment group only. Evidence of cardiac myocyte induction of TLR4 was also observed in human tissues from AIDS related cardiomyopathy. These studies demonstrate a high degree of similarity of this reproducible animal model relative to studies in human tissues; furthermore they provide first-time evidence of multi-pathogen enhancements to retrovirus related cardiac complications and implicate innate immunity responses in this setting.
REFERENCES


Figure 4.1: Development of cardiac left ventricular hypertrophy in murine AIDS and dilation in animals infected with RTV in conjunction with LPS. 

Upper panel- Representative images (magnification 25X) of equatorial cardiac cross-sections of control mouse and RTV infected mouse in combination with LPS at 10 wks. 

Middle panel- Average heart weights (normalized to body weight) at 10 weeks post-RTV infection. 

Lower panels- Average interior cardiac dimensions (systolic and diastolic) at 10 weeks post RTV. 

*, p<0.05 as compared to control. 
†, p<0.05 as compared to RTV alone.
Figure 4.2: Cardiac alterations during murine AIDS, and RTV infection in combination with LPS.

Upper panels- Average data for heart rate (HR), and fractional shortening (FS%) at 10 weeks post RTV infection.

Lower panel- Average data for cardiac output (CO), and stroke volume (SV) at 10 weeks post RTV infection. *, p<0.05 as compared to control. †, p<0.05 as compared to RTV alone.
Figure 4.3: Splenomegaly and increased viral load in cardiac and splenic tissue during the progression of murine AIDS.

**Panel A:**
Representative images of retroviral RT-PCR products from cardiac (C) and splenic (S) tissue in mice infected with LPBM5 and in combination with LPS.

**Panel B:**
- **Right panel:** Average data for total spleen weight in control vs. retrovirus infected animals at sacrifice. Data are expressed as mg per g of body weight (n = 6-12). *
  p<0.05 as compared to control. **Left panel:** Total cardiac viral load at 10 weeks post RTV infection (expressed as a percentage of splenic RTV expression) with RTV alone and in combination with LPS. Data is represented as mean ± SEM (n=3-6).
Figure 4.4: Circulating monocyte levels are significantly diminished during murine AIDS while circulating eosinophils are significantly enhanced with RTV infection in combination with LPS. Total number of monocytes, neutrophils, lymphocytes and eosinophils per µL of whole blood from control and RTV-infected mice. *, p<0.05 as compared to control. †, p<0.05 as compared to RTV alone.
Figure 4.5: Increased cardiac presence of CD68+ infiltrates during murine AIDS. Total number of infiltrating immune cells per mm² of cardiac tissue, were assessed using digital imaging approaches. *, p<0.05 as compared to control. †, p<0.05 as compared to RTV alone. Cardiac function related to cardiac CD68+ cells. [●] Control, [▲] LPS alone, [■] RTV alone, [♦] RTV and LPS]. *, p<0.05.
Figure 4.6: Increased cardiac TLR4 expression and protein prevalence during RTV infection in combination with LPS.

**Panel A:** *Upper Panel:* Representative image of cardiac TLR4 expression during murine AIDS. *Lower Panel:* Average data for total cardiac TLR4 expression (expressed as percentage of β-actin expression). *, p<0.05 as compared to control. †, p<0.05 as compared to RTV alone.

**Panel B:** *Upper Panels:* Representative images of TLR4 prevalence in left ventricular myocardium of control and RTV infected mice in combination with LPS (400x magnification). *Lower Panel:* Relative prevalence of TLR4 staining was determined by digital imaging. Average data from control, RTV alone, LPS alone and RTV and LPS in combination are depicted. *, p<0.05 as compared to control.
Figure 4.7: Increased cardiac TLR4 prevalence in HIV dilated cardiomyopathy.

*Upper Panels* - Representative images (400x magnification) of TLR4 immunoreactivity in left ventricular myocardium of a seronegative patient with no evidence of cardiovascular disease (CTRL) and a HIV+ patient with dilated cardiomyopathy (HIV-DCM).

*Lower Panel* - Relative immunoprevalence was determined by digital imaging. *, p<0.05 as compared to control.
SECTION II

COCAINE RELATED CARDIOVASCULAR DISEASE
CHAPTER 5

COCAINE INDUCED ELECTROPHYSIOLOGICAL AND CONTRACTILE
ABNORMALITIES IN MICE: EVIDENCE OF REACTIVE NITROGEN SPECIES
AND NITRIC OXIDE SYNTHASE 2 ACTIVATION
**ABSTRACT:**

Cocaine (COC) related cardiovascular toxicities include acute and chronic cardiac events. The mechanisms of these phenomena are not well defined and treatment options are limited. We tested the hypothesis that cocaine *in vivo* causes acute electrocardiographic (ECG) anomalies and chronic cardiovascular dysfunction. Female C57BL/6 mice were dosed with ip COC (30mg/Kg) and ECG effects were monitored using a non-invasive 4 lead ECG (BIOPAC, Inc.) setup. Five days post cocaine, cardiac function was assessed using non-invasive murine echocardiography and ECG. Acute atrial (P wave), atrio-ventricular (P-R interval), ventricular (QRS and QT interval) conduction abnormalities were observed. QT and corrected QT (QTc) interval prolongation along with depressed cardiac output and stroke volume was observed five days post COC. These changes are consistent with phenomena known to occur in humans. In addition, we found extensive dysregulation of NO as evidenced by increased cardiac prevalence of NOS2, reactive nitrogen species and protein-3NT. These studies demonstrate that murine cardiac function is highly sensitive to COC treatment *in vivo*, and that the mouse is an appropriate model for further mechanistic studies and pharmacological investigations.
INTRODUCTION:

Cocaine abuse remains an epidemic in the U.S. and is associated with high social and medical costs. Estimates of the annual total costs for drug abuse has been estimated to be $98 billion dollars, and a majority of these costs are related to cocaine emergency cardiac care as these incidents account for nearly 30% all drug related emergency room (ER) visits, with an annual expenditure of nearly $83 million (1, 2). For example, acute episodes of angina pectoris, cardiac arrhythmias and myocardial infarction have all been commonly reported in cocaine abusers, and occasionally occur in first-time users. Separate from these acute events, chronic conditions including myocarditis, vasculopathy, cardiomyopathy, congestive heart failure have also been demonstrated in frequent cocaine users (3-6). Despite the high incidence and medical costs, the mechanisms responsible for cocaine-related cardiovascular disease are not well defined. Furthermore traditional therapies commonly employed for acute coronary syndrome have not been demonstrated effective, and at this time there is no established therapeutic strategy proven safe and effective for this setting (7-10). Given the facts that nearly 25 million people in the U.S. have used cocaine at least once and more than 5 million people abuse the drug on a regular basis, optimized and cost-effective strategies for cardiac complications are warranted (1).

As a pharmacological agent, cocaine is known to possess complex and multi-site activities in vivo. For example, cocaine and its related metabolites are known to block catecholamine reuptake at nerve terminals (considered a
classical mechanism of action), agonize dopamine and adrenergic (alpha and beta) receptors, and inhibit Na+ channel conductance in various cells and tissues (11). Thus within a single dosing interval, all of these mechanism are likely to contribute to cardiovascular pharmacological effects to varying degrees. This complicated pharmacology may explain at least some of the acute hemodynamic responses associated with cocaine use but often fails to completely explain cardiac complications that arise from its abuse. In addition, the actual hemodynamic changes elicited by a single cocaine dose in humans have been shown to be dose, time, and context dependent (12-14). It is likely that this degree of pharmacological complexity contributes to the general lack of success for emergency care (7-10).

Over the last three decades, much of the in vivo investigations of cocaine’s cardiovascular pharmacology and toxicology have been conducted in a wide array of animal models, including sheep, dogs, rabbits, and rats. In addition, other investigators have employed murine models for investigation of cocaine related hepatotoxicity. In these studies, our goal was to define the acute and protracted cardiovascular effects in a murine model, using our established expertise measuring contractile and electrophysiological parameters in this small species.

An additional component of these investigations was to evaluate potential contributions of altered nitric oxide (NO) control and reactive nitrogen species during cocaine induced cardiac dysfunction. Under normal physiological conditions, NO is known to modulate cardiac myocyte contractility and blood flow.
distribution (15), and high levels of NO production (via inducible nitric oxide synthase, NOS2) have been associated with several forms of cardiac disease, including dilated cardiomyopathy and congestive heart failure (16, 17). Once formed, NO rapidly reacts with available superoxide anion to form peroxynitrite, a reactive nitrogen species known to conduct several oxidative reactions in a cellular environment (18). Of these, nitration of protein tyrosine residues, producing 3-nitrotyrosine, is a unique feature of reactive nitrogen species (RNS) when compared to actions of reactive oxygen species (e.g., superoxide anion, hydroxyl radical, etc.) (19). We and others have demonstrated that reactive nitrogen species formation may be an important participant in the initiation and/or progression of a wide array of cardiac pathologies and that nitration of protein tyrosine residues can inhibit cardiac enzyme function and promote cardiac myocyte death via necrosis and/or apoptosis (20-24). Although reactive nitrogen species are known cellular toxicants, their participation in cocaine-induced cardiac toxicity has not been established. Here we tested the hypothesis that cocaine administration leads to acute electrocardiographic abnormalities and chronic cardiac dysfunction as measured by non-invasive murine electrocardiography and echocardiography. We therefore tested the hypotheses that NOS2 induction and/or cardiac protein nitration are involved in cocaine-induced cardiac dysfunction.
METHODS:

Study Design

All aspects of our animal use were in accordance with the guidelines of the National Institutional of Health and approved by the Institutional Animal Care and Use Committee at the Ohio State University. Female C57BL/6 mice (25-35g; n=7) were injected with a single dose of cocaine (30 mg/Kg ip) on day 0. Vehicle treated (n=3-6) mice were injected with an intraperitoneal injection of 0.1mL saline (0.9%) on day 0. Electrocardiographic measures were made under light halothane anesthesia immediately post cocaine or vehicle administration on day 0. Cardiac performance was assessed by echocardiography (see below) and electrocardiography (see below) on day 5. Immediately following echocardiography, animals were sacrificed with an overdose of pentobarbital. Entire hearts were then rapidly isolated, rinsed in ice-cold physiological buffer, equatorially sectioned at the mitral valve, and fixed in 10% buffered formalin for later immunohistochemical analysis (21).

Non-invasive Murine Electrocardiography

Animals were placed on a heated gel pack (Instant Heat Pads from Harvard Apparatus, South Natick, Massachusetts) designed to maintain normothermia during data acquisition. Recordings were made with adhesive electrodes attached to all four paws (BIOPAC Systems, Inc, Santa Barbara, CA) with the mice in a supine position. Electrocardiograms were digitally recorded,
continuously for 10 minutes post cocaine or saline, using a physiologic data acquisition system (MP100, Biopac Systems), with a sampling rate of 2000 Hz.

Data Analysis

The ECGs were signal averaged (from Lead I) over 150-200 beats per acquisition for determination of the P wave duration, P-R interval, and QT interval (graphically displayed in Figure 1, modified from Chapter 8). P wave duration (a measure intra-atrial conduction) from the onset of the P wave to the point where the P wave returns to the baseline; the P-R interval (representing intra-atrial and atrioventricular conduction) from the P wave onset to the onset of the QRS complex (representing ventricular depolarization); the end of QRS could not be measured accurately (25, 26), and therefore an estimate (onset of QRS complex to the start of S) of QRS duration has been reported. The QT interval (representing the duration of ventricular depolarization and repolarization) was measured from the onset of the QRS complex to the end of the T wave (intersection with the baseline). The average RR interval was determined from a 30 second sample. Rate corrected QT interval, QTc, was calculated using the methods of Bazett (QTc=QT/RR^{1/2}) (27).

Non-Invasive Murine Echocardiography

Mice were placed under light anesthesia with halothane inhalation (0.5 – 1% halothane USP in a mixture of 95% O₂ and 5% CO₂). We have recently demonstrated that this anesthetic approach provides more reliable and more
physiologically relevant cardiac function parameters when compared to injectable anesthesia in mice (28). Two-dimensional and M-mode echocardiographic images were recorded and analyzed by a SONOS 1000 echocardiograph and a 7.5 MHz ultrasonic probe (Hewlett-Packard Company, Andover, MA). Two-dimensional transverse LV imaging was used to position the probe just distal to the mitral valve leaflets and M-mode images were then captured. Three loops of M-mode data were captured from each animal at approximately 5-minute intervals and stored on digital disk until analysis. Each of these captured image loops provided 7-12 heart cycles, data were averaged from at least 5 cycles/loop.

LV systolic (LVIDs) and diastolic (LVIDd) internal dimensions were measured according to the American Society for Echocardiography leading-edge technique by a blinded investigator (29). These parameters allowed the determination of LV fractional shortening (%FS), a measure of systolic function, by the equation:

\[
%FS = \frac{(LVIDd - LVIDs)}{LVIDd} \times 100%.
\]

Ascending aortic flow velocity was determined using the continuous Doppler wave mode. Peak aortic flow velocity and velocity-time integral (VTI) were determined for at least 5 beats/loop for each animal. At sacrifice, the aortic outflow tract (aortic root) was isolated and cross-sectional area was measured via light microscopy with area-calibrated digital image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD) using methods we have previously described (28). Stroke volume (SV) was calculated by VTI x aortic root cross-sectional area (28). Cardiac output (CO) was calculated by SV x heart rate.
**Histology and immunohistochemistry**

Following formalin fixation (48hrs) cardiac tissues were embedded in paraffin for histological studies (cross-sectional orientations were used). Tissue sections (5µm) were mounted onto microscope slides and prepared for histological and immunohistochemical analyses, as we have previously described. Cardiac and splenic cross-sections were stained using hematoxylin/eosin and Masson's trichrome for routine morphologic and histologic assessments. Cardiac 3-nitrotyrosine (anti-3NT antibody, Upstate Biotechnology, Lake Placid, NY, 1:400 dilution) and nitric oxide synthase 2 (anti-NOS2, Transduction Labs, Lexington, KY, 1:400 dilution) were assessed in cardiac cross-sections, as previously described (21). Diaminobenzidine (DAB, 0.06% w/v, DAKO, Carpinteria, CA) was used to provide visualization of immunoreactivity, with methyl green counterstaining.

**Digital image analysis**

Digital images were acquired using a Polaroid DMC camera and Olympus microscope (model BX40) and transferred to Image Pro Plus software (Media Cybernetics, Silver Spring, MD) for both area and intensity analyses. Images were captured using identical light and software settings for control and infected tissues. In NOS2 and 3NT studies, left ventricular images were captured at 400x, and relative intensity was determined using image threshold analysis, as we have previously described (21, 30). Colored images were converted to gray-scale via extraction of the green channel to remove interference from methyl
green counterstaining. Intensity thresholds were predetermined as background staining in control tissues (eg, intensity of 160 for NOS2 stain); therefore, the percentage of total pixels in an image falling into the 0 to 160 range was used as a relative measure of positive immunoreactivity.

**Statistical Analysis**

All statistical analyses were performed using SigmaStat statistical software (Jandel Scientific, San Rafael, CA). Statistical comparisons were made using student t-test. In all cases p<0.05 was considered statistically significant.
RESULTS:

**Acute conduction abnormalities associated with cocaine administration**

Cocaine abuse, in humans, has been associated with acute and chronic electrical abnormalities (31-34). Therefore, we evaluated the effect of a single intra-peritoneal dose of 30mg/Kg cocaine on murine electrocardiograms acutely. Shown in Figure 2 (Panel A) are representative signal averaged Lead I recordings, at different time points (0-10min) following saline or (30mg/kg) cocaine administration. Cocaine caused P wave abnormalities (wider and split P wave), P-R interval elongation and QRS widening within 30 seconds of dosing (Figure 2A, Right Panel). Saline administration did not alter the murine electrocardiogram (Figure 2A, Left Panel). Shown in Figure 2 (Panel B) are the RR, P-R interval, QRS, and corrected QT interval measures with cocaine or saline over a span of 10 minutes post dosing. Cocaine caused an immediate increase in heart rate (Figure 2B, upper left panel) along with a sustained widening of the QRS complex (Figure 2B, lower left panel). Atrio-ventricular conduction (P-R interval) was initially retarded but regained normalcy 5 minutes post cocaine (Figure 2B, upper right panel). The corrected QT interval was slightly elongated after cocaine administration (Figure 2B, lower right panel).
**Altered electrical cardiac conduction and contraction in mice 5 days post cocaine**

Shown in Figure 3 are averaged electrocardiographic measures, in mice, 5 days after a single dose of cocaine or saline control. Mice, administered cocaine exhibited slower ventricular conduction as compared to saline controls (Figure 3, lower panels). Heart rate remained unaltered with cocaine administration (Figure 3, upper left panel). Shown in Figure 4 are averaged echocardiographic measures, in mice, 5 days after a single dose of cocaine or saline control. Cocaine caused significant reductions in cardiac output and stroke volume as compared to saline controls (Figure 4, middle and lower panels).

**Increased cardiac RNS, attendant protein nitration and NOS2 presence five days post cocaine**

Representative photomicrographs of cardiac protein-3NT immunoprevalence in mice treated with cocaine or saline are shown in Figure 5, where brown staining indicated positive immunoprevalence. Extensive cardiac myocyte protein nitration was observed in cocaine treated mice relative to saline treated controls (Figure 5, lower panel). Shown in Figure 6 are representative photomicrographs of cardiac NOS2 presence in mice treated with cocaine or saline. Immunohistochemical staining showed statistically significant evidence of NOS2 expression in cocaine treated mice relative to saline controls (Figure 6, lower panel).
**DISCUSSION:**

Cardiac complications are an established problem associated with cocaine abuse and include both acute cardiac incidents and chronic cardiac conditions (3-6). Traditional cardiovascular therapies when used to treat cocaine related cardiac events is often not reliable and fails to alleviate the cocaine related cardiac dysfunction (7, 10). Even though cocaine has known cardiovascular problems, it is still the most frequently abused drug in the United States (1). Despite the high costs and increasingly problematic cardiac toxicity associated with cocaine abuse, the mechanism involved in cocaine related cardiac toxicity is not completely understood. Therefore, we have developed a murine model of cocaine related cardiac toxicity to further investigate the mechanisms involved.

Here we have demonstrated that a single relevant dose of 30mg/kg cocaine caused acute electrical abnormalities along with chronic cardiac conduction and contractile abnormalities. Cocaine use, in humans is often associated with potentially fatal electrical anomalies and arrhythmias (31-34). Our murine model of cocaine cardiac toxicity recapitulates the human phenomenon *in vivo*, as cocaine administration resulted in altered cardiac conduction. Interestingly, five days post cocaine, ventricular conduction was significantly slower in cocaine treated mice, predisposing the animals to potential fatal ventricular arrhythmias (analogous to cocaine abuse in humans) (33, 35). Our murine model also exhibited cardiac contractile abnormalities (reductions in cardiac output and stroke volume), as measured by echocardiography. The dose of cocaine used here was similar to doses employed by several other groups for
the study of mouse behavioral effects (range 10-50mg/Kg) (36, 37). In initial experiments, we determined that this dose causes transient increases in heart rate and cardiac output (maximal increases 40-80%) (38). These cardiovascular effects are qualitatively similar to those achieved in humans (12). Furthermore, peak blood concentrations in mice at this dose are about 1-3µg/mL, similar to concentrations achieved in humans (13, 36). Thus, our dose appears to be "pharmacologically relevant" to the study design. In summary, these cardiac changes, produced by cocaine, are consistent with clinical observations, suggesting that this murine model appears appropriate for mechanistic evaluations.

Nitric oxide (NO) is a well-established participant in the regulation of vascular tone and may play important roles in cardiac function and disease (15). In settings of cardiac injury and/or inflammation, elevated NO production can occur via induction of NOS2 (16, 17). High concentrations of NO achieved through NOS2 induction may participate in further cardiac oxidative damage, apoptosis, and/or necrosis (39). Using immunohistochemical analysis, we observed widespread induction of NOS2 in cardiac tissue from cocaine treated mice compared to saline controls. Cocaine-induced liver injury, in mice, has also been shown to be mediated by NOS2 induction in hepatocytes (40). Aoki’s murine model of liver injury also suggests that reactive nitrogen species might be involved in cocaine-induced hepatotoxicity, but they do not demonstrate the presence of RNS in cocaine-injured livers (40). Here we tested the hypothesis that cardiac RNS formation was increased with cocaine administration.
The reactivities of NO in vivo are highly dependent upon its interactions with other oxidants and its capacity to participate in the production of reactive nitrogen species. Reactive nitrogen species (RNS) are a family of biologically relevant oxidants derived from the interaction of nitrogen based intermediates (most notably NO) with reactive oxygen species (e.g. superoxide anion, hydroxyl radical, hydrogen peroxide) (41). RNS can have profound cellular effects and toxicities due to the distinct reactivities of RNS relative to their reactive oxygen precursors (42). These reactivities include the avid capacity to cause nitration of tyrosine residues, both protein bound and free, resulting in the stable formation of 3-nitrotyrosine residues (19). Protein-3NT formation has been demonstrated to be a potent structural and functional post-translational protein modification, has deleterious effects on cardiac contractility, and has been observed in a panoply of cardiovascular disease states (21-24, 43).

We observed a progressive increase in the cardiac presence of protein-3NT in cocaine treated mice as compared to saline controls. These findings suggest that cardiac RNS formation and attendant protein nitration may participate in cocaine-related cardiac pathology. Staining patterns indicate that cardiac myocytes themselves carried a majority of the heart’s protein nitration burden—staining was widespread throughout the myocardium and was not confined to focal immune infiltrates. The rates of RNS formation are highly dependent on both NO and the reactive oxygen intermediates (bimolecular reaction kinetics), and thus increases in either NO or reactive oxygen species
might promote RNS formation (41). In this study, the increases in NO formation (via induction of NOS2) might be the promoter of cardiac RNS production.

In summary, a single dose of cocaine caused acute electrical abnormalities in mice, analogous to clinical phenomenon observed in humans. Cocaine administration also resulted in protracted cardiac dysfunction (slower conduction capabilities and depressed cardiac output and stroke volume) as measured by electrocardiography and echocardiography. Our murine model has apparent value in investigating cocaine related cardiac toxicity as the mouse exhibits cardiac insufficiencies similar to human cocaine abusers. Furthermore, the mouse has definable cardio toxic endpoints that allow for the evaluation of novel therapeutics. In addition, we observed extensive dysregulation of NO as evidenced by increased cardiac prevalence of NOS2, reactive nitrogen species and protein-3NT. These initial findings, in mice, suggest that novel therapeutics to control excessive prevalence of cardiac NOS2 and reactive nitrogen species might be useful in managing cocaine related cardiac dysfunction.
REFERENCES


ventricular systolic and diastolic function in humans. *Circulation* 97:1270-1273.


Figure 5.1:
A representative signal averaged Lead I recording from an untreated C57BL/6 mouse (modified from Chapter 8).
Figure 5.2:  
Acute electrocardiographic abnormalities post a single intra-peritoneal injection of 30mg/kg cocaine.

A – **Left Panel:** Representative signal averaged Lead I recordings from a vehicle (saline)-administered mouse.  **Right Panel:** Representative signal averaged Lead I recordings from a mouse administered 30mg/kg cocaine.

B – **Left Panel:**  
**Upper Panel:** Averaged RR interval post cocaine or saline measured over a span of 10 minutes post the injection. Data is represented as mean – SEM.  
**Lower Panel:** Averaged QRS interval post cocaine or saline measured over a span of 10 minutes post the injection. Data is represented as mean + SEM.

**Right Panel:**  
**Upper Panel:** Averaged P-R (upper) and corrected (lower) QT interval post cocaine or saline measured over a span of 10 minutes post the injection.  
**Lower Panel:** Averaged corrected QT (QTc) interval post cocaine or saline measured over a span of 10 minutes post the injection.  
Data is represented as a percentage of basal responses for each mouse. Vehicle (saline) administered (●), cocaine (○).
A

SALINE

COCOAINE

TIME (msec)

50 100 150

+0.5min

+2.5min

+5min

+7.5min

+10min

B

RR INTERVAL

P-R INTERVAL

QRS INTERVAL

QTC (Bazett)

TIME POST DOSE

(minutes)

SALINE

COCOAINE

(%) BASAL RESPONSE

(%) BASAL RESPONSE
Figure 5.3: Protracted electrocardiographic dysfunction in mice 5 days post cocaine (30mg/kg, ip.) administration.

Upper Panel: Averaged RR and P-R interval measures 5 days post cocaine or saline.

Lower Panel: Averaged QT and corrected QT interval measures 5 days post cocaine or saline. Data is represented as mean±SEM.

Data is represented as mean±SEM (n=3-7). Statistical significance *, p<0.05.
Figure 5.4: Protracted echocardiographic dysfunction in mice 5 days post cocaine (30mg/kg, ip.) administration.
Averaged cardiac function (as measured by echocardiography) in mice 5 days post cocaine or saline. Data is represented as mean±SEM (n=3-7). Statistical significance *, p<0.05.
Figure 5.5: Increased cardiac RNS formation and attendant protein nitration five days post cocaine (30mg/kg, ip).

**Upper Panels:** Representative images of 3-nitrotyrosine immunoreactivity in left ventricular myocardium of a vehicle control (saline) and virus-infected mouse (400x magnification).

**Lower Panel:** Relative immunoprevalence of 3-nitrotyrosine staining was determined by digital imaging and thresholding analyses. Data are represented as mean ± SEM. Statistical significance *, p<0.05 as compared to vehicle control (saline).
Figure 5.6: Increased cardiac presence of NOS2 five days post cocaine (30mg/kg, ip.).

Upper Panels: Representative images of NOS2 immunoreactivity in left ventricular myocardium of a vehicle control (saline) and virus-infected mouse (400x magnification).

Lower Panel: Relative immunoprevalence of NOS2 staining was determined by digital imaging and thresholding analyses. Data are represented as mean ± SEM. Statistical significance *, p<0.05 as compared to vehicle control (saline).
CHAPTER 6

COCAINE INDUCED ENDOTHELIAL DYSFUNCTION IN MICE AND OXIDANT PRODUCTION IN MURINE ENDOTHELIAL CELLS
ABSTRACT

Cocaine (COC) related cardiovascular toxicities include acute cardiovascular events and chronic cardiac and vascular dysfunction. The mechanisms of these phenomena are not well defined, and treatment options are limited. We tested the hypothesis that cocaine in vivo causes altered vascular responses. Five days after a single cocaine dose (30 mg/kg ip), mice (25-35g) were sacrificed and isolated thoracic aorta responses were evaluated. Selective impairment of acetylcholine induced (NO mediated) endothelium dependent relaxation was observed (Emax(%): 72±5 vs. 53±4, p<0.05), while responses to sodium nitroprusside remained unchanged (NS). In subsequent studies, isolated endothelial cells were treated with cocaine in vitro and intracellular oxidant production was evaluated by microscopy and digital image analysis (DCF fluorescence). Cocaine incubation caused time and concentration dependent production of reactive oxygen species relative to control (0-1mM COC, p<0.05); this signal was inhibited by the antioxidant N-acetylcysteine and the sigma receptor antagonist haloperidol. These studies demonstrate that endothelial performance is highly sensitive to cocaine treatment in vivo, and that increased cellular oxidative events may participate in cocaine related vascular toxicities.
INTRODUCTION

Cocaine abuse remains an epidemic in the U.S. and is associated with high social and medical costs. Estimates of the annual total costs for drug abuse has been estimated to be $98 billion dollars and a majority of these costs are related to cocaine emergency cardiovascular care, as these incidents account for nearly 30% all drug related emergency room (ER) visits, with an annual expenditure of nearly 100 million dollars (1, 2). Vascular complications associated with cocaine abuse include both acute events (hypertensive crisis, myocardial infarction, coronary vasospasms, and stroke) and chronic conditions (vasculopathy/vasculitis, hypertension, thrombosis, premature atherosclerosis and coronary artery disease) (3-6). Despite the high incidence and medical costs, the mechanisms responsible for cocaine-related vascular complications are not well defined. Furthermore, traditional therapies commonly employed for acute coronary syndrome have not been proven effective for cocaine-induced ischemia, and at this time there is no established therapeutic strategy proven safe and effective for this setting (7-10). Given the facts that nearly, 25 million people in the U.S. have used cocaine at least once and more than 5 million people abuse the drug on a regular basis, optimized and cost-effective strategies for cardiac and vascular complications are warranted (1).

As a pharmacological agent cocaine is known to possess complex and multi-site activities in vivo. For example, cocaine and its related metabolites are known to block catecholamine reuptake at nerve terminals (considered a
classical mechanism of action), agonize dopamine and adrenergic (alpha and beta) receptors, and inhibit Na+ channel conductance in various cells and tissues (11). Thus within a single dosing interval, all of these mechanism are likely to contribute to vascular pharmacological effects to varying degrees. This complicated pharmacology may explain at least some of the acute hemodynamic responses associated with cocaine use but often fails to completely explain vascular complications that arise from its abuse. In addition, the actual hemodynamic changes elicited by a single cocaine dose in humans have been shown to be dose, time, and context dependent (12-14). Cocaine’s intricate pharmacology probably complicates and adds to the inadequacy of traditional cardiovascular therapy in treating cocaine related conditions (7-10). Thus, there is an opportunity to improve therapy through enhanced mechanistic insight into the vascular complications.

One critical component of vascular integrity and function is the endothelial monolayer, an important component of normal endothelial cell function is the production of nitric oxide (NO) via metabolism of arginine. NO is now recognized as a critical component to a healthy endothelium, providing local modulation of vascular tone and inhibiting blood cell adhesion (15, 16). An impaired capacity of the endothelium dependent and NO mediated relaxant response has been observed in a wide variety of patients with vascular risk factors, including diabetes, smoking history, elevated cholesterol, and familial history (17). Cocaine has been shown to cause chronic endothelial dysfunction in humans as measured by forearm plethysmography (18).
Cocaine induced impaired endothelial function has been theorized to be related to increased levels of endothelin-1 mediated by direct influence of cocaine on sigma receptors (19). However, the exact mechanisms involved in cocaine induced vascular dysfunction are not completely understood.

Over the last three decades, much of the in vivo investigations of cocaine’s cardiovascular pharmacology and toxicology have been conducted in a wide array of animal models, including sheep, dogs, rabbits, and rats. In addition, other investigators have employed murine models for investigation of cocaine related hepatotoxicity. Here we employed a murine model to evaluate cocaine induced vascular endothelial dysfunction. In additional studies we tested the hypotheses that oxidants were involved in cocaine mediated vascular function. In parallel studies, we investigated mechanistic aspects using isolated murine endothelial cells to evaluate direct cellular effects, testing the hypotheses that sigma receptor activation, oxidants and/or apoptosis are affected by cellular exposures to cocaine.
METHODS

*In vivo vascular function in mice*

*Study design and dose selection*

All aspects of our animal use were in accordance with the guidelines of the National Institutional of Health and approved by the Institutional Animal Care and Use Committee at the Ohio State University. Female C57BL/6 mice (25-35g; n=6) were injected with a single dose of cocaine (30 mg/Kg ip). Initial non-invasive murine echocardiographic studies were performed to evaluate the acute hemodynamic effect of 30mg/kg *in vivo*. Five days after a single cocaine dose, mice were sacrificed, and isolated thoracic aorta responses were evaluated.

*Non Invasive murine echocardiography*

Mice were placed under light anesthesia with halothane inhalation (0.5 – 1% halothane USP in a mixture of 95% O₂ and 5% CO₂). Two-dimensional and M-mode echocardiographic images were recorded and analyzed by a SONOS 1000 echocardiograph and a 7.5 MHz ultrasonic probe (Hewlett-Packard Company, Andover, MA). Ascending aortic flow velocity was determined using the continuous Doppler wave mode. Peak aortic flow velocity and velocity-time integral (VTI) were determined for at least 5 beats/loop for each animal. At sacrifice, the aortic outflow tract (aortic root) was isolated and cross-sectional area was measured via light microscopy with area-calibrated digital image analysis (Image-Pro Plus, Media Cybernetics,
Silver Spring, MD) using methods we have previously described (20). Stroke volume (SV) was calculated by VTI x aortic root cross-sectional area (20). Cardiac output (CO) was calculated by SV x heart rate. Heart rate was measured electrocardiographically (Lead I) throughout the imaging session. Cardiac output and heart rate were measured prior to cocaine (30mg/Kg ip.) and fifty minutes post cocaine.

**Isolated Vascular Function:**

Five days after the single dose of cocaine, mice were euthanized by a pentobarbital overdose, and the thoracic aorta was rapidly isolated as previously described (21, 22). Vascular segments (2-3mm), dissected free of fat and loose connective tissue, were suspended by stainless hooks in a 10mL tissue bath containing Krebs’ buffer at 37°C, oxygenated by constant bubbling of a 95/5% O2/CO2 mixture. The resting tension was maintained at 1.0g. Following a 45min equilibration in oxygenated Krebs’ buffer, aortic segments (n=3-5 segments per animal) from saline and cocaine treated animals were pre-contracted with 0.5µM phenylepinephrine (PE). PE pre-contraction was unchanged between the two groups (SAL: 185±15mg vs. COC: 198±12mg, NS). After pre-contraction, concentration-effect data was obtained by cumulative addition of acetylcholine (Sigma Chemical Co., St. Louis, MO) in 100µL aliquots over a concentration range of 9X10^{-9} to 9X10^{-6} M. Similar concentration-effect data was obtained by cumulative additions of sodium nitroprusside (Sigma Chemical Co., St. Louis, MO) over a concentration range
of 3X10^{-10} to 1X10^{-6} M. Vessel tension data were collected by DigiMed Tissue Force Analyzer and System Integrator model 210 (Micro-med, Louisville, KY). Cumulative relaxation data were expressed as percentage of initial pre-contraction. Relaxation data from each vessel segment was fitted to a sigmoidal $E_{\text{max}}$ model, using Graph Pad Prism Software (San Diego, CA). $EC_{50}$, $E_{\text{max}}$, and Hill slope were determined for each treatment group. All curve fits had $r^2$ values >0.8.

**Isolated Endothelial Cell Experiments**

**Isolation and Culture of Murine Endothelial Cells**

Endothelial cells were isolated from C57BL mice, as previously described (23), and maintained in modified murine endothelial cell (MEC) medium containing (per 500 mL): DMEM, 200 mL; Hams F12, 200 mL; Penn/Strep, 8 mL of 200U+200$\mu$g/mL; ECGS, 15mg; Heparin 5,000 U (Gibco, BRL, Grand Island, NY).

**DCF Fluorescent Measurement of Reactive Oxygen Species in Cultured Endothelial Cells:**

Endothelial cells (passage 16-20) were cultured on glass coverslips affixed to six-chambered wells. Once the cells had reached approximately at 80% confluence, they were exposed to varying concentrations of cocaine (COC: 0, 1, 10,100,1000 $\mu$M) for various incubation times (0.75, 24, 48 hr). At
each selected time point intracellular reactive oxygen species production was measured using a fluorescent dye (Molecular Probes, Inc.). Endothelial cells were incubated, in a dark room, with 5-(and 6-)chloromethy-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA: 5 µM, 30 min, 37°C) in serum-free media. Thirty minutes after the dye (CM-H$_2$DCFDA) was allowed to passively diffuse through the cells, where its acetate groups were cleaved by intracellular esterases, the cells were washed free of excess dye and prepared for imaging. Free radical production was measured by changes in fluorescence due to the oxidation of CM-H$_2$DCF by reactive oxygen species to produce CM-DCF (a fluorescent product). Bright field and fluorescent images (n=50-100 cells) were captured immediately using a fluorescent microscope (Olympus microscope equipped with a fluorescent image capture system - Magna Fire Software). Cells were identified and located using bright field images and corresponding fluorescent intensity, in the identified cells, was measured within each cellular boundary using an imaging software program (Image Pro Plus Software). Oxidant production was also evaluated in endothelial cells exposed 1000µM cocaine in combination with 20mM N-acetylcysteine (NAC – free radical scavenger) for a 24-hour incubation period. Free radical production by 100µM cocaine, in endothelial cells, in the presence of 10 µM haloperidol (sigma receptor antagonist) was also evaluated in later studies.
**Detection of Apoptosis and Necrosis in Cultured Cells**

Endothelial cells (passage 16-20) were cultured on 8-well chambered glass slides. Once the cells had reached approximately at 80% confluence, they were exposed to varying concentrations of cocaine (COC: 0, 1, 10, 100, 1000 µM) for twenty-four hours. Cell death (apoptosis and/or necrosis) was assessed by Alexa 488 annexin V and propidium iodide (PI) labeling of the cells (Vybrant Apoptosis Assay Kit, Molecular Probes Inc.). Endothelial cells were incubated with Alexa 488 annexin V (fluorescein (FITC)-conjugated annexin V) and PI for 15min at room temperature. Cells were then washed free of excess dye and fixed in 4% ice-cold paraformaldehyde (3min). Annexin V labeling of phosphatidylserine translocation across cell membrane (inside to the outside) and PI labeling was captured using a fluorescent microscope (Olympus microscope equipped with a fluorescent image capture system - Magna Fire Software). Cells in late stage apoptosis were identified by PI+ stained nucleus (red fluorescence) and annexin V labeled – phosphatidylserine (green fluorescence). Necrotic cells were identified by PI+ stained nuclei only. Cells demonstrating either feature of fluorescent labeling were then totaled and represented as a percentage of total cells per treatment group.
**Statistical Analysis**

All data are represented as mean ± SEM, except where noted. Student t-tests were used to evaluate relaxation responses between vascular segments from cocaine and saline treated animals (Sigmastat, Jandel Scientific, San Rafael, CA). Simultaneous comparisons of groups were handled by one-way analysis of variance, with Student Newman – Keuls post – hoc analysis (Sigmastat, Jandel Scientific, San Rafael, CA). Statistical significance was assigned as p<0.05 for all comparisons.
RESULTS

**Cocaine cause protracted impairment of endothelial function**

Cocaine abuse has been associated with endothelial dysfunction in humans (18). Here we tested the hypothesis that a single dose of 30mg/Kg of cocaine would cause protracted (5 days) endothelial dysfunction in mice. Shown in Figure 2 are the averaged concentration-effect data for acteylcholine (endothelium mediated) and sodium nitroprusside (non-endothelium mediated) in vascular segments from saline (SAL) and cocaine (COC) treated animals. There was no significant difference in PE pre-contraction between the two treatment groups (SAL: 185±15mg vs. COC: 198±12mg, NS). Endothelium dependent, nitric-oxide (NO) mediated maximal relaxation to acetylcholine was significantly depressed in vascular segments from cocaine treated animals as compared to saline controls (E$_{max}$ (%): SAL: 72±5 vs. 53± 4, *, p<0.05) (Figure 2, Panel A). There was no significant difference, in ACH potency as determined by comparisons of fitted EC$_{50}$ values (EC$_{50}$ (nM): SAL: 52±16 vs. COC: 175±52, NS). Endothelium-independent relaxation response to sodium nitroprusside was not significantly affected (Figure 2, Panel B). There was no significant change in fitted E$_{max}$ or EC$_{50}$ values between the two groups (E$_{max}$ (%) - SAL: 114±2 vs. COC: 112±2; EC$_{50}$ (nM) – SAL: 6±1 vs. COC: 7±1). Thus, there was a selective impairment in endothelial NO production or availability in vessels obtained from cocaine treated animals.
**Intracellular oxidant production in murine endothelial cells**

Isolated endothelial cell experiments were designed to further evaluate the effect of cocaine on endothelial cells in vitro. Shown in Figure 3 (Panel A) are relative levels of intracellular oxidants produced in response to cocaine ([COC]: 0, 1, 10, 100, 1000 µM) for twenty-four hours determined by DCF fluorescence experiments. Shown in Figure 3A (Left panel) are representative images of the DCF fluorescence when endothelial cells were exposed to cocaine. Intracellular oxidant production was significantly increased with cocaine exposure and was dependent on the concentration of cocaine (Figure 3A, Right Panel). Oxidants were produced with relevant concentrations of cocaine \( C_{\text{max}} \) in vivo \(< 50\mu\text{M cocaine} \) (Figure 3A, Right Panel). Oxidants produced by endothelial cells exposed to 1000µM cocaine were inhibited by 20mM NAC. Shown in Figure 3 (Panel B) is intracellular oxidant production (24hr) in endothelial cells alone and in conjunction with haloperidol (10µM). The endothelial oxidant signal produced by 100µM cocaine was suppressed in the presence of a sigma receptor antagonist (haloperidol) (Figure 3, Panel B). Shown in Figure 4 are levels of oxidants produced by endothelial cells due to cocaine exposure over a period of 0.75, 24, and 48 hours. An acute exposure (45min) of 1000µM cocaine produced a significant increase in intracellular oxidants in endothelial cells (Figure 4, Panel A). Oxidant production in endothelial cells was observed at even lower concentrations of cocaine (0.01,
0.1, 1, 10 μM) after a 48 hours (Figure 4, Panel B). Cocaine mediated oxidant production was both time and concentration dependent (Figure 4, Panel C).

_Cocaine causes apoptosis and necrosis in isolated murine endothelial cells_

Cocaine’s role in endothelial cell death (apoptosis and necrosis) was evaluated in isolated cultures using a fluorescent labeling system (annexin V and propidium iodide). Shown in Figure 5, upper panel are representative images of endothelial cells exposed to cocaine undergoing both apoptosis and necrosis after 24hr. Shown in Figure 5 are the effects of cocaine (0, 10, 100, 1000 μM for 24hr) exposure on endothelial cell viability in vitro. Apoptosis and necrosis was evident at both 100μM and 1000μM cocaine (Figure 5, lower panel). Even though most apoptotic and necrotic events were observed with high concentrations of cocaine (100μM and 1000μM), a small percentage of cells underwent apoptosis with 10μM cocaine (Figure 5, upper left panel).
DISCUSSION

Cocaine abuse has been associated with acute and potentially fatal vascular incidents (hypertensive crisis, myocardial infarction and vasospasms) and chronic vascular dysfunction (vasculopathy/vasculitis, hypertension, thrombosis, premature atherosclerosis and coronary artery disease, and stroke) (3, 5, 6, 24). Cocaine’s complicated pharmacology has often influenced the reliability of traditional cardiovascular therapy for cocaine related vascular disease (6-10). Cocaine abuse is on the rise even though cocaine abuse has been linked to several cardiovascular insufficiencies (1). The mechanism involved in cocaine related vascular dysfunction is not completely understood. Thus, relevant models (in vivo and in vitro) of cocaine related vascular dysfunction are becoming increasingly important in investigating the putative mechanisms involved in cocaine related toxicities. Murine models are gaining validity and usefulness in defining mechanisms involved in cardiovascular disease and also provide with an opportunity to test novel therapeutics. Here we investigated the value and appropriateness of mouse models in cocaine related vascular dysfunction.

Endothelial dysfunction is an important initiator of chronic vascular disease in both humans and animals. C57BL/6 mice administered 30mg/kg cocaine intraperitoneally had significantly impaired vascular function as compared to the saline administered controls. Vascular impairment was selective for the endothelium as responses to acteylcholine (endothelium mediated NO release) were depressed while responses to sodium
nitroprusside (an exogenous NO donor) remained unaltered. These results were in accordance with a clinical study by Havarenek and his colleagues, which demonstrated that endothelial function, as measured by forearm plethysmography, in cocaine addicts was significantly impaired when compared to people who did not use cocaine (18). The dose of cocaine used here was similar to doses employed by several other groups for the study of mouse behavioral effects (range 10-50mg/Kg) (25, 26). In initial experiments we determined that this dose causes transient increases in heart rate and cardiac output (maximal increases 40-80%). These cardiovascular effects are qualitatively similar to those achieved in humans (12). Furthermore, peak blood concentrations in mice at this dose are about 1-3μg/mL, similar to concentrations achieved in humans (13, 25). Thus our dose appears to be "pharmacologically relevant" to the study design.

The mechanistic study of cocaine related vascular dysfunction was further investigated in isolated murine endothelial cells. Oxidant pathways have been shown to be involved in several states of cardiovascular disease including endothelial dysfunction (27). However, the role of oxidants in cocaine mediated endothelial toxicity has never been studied. We found that relevant concentrations of cocaine were able to enhance oxidant production in endothelial cells as measured by DCF fluorescence. Plasma concentrations of cocaine in humans are normally less than 100μM, but concentrations higher than 100μM have been found to be present in people who abuse and overdose on cocaine (28-31). Cocaine concentrations used in the isolated
murine endothelial cells encompassed both ends of the spectrum of cocaine plasma concentrations in humans. Interestingly, even low concentrations of cocaine (<10μM cocaine) were able to promote oxidant production in endothelial cells (48 hour exposure). DCF fluorescence is a sensitive method to detect intracellular oxidant production, but its value is limited by the inability to detect the exact nature of oxidant species (32). However, this method was still useful in measuring the overall oxidant stress invoked by cocaine in endothelial cells.

The exact mechanism by which cocaine enhanced intracellular oxidant production in endothelial cells, has not been completely elucidated. However, our investigations suggest that cocaine might mediate oxidant production via sigma receptors. Recent data has demonstrated the presence of sigma receptors (non-opioid) on endothelial cells (19). Inhibition of sigma receptors (via haloperidol) on bovine coronary artery cells resulted in abrogation of cocaine stimulated increase in endothelin (19). Endothelin is a vaso-constrictive peptide that has been shown to be elevated in several vascular disease settings, including cocaine related vascular dysfunction (15). Intracellular oxidants might promote endothelin release, which suggests that cocaine might increase intracellular oxidants, and these oxidants may stimulate endothelial cells to release endothelin (33-35). Sigma receptors have recently been shown to mediate events in endothelial cells, but the exact mechanisms by which cocaine mediates its toxicity through them is still not understood. Neuronal-inhibition, of sigma receptors, have shown some
promise in alleviating psychotic conditions associated with cocaine abuse, but its exact usefulness to vascular disease is a still unknown (36). However, there is hope that these receptors might be novel targets for cocaine related vascular toxicity.

Cocaine has been shown to elicit its cytotoxic effects in neurons, thymocytes, hepatocytes and myocytes by stimulating apoptosis (37-40). Apoptosis or programmed cell death is an efficient physiological process by which the body rids itself of unwanted and damaged cells (41). Here we have demonstrated that cocaine exposure (24hr) causes endothelial cells to die via both apoptosis and necrosis. Our results are in accordance with experiments performed in human and bovine coronary artery endothelial cells. Both these studies illustrate that in vitro cocaine exposure causes endothelial cells to undergo apoptosis (42, 43). Cocaine has been shown to mediate endothelial cell apoptosis by suppressing nitric oxide release from bovine coronary artery endothelial cells (43). Studies in human coronary artery endothelial cells suggest that cocaine might be mediating apoptosis via release of mitochondrial cytochrome c and further activation of caspases-9 and caspases-3 (44). Our study illustrates the fact that fairly high concentrations of cocaine (100µM and 1000µM), analogous to plasma concentrations in people who died from an overdose of cocaine, are required to cause endothelial cell death (31). Endothelial cell death (via both apoptosis or necrosis) can lead to endothelial dysfunction, which may be mechanism by which cocaine abuse results in vascular toxicity (41).
In summary the mouse seems to be an appropriate model to study cocaine related vascular dysfunction especially since a 30mg/kg dose of cocaine cause protracted endothelial dysfunction. Cocaine related endothelial toxicities involve a concentration and time dependent production of oxidants. Antioxidants may have value ameliorating endothelial dysfunction due to cocaine induced vascular toxicity, and preliminary evidence suggests that cocaine may mediate oxidant production through sigma receptors. Cocaine induced endothelial dysfunction and toxicity *in vitro* involves both apoptotic and necrotic events that may explain protracted endothelial dysfunction with cocaine use and abuse.
REFERENCES


ventricular systolic and diastolic function in humans. *Circulation* 97:1270-1273.


36. Maurice, T., Martin-Fardon, R., Romieu, P., and Matsumoto, R.R. 2002. Sigma(1) (sigma(1)) receptor antagonists represent a new strategy


Figure 6.1:
Cocaine (30 mg/kg, ip.) caused acute increases in heart rate and cardiac output (maximal increases of 40-80%) lasting for 40-60 minutes after injection in vivo.

**Upper Panel:**
Heart rate, as measured by electrocardiography, was significantly increased with cocaine (30mg/kg, ip.).

**Lower Panel:**
Cardiac output, as measured by electrocardiography and echocardiography, was significantly increased with cocaine (30mg/kg, ip.). Data is represented as mean±SEM (n=3-6).
Figure 6.2:
Cocaine caused protracted (5 days) endothelial dysfunction in mice - evidence of selective reduction in endothelial NO production or availability.

**Panel A:**
Endothelium dependent, NO mediated relaxation in response to acetylcholine (ACH) was significantly depressed ((E\textsubscript{max} (%): SAL: 72±5 vs. 53± 4, *, p<0.05), while EC\textsubscript{50} were not significantly different between the two treatment groups (EC\textsubscript{50} (nM): SAL: 52±16 vs. COC: 175±52).

**Panel B:**
Endothelium-independent relaxation in response, to sodium nitroprusside (exogenous NO donor), in vascular segments from saline and cocaine treated animals were not significantly different. Fitted E\textsubscript{max} and EC\textsubscript{50} responses were unaffected by the prior cocaine treatment (E\textsubscript{max} (%): SAL: 114±2 vs. COC: 112±2; EC\textsubscript{50} (nM) – SAL: 6±1 vs. COC: 7±1).

Segments from saline administered mice (●) and cocaine administered mice (○). Data is represented as mean ± SEM (n=4-6). Statistical significance *, p<0.05.
Figure 6.3: *In vitro* cocaine exposure caused an increase in murine endothelial cell oxidant production (as measured by DCF fluorescence).

**A:** **Left Panel:** Representative images of oxidant production, in endothelial cells, exposed to a control solution and cocaine (10 and 100 µM). Note the suppression of the DCF fluorescent signal in the cells exposed to 1000 µM cocaine in conjunction with 20 mM N-acetyl cysteine (NAC). **Right Panel:** Statistically significant concentration-dependent increases in intracellular (endothelial cells) oxidants following a 24 hr exposure to cocaine (1, 10, 100, 1000 µM) compared to control (*, p<0.05; n=50-100 cells). The oxidant signal was significantly inhibited by the antioxidant NAC, (20 mM, †, p<0.05, n=40-78 cells). Data are represented as mean ± SEM. Statistical significance *, p<0.05 compared to 0 µM cocaine and †, p<0.05 compared to 1000 µM cocaine.

**B:** Intracellular oxidant production due to 100 µM cocaine was inhibited by 10 µM haloperidol (sigma receptor antagonist). Data are represented as mean ± SEM. Statistical significance *, p<0.05 compared to 0 µM cocaine and †, p<0.05 compared to 100 µM cocaine.
Figure 6.4: 
*In vitro* cocaine exposure caused a time-dependent increase in intracellular oxidant production in murine endothelial cells. 

**Panel A:** An acute exposure (45min) of 100µM cocaine caused a significant increase in intracellular oxidant production as compared to 0µM cocaine (*, p<0.05, n=50-75 cells).  

**Panel B:** In vitro exposure (48hr) of cocaine (0.1, 1, 10 µM) caused a significant increase in intracellular oxidant production as compared to 0µM cocaine (*, p<0.05, n=50-75 cells).  

**Panel C:** Intracellular oxidant production modulated by cocaine is both time and dose dependent in isolated murine endothelial cells.
Figure 6.5:
Cell death (apoptosis/necrosis) due to an in vitro exposure (24hr) of cocaine (10, 100, 1000 µM) (as measured by annexin V and propidium iodide labeling.

Upper Panels: Representative images of murine endothelial cells co-labeled with annexin V (green) and propidium iodide (PI) after exposure (24hr) to cocaine (0, 10, 100, 1000 µM). Cells undergoing an early stage of apoptosis cells were stained with annexin V alone, while late cells at later stage of apoptosis were stained with both annexin V and propidium iodide. Cells primarily labeled by propidium iodide (nuclear stain) were identified as being necrotic cells (1000µM cocaine).  Lower Panels: Cocaine exposure (24hr) caused a significant increase in cells undergoing apoptosis and/or necrosis. Data is represented as an average percentage of cells undergoing apoptosis or necrosis per treatment group.
SECTION III

MURINE MODELS IN CARDIOVASCULAR DISEASE
(MURINE ECHOCARDIOGRAPHY AND ELECTROCARDIOGRAPHY)
CHAPTER 7

NON-INVASIVE ECHOCARDIOGRAPHIC STUDIES IN MICE: INFLUENCE OF ANESTHETIC REGIMEN

This chapter has been published in Life Sciences (Life Sci 72:2401-2412.) and has been presented here as per the requirements of the journal.
ABSTRACT

Transgenic murine models of cardiovascular disease offer great potential insights regarding mechanisms of human disease, but efficient and reliable methods for phenotype evaluation are necessary. We employed non-invasive echocardiography to evaluate hemodynamic parameters in mice, and evaluated statistical reliability of these parameters with respect to anesthesia regimen. Male CF-1 mice received inhaled halothane (0.25-0.75% in 95% O₂) or ketamine/xylazine (80/10mg/kg i.p.) and 2-dimensional, M-mode, and Doppler ultrasound imaging were used to assess cardiac contractility and aortic flow velocities. Halothane was more convenient and reliable with respect to rate of induction, reversal, and control of anesthetic depth. At comparable levels of anesthesia, ketamine/xylazine produced significant reductions in heart rate (308 ± 14 vs. 501 ± 14 bpm, p<0.001), left ventricular fractional shortening (41.7 ± 1.3 vs. 49.3 ± 1.0 %, p<0.001), and cardiac output (7.6 ± 0.5 vs. 11.5 ± 0.6 ml/min, p<0.001) when compared to halothane inhalation. No change in stroke volume or peak aortic velocity was observed. Correlation analyses revealed highly significant positive relationships between heart rate and fractional shortening (r= 0.61, p<0.002) and cardiac output (r= 0.88, p<0.001) but no relation to stroke volume or aortic velocity. Variability of intra-animal and intra-group parameter estimation were frequently 2-fold larger for ketamine/xylazine anesthesia vs. halothane. Statistical power analysis showed the increased measurement error for ketamine/xylazine leads to much larger numbers of mice/group to achieve identical statistical sensitivity. These data
further illustrate the feasibility of echocardiography for rapid, non-invasive cardiovascular assessment in mice. However, several obtainable parameters are highly sensitive to both heart rate and anesthetic used and the choice and control of anesthetic are critical for physiologically relevant performance parameters and maximal ability to detect statistical differences among groups. Thus, for these non-invasive studies, inhalation anesthesia with agents such as halothane is superior to anesthesia induced by ketamine/xylazine administration.

**Keywords:** mice, echocardiography, anesthesia, halothane, ketamine, xylazine, cardiovascular
INTRODUCTION

Transgenic animal techniques have greatly expanded studies of cardiovascular disease mechanisms and pharmacological research. These animal models, primarily developed in mice, include selective gene deletion ("knock-out"), tissue-specific gene promotion, and selective gene induction strategies [1-3]. While these strategies have enhanced research potential in the identification of molecular and genetic factors contributing to disease, they have also created significant challenges for reliable physiological parameter measurements in these small rodents.

Regardless of the murine model(s) employed, accurate, reliable, and convenient measurement of cardiovascular function is critical for characterizing phenotypes and relating these to human disease. Several invasive approaches have been previously employed for the study of cardiac function in mice, including in vivo open-chested working heart preparations or ex vivo techniques [4-7]. These methods have been valuable, but technical difficulties, non-physiological conditions, and limited throughput make them difficult for routine investigations and efficient phenotypic or pharmacological screening.

Trans-thoracic ultrasound technologies (echocardiography) may be useful for rapid and non-invasive cardiac evaluations in mice [8-12]. Thus far, most published reports using such methods have focused on cardiac size
estimations rather than functional performance and have employed a wide array of instrumentation, animal handling conditions, and anesthesia regimens. Consequently, reported parameter values for cardiac performance are highly variable and may not be physiologically relevant. For example, reported heart rate values in control mice have ranged from 250 to 460 beats/min in various studies employing echocardiographic methods [8,10,11], whereas rates in conscious, unrestrained animals of 450-650 bpm are considered physiologically relevant [13,14].

Given the limited availability and high cost associated with transgenic animal models, optimal conditions for valid echocardiographic measures are important. While other reports have demonstrated the potential value of these methods, conditions required for reliable measurements have not been defined. This aspect is particularly important for long-term serial study designs and drug trials, as well as the rapid screening of large numbers of animals.

In evaluating the optimal conditions for murine echocardiography, investigators must consider not only animal and instrumentation conditions but also which cardiac functional parameters are most reliable in detecting differences among groups. Here we have compared the utility of an injectable mixture of ketamine/xylazine, widely used for small animal research and previously used for echocardiographic measures [8-10], to that of a halothane inhalation approach. In addition, we considered the potential impact of anesthetic choice on statistical power, contrasting several traditional
parameters of cardiac performance. The goals of our investigations were to identify conditions and parameters that provide reliable, convenient, and relevant echocardiographic measurements in mice.
MATERIALS AND METHODS

Study Design

A randomized crossover study design was employed to evaluate the impact of anesthetic on cardiac functional parameters. Male mice (20-25g, CF1 strain, Harlan, Indianapolis, IN) were anesthetized by either ketamine/xylazine or halothane inhalation and each regimen was separated by 24-36 hours. In preliminary studies we found treatment order did not affect parameter measurements. All aspects of this study were approved by the Institutional Animal Care and Use Committee.

A mixture of ketamine HCl (Ketaset, Fort Dodge Labs, Fort Dodge, IA) and xylazine HCl (TranquiVed, Vedco Inc., St. Joseph, MO) was prepared in sterile 0.9% saline. A dose of 80/10 mg/kg (ketamine/xylazine) was injected intraperitoneally in an approximate volume of 0.25 ml. This drug combination is commonly employed as a short-acting rodent anesthetic and has been previously used for murine echocardiography. The dosages used were approximately the average of previously published reports, in preliminary trials it provided approximately 30-60 minutes of anesthesia in mice.

Halothane USP (Halocarbon Labs, River Edge, NJ) was administered via a vaporizer (Bickford Vapomatic, A.M. Bickford Inc, Wales Center, NY) specially modified for low gas flow rates (100-300 ml/min). Following induction in an isolation chamber (3.5-4.5% halothane in 95% oxygen), anesthesia was maintained by spontaneous breathing of 0.5-0.75% halothane, delivered by a small nose cone.
Echocardiography and Doppler Flow Analysis

Following anesthetic induction, mice were gently restrained in the left lateral decubitus position with elastic bands attached to a heated pad to maintain normothermia. The chest area was shaved and ultrasound coupling gel was liberally applied to the left chest wall. Two-dimensional and M-mode echocardiographic images were recorded and analyzed by a Sonos 1000 echocardiograph and a 7.5 MHz pediatric ultrasonic probe (Hewlett-Packard Company, Andover, MA). Two-dimensional transverse LV imaging was used to position the probe just distal to the mitral valve leaflets and M-mode images were then captured. Three loops of M-mode data were captured from each animal at approximately 5 minute intervals and stored on digital disk until analysis. Each of these captured image loops provided 7-12 heart cycles, data was averaged from at least 5 cycles/loop. LV systolic (LVIDs) and diastolic (LVIDd) internal dimensions were measured according to the American Society for Echocardiography leading-edge technique by a blinded investigator. These parameters allowed the determination of LV fractional shortening (%FS), a measure of systolic function, by the equation: %FS = [(LVIDd – LVIDs) / LVIDd] x 100%.

Ascending aortic flow velocity was determined using the ultrasonic probe in continuous Doppler wave mode. The probe was maintained in the parasternal short axis orientation, but moved horizontally along the chest wall toward the suprasternal notch. While monitoring real time color flow, the probe was slowly angled towards the head of the animal to approximately 45°
relative to the spinal axis (probe face pointing towards the heart). This probe position provided color-enhanced definition of blood flow around the aortic arch. The Doppler beam was centered on the ascending flow tract approximately 2 mm distal from the aortic valves and beat-to-beat cycles of aortic blood flow velocity were then recorded in three captured loops as described above. From these recordings, peak aortic flow velocity and velocity-time integral (VTI) was determined for at least 5 beats/loop for each animal. At the end of this crossover study each animal was sacrificed for isolation of the aortic outflow tract (aortic root). This tissue provided accurate measurement of aortic root diameter via light microscopy with area-calibrated digital image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). Aortic root cross-sectional area was then calculated and stroke volume (SV) was calculated by VTI x aortic root cross-sectional area. Cardiac output (CO) was calculated by SV x heart rate.

**Statistics**

All statistical evaluations were performed using SigmaStat statistical software (Jandel Scientific, San Rafael, CA). Statistical comparisons of cardiac functional parameters between anesthetic treatment groups were made using paired t-tests. Pearson's correlation analysis was used to test statistical relationships between measured heart rate and other cardiac parameters. Variation of parameter estimates were compared using coefficients of variation (CV%), calculated as standard deviation/mean x 100%. The intra-animal and
intra-group variability of cardiac parameters was evaluated for each anesthetic regimen and statistical power analysis was performed to determine the animals required for detectable differences. In all tests p<0.05 was considered significant.
RESULTS

In general, intraperitoneal injection of ketamine/xylazine (80/10 mg/kg) required 5-20 minutes post-injection to provide adequate depth of anesthesia (minimal response to hind-foot pinch) and the duration of this effect ranged from 30-60 minutes in normal mice (n=12). In contrast, halothane induction consistently required less than 5 minutes. Depth of halothane anesthesia was conveniently stabilized through slight adjustment of inhalant concentration (0.25-0.75%) and mice quickly recovered to ambulatory activity within 2 minutes after removing the nose cone. One animal died during the echocardiography procedure following ketamine/xylazine anesthesia, presumably due to respiratory difficulties. No complications were observed with halothane anesthesia.

Shown in Figure 1 are representative LV M-mode and ascending aorta Doppler flow images following ketamine/xylazine versus halothane anesthesia. Images were readily obtained in all animals using the 7.5 MHz pediatric probe. Both sets of images clearly illustrate the markedly reduced heart rate caused by ketamine/xylazine. Shown in Figure 2 are statistical relationships between cardiac functional parameters and heart rate, using Pearson’s Product Moment correlation analyses. Fractional shortening and cardiac output were statistically correlated with measured heart rate, whereas stroke volume or peak aortic velocity were not. Table 1 shows the intra-animal and intra-group variability of measured functional parameters following ketamine/xylazine
versus halothane anesthesia (expressed as coefficients of variation, CV%).

Intra-animal variability was determined by the average and standard deviation of the triplicate measurements made (at approximately 5 minute intervals) while anesthetized by each treatment (e.g., 12 observations for each treatment group). Group variability was determined by the standard deviation and mean of the population following each treatment. Frequently, CV% values were nearly 2-fold higher in the ketamine/xylazine treatment group relative to corresponding values following halothane anesthesia.

Using the measured variance (standard deviation) in cardiac functional parameters, statistical power analyses were conducted (SigmaStat) to determine the number of animals required for detecting differences among groups. Shown in Figure 3 are relationships of statistical power and numbers of animals required to detect statistically meaningful differences between two groups (assuming an unpaired student’s t-test). For each anesthetic regimen, an exponential relationship between number of animals/group and statistical power was observed. Additionally, the use of ketamine/xylazine anesthesia was associated with an increased number of animals required to detect differences relative to halothane use. Thus, for each parameter measured, additional animals are required when using ketamine/xylazine to achieve the same statistical sensitivity compared to halothane. Depending on the statistical power desired, the animal requirements often differed by more than 2-fold between anesthetic treatments and were most obvious for fractional shortening and heart rate parameters (Figure 3).
DISCUSSION

Our cross-over study demonstrated that anesthetic choice can have a profound effect on cardiovascular parameter estimation in mice. These anesthetic regimens were studied because ketamine/xylazine is a well-established and commonly used rodent injectable anesthetic combination and halothane inhalation has also been valuable for rat use in our laboratory [15]. Despite comparable depth of anesthesia (non-response to tail or foot pinch), statistically significant differences in heart rate, cardiac output, and LV fractional shortening were observed between anesthetics. Consistent with this, previous studies using injectable anesthetics and murine echocardiography have frequently reported heart rates in control (wild type) strains as low as 200-300 beats/min [3,8,10]. In contrast, heart rates in conscious normal mice typically range from 450-650 beats/min using telemetry methods [13,14]. Here we found that inhalation anesthesia was easier to control and provided more stable and physiologically relevant heart rates. We also observed a statistically significant correlation between heart rate and other functional parameters for both anesthetic regimens. While such a correlation is expected for cardiac output under normal physiologic ranges, we also found that LV fractional shortening was positively correlated to heart rate. This phenomenon is supported by human echocardiographic investigations [16], and demonstrates the importance of comparing cardiac performance in animal groups with similar and physiologically relevant heart rates.
In addition to increased convenience and more physiologically relevant parameters, less intra-animal and intra-group variability was observed with halothane inhalation. Though the difference in CV% values was dependent on the parameter measured, error estimates were often nearly 2-fold higher in the ketamine/xylazine group. These error discrepancies were evident not only between groups, but also between animals within treatment groups. This phenomenon most likely illustrates the inherent variables associated with a systemically administered drug combination, which include many pharmacokinetic variables (e.g., absorption, distribution, elimination). Based on statistical power analyses, the lower variability associated with the use of halothane translates into fewer numbers of mice required to sensitively detect differences in measured parameters. This statistical issue is an important consideration for long-term or serial-measurement trial designs as well as for investigations of transgenic animals of limited supply or high cost.

The dissociative anesthetic ketamine may be the most widely used injectable anesthetic in veterinary practice, with reported uses in more than 80 species [17]. It is commonly combined with xylazine to provide prolongation of anesthetic and analgesic effects [17,18]. These agents are capable of delivering a surgical plane of anesthesia with a wide safety margin and relatively few side effects [19]. However, these drugs have each been reported to induce a variety of cardiovascular alterations in mammals, including decreased cardiac output, reduced myocardial contractility, baroreceptor changes, hypotension, bradycardia, and arrhythmias [20,21].
In this study, a dose of ketamine/xylazine for mice was chosen based on an average of surveyed literature reports [3,9-11,22,23]. While lower dosage combinations have been used, this dose was generally sufficient to induce a depth of anesthesia adequate for proper restraint of the animal, permitting measurements to be made. However, there was significant variability in the time to onset, maintenance and recovery from anesthesia. In addition, cardiovascular performance was clearly blunted in mice receiving ketamine/xylazine relative to halothane, consistent with previously described reports of cardiodepression. For example, heart rates in mice receiving ketamine/xylazine were nearly half of those obtained from halothane-treated mice and telemetry values [13,14]. Associated with the substantially reduced heart rates were markedly reduced fractional shortening and estimated cardiac output values. These effects represent severe suppression of cardiac performance considering that these were normal, healthy mice. This issue of anesthetic induced cardiac impairment is likely to be increased in the setting of mouse models of heart failure, since increased sensitivity to anesthetics is a well known challenge for clinical anesthesia of cardiac patients [24,25]. Thus, preservation of relevant hemodynamic variables through the use of light inhalation anesthesia should help to reduce this potentially confounding variable.

Yang et al have recently proposed the use of animal training for conscious mouse echocardiography assessments. Interestingly, the parameters we obtained using light inhalation anesthesia were identical to
those determined for conscious and highly trained normal mice [12]. Halothane is documented to have direct cardiodepressive actions, but these effects are generally dose dependent and only significant at surgical planes of anesthesia (plane 2 or lower) [18]. Furthermore, the inhaled route of administration could be readily controlled. In these studies, the level of anesthesia was quickly and easily titrated. Mice could be rapidly anesthetized, assessed, and recovered in as little as 15 minutes, relative to 1-1.5 hours frequently required for ketamine/xylazine. Furthermore, the preservation of nearly resting cardiovascular performance indices and lower measurement error illustrates the significant advantage of using halothane over ketamine/xylazine. Relative to conscious animal studies this anesthetic regimen seems to have little physiological impact under our conditions and has an advantage of no extensive training of animals, as required for fully conscious studies (therefore enhancing throughput).

In summary, echocardiography offers great potential as a non-invasive tool for cardiac performance measures in mice. However, important considerations regarding anesthetic selection exist for physiologically relevant and statistically reliable performance evaluations. We found that significant advantages exist for inhalation anesthesia with halothane (or other similar agents) relative to injectable agents (ketamine/xylazine). These include increased convenience and throughput, maintenance of physiologically relevant hemodynamics, and decreased measurement variability leading to fewer animals required for detection of differences among groups.
REFERENCES


FIGURES
212
Figure 7.1: Cardiac function assessment via echocardiography.

*Left panels:* Representative M-mode and Doppler images. Data is representative of mice under halothane (top panels) or ketamine/xylazine (bottom panels) anesthesia.

*Right Panels:* Cardiac functional parameter values following ketamine/xylazine versus halothane anesthesia (mean ± SE, n=12, p<0.05).
Figure 7.2: Correlation analyses of hemodynamic variables.

Pearsons Product Moment correlation analyses were conducted on measures of LV contractile performance in relation to heart rate. Data is represented as 1 data point/animal, n=12 each for halothane (closed circles) and ketamine/xylazine (open squares).
Figure 7.3: Power analysis to determine sample size.

Statistical power analyses (SigmaStat) were conducted using the variability (SD) derived from parameter estimates for halothane-treated (solid line) or ketamine/xylazine-treated mice (dashed line). The analyses was conducted at specified power levels to determine the number of mice/group required to significantly detect differences of 10% (top panels) or 20% (bottom panels) from group mean values. Significance was defined as p< 0.05.
<table>
<thead>
<tr>
<th></th>
<th>Intra-animal (CV%)</th>
<th>Intra-group (CV%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Halothane</td>
<td>Ket/Xyl</td>
</tr>
<tr>
<td>FS</td>
<td>6.8</td>
<td>11.0</td>
</tr>
<tr>
<td>HR</td>
<td>3.1</td>
<td>6.6</td>
</tr>
<tr>
<td>AV&lt;sub&gt;max&lt;/sub&gt;</td>
<td>5.5</td>
<td>4.4</td>
</tr>
<tr>
<td>CO</td>
<td>6.8</td>
<td>11.0</td>
</tr>
</tbody>
</table>

Table 7.1: Variability of cardiovascular parameter measurement as a function of anesthesia. Values represent the mean (of 12 mice/group) coefficient of variation (CV%), calculated as described in Materials and Methods. FS, % left ventricular fractional shortening; HR, heart rate; AV<sub>max</sub>; peak aortic velocity; CO, estimated cardiac output.
CHAPTER 8

AGE AND ANESTHETIC EFFECTS ON MURINE ELECTROCARDIOGRAPHY

This chapter was published in Life Sciences (Life Sci 72:2401-2412) and has been presented here as per the requirements of the journal.
ABSTRACT

Murine Models offer potential insights regarding human cardiac disease, but efficient and reliable methods for phenotype evaluation are necessary. We employed non-invasive electrocardiography (ECG) in mice, investigating statistical reliability of these parameters with respect to anesthetic and animal age. Mice (C57BL/6, 8 or 48 weeks) were anesthetized by ketamine/xylazine (K/X, 80/10 mg/kg ip) or by inhalation anesthetic (halothane, HAL; sevoflurane, SEV) and 6 lead ECGs were recorded. P wave duration and QT interval was significantly prolonged with K/X compared to HAL and SEV, indicating slowed atrial and ventricular conduction. P-R interval (atrio-ventricular conduction) was significantly increased in aged mice under all anesthetics. Heart rate was inversely correlated to QT interval and P wave duration. We also detected significant age effects with respect to optimal approaches for QT interval corrections. Power analysis showed 4-fold higher number of mice/group, were required for K/X, to achieve identical statistical sensitivity. These data demonstrate the importance of anesthetic selection for relevant and reliable ECG analysis in mice and illustrate the selective influences of anesthetics and age on cardiac conductance in this species.

Key words: murine, electrocardiography, age, anesthesia
INTRODUCTION

Within the last decade mice have become an increasingly important species in pharmacological and toxicological research. Transgenic mouse technologies have enhanced research potential in the identification of molecular and genetic factors contributing to disease (Babij et al., 1998; Wakimoto et al., 2001). In addition, the use of mice as screening tools offers excellent potential for investigating drug efficacy and/or toxicities (Lande et al., 2001; Anderson et al., 2001; Wang et al., 2000). However, the “downsizing” to this species has also created significant challenges for reliable physiological parameter measurements that are both interpretable and clinically relevant. Regardless of the murine model(s) employed, accurate, reliable, and convenient measurement of cardiovascular function is critical for characterizing phenotypes and relating these to human disease.

We, and others, have recently demonstrated the potential value of electrophysiological recordings in mice to characterize transgenic models of cardiac disease or dysfunction (Okamoto et al., 2001; Tamaddon et al., 2000; London et al., 1998). Thus far, most published reports using such methods have focused on qualitative evidence of conduction disturbances and in many cases the abnormalities are overt and beyond changes typically observed in patients (owing to the severe cardiac pathologies often reported in transgenic models) (Lee et al., 2000; Biben et al., 2000; Valencik et al., 2000). In addition, the quantitative ECG measures, when reported, often vary widely in a given treatment group. For example, the reported QT interval (an index of ventricular
conduction) for a “wild type” mouse strain has varied roughly three-fold in prior publications; this discordance still remains in the published literature even if this parameter is heart rate corrected (QTc, rate corrected QT interval) (Berul et al., 1996; Xu et al., 1999). This parameter is now recognized as an important toxicological endpoint for assessing risk of cardiac arrhythmias in vivo (Napolitano and Priori, 2002; Haddad and Anderson, 2002; Vos et al., 2001), suggesting that reliable measurement in rodent models may enhance safety evaluations. Given the limited availability and high cost associated with transgenic animal models, optimal conditions for valid electrophysiological measures are important. This aspect is particularly important for long-term serial study designs and drug trials, as well as the rapid screening of large numbers of animals. The ability to detect relatively small changes in ECG parameters is also important for toxicity screening; for example, a less than 20% prolongation of QT interval is considered clinically relevant (Algra et al., 1991).

The wide variability in the previously published studies may have resulted from differences in data acquisition, measurement technique, sex, age, and anesthetic regimens. In evaluating the optimal conditions for murine ECG’s, investigators must consider not only animal and instrumentation conditions but also statistical considerations for in detecting differences among groups. Here we tested the hypothesis that anesthesia selection has an influence on the measured ECG parameters and potentially influences study conclusions. We systematically compared the utility of an injectable mixture of ketamine/xylazine, (a combination widely used for small animal research and previously used for
many cardiovascular studies in mice), to that of two inhalation anesthetics (halothane and sevoflurane). We focused on several clinically relevant ECG parameters in vivo and an additional component of our study was to assess the influence of animal age on these effects. We also considered the potential impact of anesthetic choice and age on statistical power to detect clinically relevant ECG changes.
METHODS

Study Design

This study was approved by the Institutional Animal Care and Use Committee at the Ohio State University. Female C57BL/6 mice were studied at 8 and 48 weeks of age. Response to anesthetic regimen (halothane, sevoflurane or ketamine/xylazine combination) was assessed in a crossover fashion. A ≥ 24 hour washout period separated the observations. Recordings were made between 12 noon and 6 pm to minimize any diurnal variation in electrophysiological parameters.

Anesthesia

Anesthesia induction with sevoflurane (Abbott Laboratories, Chicago, IL) and halothane (Halocarbon Labs, River Edge, NJ) was achieved by placing the animals in an isolation chamber with a mixture of 95% oxygen and the anesthetic. Inhalational anesthesia was maintained using a modified low-flow vaporizer with a nose cone (Bickford Vapomatic, A.M. Bickford Inc., Wales Center, NY) at a minimally effective concentration of sevoflurane or halothane. Ketamine (Ketaset, Fort Dodge Labs, Fort Dodge, IA) and xylazine (TranquiVed, Vedco Inc., St. Joseph, MO) was prepared in sterile 0.9% saline. The ketamine/xylazine mixture was administered intraperitoneally at a dose of 80 and 10 mg/kg, respectively.

Electrocardiography

Animals were placed on a heated gel pack (Instant Heat Pads from Harvard Apparatus, South Natick, Massachusetts) designed to maintain
normothermia during data acquisition. Recordings were made with adhesive electrodes attached to all four paws (BIOPAC Systems, Inc, Santa Barbara, CA) with the mice in a supine position. Electrocardiograms were digitally recorded using a physiologic data acquisition system (MP100, Biopac Systems), with a sampling rate of 2000 Hz.

**Data Analysis**

The ECGs were signal averaged (from Lead I) over 150-200 beats per acquisition for determination of the P wave duration, P-R interval, and QT interval (graphically displayed in Figure 1). P wave duration (a measure intra-atrial conduction) from the onset of the P wave to the point where the P wave returns to the baseline; the P-R interval (representing intra-atrial and atrioventricular conduction) from the P wave onset to the onset of the QRS complex (representing ventricular depolarization); the end of QRS could not be measured accurately (Li et al., 2000; Eloff et al., 2001), and therefore the QRS duration has not been reported. The QT interval (representing the duration of ventricular depolarization and repolarization) was measured from the onset of the QRS complex to the end of the T wave (intersection with the baseline). Two independent observers, blinded to the animal treatment groups, made all measurements. The average intra-observer variability for all parameters measured with all anesthetics was < 2%.

Since the QT interval is highly dependent on heart rate, the appropriateness of classical QT correction factors was tested. The average RR interval was determined from a 30 second sample. Rate corrected QT interval,
QTc, was calculated using the methods of Bazett (QTc=QT/RR^{1/2}) and Fridericia (QTc=QT/RR^{1/3}) (Matsunaga et al., 1998). The slope of the QTc vs.RR relationship was determined for each correction method; the method resulting in a slope closest to zero was determined to be most appropriate for rate correction.

**Statistical Analysis** *(Sigmastat, Jandel Scientific, San Rafael, CA)*

All data is reported as mean ± SE, except where noted. Simultaneous comparisons of groups were handled by one way analyses of variance, with Student-Newman-Keuls post-hoc analysis. Pearson Product Moment Correlation was used to evaluate the relationship between RR interval and other ECG parameters. Statistical significance was defined as a p<0.05 for all comparisons. Inter-observer variability was determined by calculating the % coefficient of variation. The intra-animal and intra-group variability in each ECG parameter was evaluated by anesthetic regimen and statistical power analysis was performed to determine the number of animals required to detect significant differences.
RESULTS

In general, intraperitoneal injection of ketamine/xylazine (80/10 mg/kg) required 5-20 minutes post-injection to provide adequate depth of anesthesia (minimal response to hind-foot pinch); the duration of anesthesia ranged from 30-60 minutes whereas sedation lasted 90-120 minutes. In contrast, halothane or sevoflurane induction consistently required less than 5 minutes, depth of anesthesia was conveniently stabilized via slight adjustment of inhalant concentration and mice quickly recovered to ambulatory activity within 2 minutes after removing the nose cone. The added convenience and reliability of inhalation anesthesia thus provided an enhanced throughput for these mouse ECG studies.

Shown in Figure 2 are representative recordings of well-defined six-lead ECGs obtained for all animals investigated. Signal averaged ECGs from lead one (Figure 2) provided adequate clarity for data parameter identification. Qualitative age- and anesthetic differences in the P, P-R, and QT interval were observed in signal averaged Lead I recordings (Figure 2: Right Panel). The QRS interval was not included in parameter investigations; similar to other investigator's observations, it was found to be coincident with early repolarization and could not be reliably determined (Li et al., 2000; Eloff et al., 2001).

Figure 3 compares ECG parameter values following ketamine/xylazine, halothane and sevoflurane at both ages (mean ± SE). Ketamine/xylazine significantly reduced the heart rate compared to either inhalational anesthetic at both ages (Figure 3). There are age-dependent differences in heart rate response to ketamine/xylazine and halothane (Figure 3). Atrial conduction (P
wave duration) was significantly slower with ketamine/xylazine at both ages (Figure 3). In addition, age-dependent differences in the PR interval (intra atrial and atrio ventricular conduction) occurred with all anesthetics tested (Figure 3). Age-dependent differences in ventricular repolarization (QT) only occurred with ketamine/xylazine (Figure 3).

Shown in Figure 4 are statistical relationships between ECG parameters and RR interval, using Pearson’s Product Moment correlation analyses. QT interval and P wave duration, at both ages, were statistically correlated with measured RR interval, whereas P-R interval was not (Figure 4). Two classical correction equations (Bazett vs. Fridericia correction factor methods) were compared with respect to their ability to correct for the observed QT heart rate dependence (Figure 5). At 8 weeks the slope of QTc vs. RR was closer to zero with the Bazett correction, but at 48 weeks the Fridericia correction yielded a slope closer to zero.

Using the measured variance (standard deviation) in cardiac functional parameters, statistical power analyses were conducted (SigmaStat) to determine the number of animals required for detecting differences among groups. Shown in Figure 6 are relationships of statistical power and numbers of animals required to detect statistically meaningful differences between two groups (assuming an unpaired student’s t-test). For each anesthetic regimen, an exponential relationship between number of animals/group and statistical power was observed. Additionally, the use of ketamine/xylazine anesthesia was associated with an increased number of animals required to detect differences relative to
inhalation anesthetics. The choice of anesthetic appears to influence the number of animals needed by 2-fold or more. In addition, the selection of an “optimal” anesthetizing agent is apparently age-dependent (Figure 6).
DISCUSSION

Mice have become an increasingly important species in pharmacological and toxicological research and the development of transgenic murine models of cardiovascular disease has rapidly expanded, offering great potential insight regarding mechanisms of human disease (Babij et al., 1998; Wakimoto et al., 2001). The use of mice as screening tools also offers excellent potential for investigating drug efficacy and/or toxicities (Lande et al., 2001; Anderson et al., 2001; Wang et al., 2000). To fully and efficiently utilize murine models for drug trial and transgenic characterization, routine and accurate evaluations of animal phenotypes are crucial.

The central focus of our studies was to investigate some key issues that may influence the reliability and detectability of ECG parameters in mice. Several investigations have demonstrated the value of ECG methods for murine studies, but no previous studies have systematically tested the hypotheses presented herein. Separate investigations have studied the effect of sex (Chu et al., 2001) and age (Maguire et al., 2000) on ECG parameters, but few attempts have been made to study the effect of age- and anesthetic in evaluating optimal conditions for ECG parameter evaluations, particularly with respect to statistical reliability. The issues of parameter reliability and statistical power are particularly important for large scale or high throughput screening and/or experimental therapeutic studies. Here we have found that six-lead ECGs can be reliably performed in mice. Lead I was signal averaged, rather than filtered, to calculate the ECG parameters commonly measured in humans and routinely used in diagnosis of
conductance disturbances in vivo. In our preliminary trials we found that inadequate sampling or over-filtering can reduce the quality of the signal and introduce significant artifact into the measurements.

We focused on the relative effects of 3 anesthetic regimens with respect to mouse ECG effects. In contrast, Chu et al have recently proposed the use of animal training for conscious mouse ECG assessments (Chu et al., 2001). In some ways the use of conscious animal conditions is likely to have some potential advantages, perhaps most importantly it avoids the use of an anesthetic, which may itself influence ECG parameters. The use of conscious mice for ECG recordings may have value in some settings, but movement artifacts, the necessity for animal acclimatization, and the practical capability of only capturing very short bursts of ECG recordings (typically only ~2 seconds rather than several minutes in our experimental approach) are all potential limitations for high throughput screening. Furthermore, the inhaled route of administration could be readily controlled and easily titrated, similar to our previous echocardiographic studies (Chaves et al., 2001). Mice could be rapidly anesthetized, assessed, and fully recovered in as little as 15 minutes, relative to 1-2 hours frequently required for ketamine/xylazine. Lower measurement error illustrates the significant advantage of using inhalation anesthetics (halothane and sevoflurane) over ketamine/xylazine. Our approach using very light anesthesia in mice provided the opportunity to capture high fidelity ECGs for several minutes using a six-lead configuration with a low degree of intra- and inter-animal variability, especially with stable inhalation anesthesia. This coupled
with, high throughput capabilities and high statistical power, were considered advantageous.

Halothane is documented to have direct cardiodepressive actions in other species and has been shown to influence sodium (Weigt et al., 1997; Eskinder et al., 1993), potassium (Davies et al., 2000) and calcium (Davies et al., 2000) currents in isolated myocytes. Interestingly, halothane has been found to shorten the QT interval (Michaloudis et al., 1996), while sevoflurane has been documented to slightly lengthen the QT interval in humans (Paventi et al., 2001). However, these effects have been found to be highly dose- and concentration-dependent and significant effects are only observed at very deep surgical planes of anesthesia (Fish, 1996). Interestingly, the QT interval we obtained using light inhalation anesthesia, in young female mice, were similar to those determined for conscious and highly trained normal mice (Chu et al., 2001), suggesting the stable and light anesthesia plane we employed had no significant impact on ECGs collected.

Our cross-over study demonstrated that anesthetic choice can have a important effects on the measured ECG parameters in mice. Despite comparable depth of anesthesia (non-response to tail or foot pinch), statistically significant differences in heart rate, P-wave duration, P-R interval, and QT interval were observed between anesthetics, and these differences were further complicated by the age of the animals studied (8 weeks vs. 48 weeks of age). Previous studies using injectable anesthetics and murine ECGs have frequently reported heart rates in wild type strains as low as 200-300 beats/min (Sadeghi et al.,
2002; Berul et al., 1996). In contrast, heart rates in conscious wild-type mice typically range from 450-750 beats/min using telemetry methods (Desai et al., 1997; Johansson et al., 1997; Xu et al., 1999; Korte et al., 2002). Here we found that inhalation anesthesia was easier to control and provided more stable and physiologically relevant heart rates.

An additional component of our study was to determine which of the measurable ECG parameters were sensitive to changes in heart rate (and thus likely to be subject to experimental artifacts). We observed statistically significant correlations between P-wave duration, QT interval and RR interval, demonstrating that these parameters. Such a correlation is expected for QT interval and therefore, several correction factors are used to correct the QT interval. The relationship between QTc and RR interval has been extensively studied in several species (Matsunaga et al., 1998), but only recently investigated in mice and statistical issues were not evaluated (Mitchell et al., 1998). Therefore, we used two classical approaches to QT interval correction and evaluate their appropriateness in our data set. We found that the method of Bazett (QTc=QT/RR^{1/2}) worked best in the 8 week old mice, while the method of Fridericia (QTc=QT/RR^{1/3}) worked best in the 48 week old mice. Our observations suggest that the optimal method for rate correcting the QT interval is dependent on age, highlighting the importance and difficulties in rate correcting the QT interval in mice, especially when using this parameter in toxicological evaluations and screening of drugs.
The rate dependence of P wave duration was an unexpected finding. Atrial conduction velocity was slower at slower heart rates. As described above, halothane is known to reduce sodium current but maintains a physiologic resting heart rate. Ketamine is also known to block sodium current and has been shown to reduce ventricular conduction velocity in isolated rabbit hearts (Aya et al., 1997; Hara et al., 1998). Thus, ketamine may reduce atrial conduction velocity (prolonged P wave duration) in addition to reducing the heart rate. These combined effects may contribute to the apparent rate-dependence of atrial conduction. An alternative explanation for the rate-dependence of atrial conduction is that atrial size may be increased at lower heart rates. Increased atrial dimension could potentially lengthen the conduction pathway resulting in P wave prolongation.

In our cross-over study, we evaluated the effect of age on ECG functional parameters during injectable (ketamine/xylazine) versus inhalation (halothane and sevoflurane) anesthesia. Multiple aspects of myocardial function are changed as a function of aging (Lakatta et al., 2002). Age-dependent changes in cardiac electrophysiology (ketamine/pentobarbital anesthesia) have been described in 129SvEv mice at mean ages of 7 and 28 weeks (Maguire et al., 2000). Older mice had significantly lower heart rates and longer P-R and QRS intervals. Our study demonstrated a similar observation, in the aged mice (48 weeks old), of the lengthening of the P-R interval under all anesthetics.
Though the difference in CV% values was dependent on the parameter measured, error estimates were often nearly 2-fold higher in the ketamine/xylazine group, especially with respect to QT interval. This phenomenon most likely illustrates the inherent variables associated with a systemically administered drug combination, which include many pharmacokinetic variables (e.g., absorption, distribution, elimination, and timing). Based on statistical power analyses, the lower variability associated with the use of inhalation anesthesia translates into fewer numbers of mice required to sensitively detect relevant differences in measured parameters; at greater than 85% power this difference was often more than 4-fold (Figure 6). This statistical issue is an important consideration for toxicological evaluations of drugs where the ability to detect relatively small differences, such as a 20% change in QT interval, is crucial in evaluating drug-induced arrhythmia potential.

In summary, we found that six-lead measurements provided high quality ECG recordings in lightly anesthetized mice and that the choice of anesthetic played an important role in the observed electrophysiological parameters and in the intra-group variability of these parameter estimates. This effect on variability had important impact on the statistical power and greatly influenced the numbers of animals per group to detect differences. In general, we found inhalation anesthetics to be advantageous in that they provided rapid and convenient onset and offset and an appropriately stable depth of anesthesia for data collections. We also detected significant age effects both with respect to baseline ECG parameters and with respect to optimal approaches for QT interval corrections.
These observations demonstrate that noninvasive and high fidelity measurements of ECG's are possible in mice but that careful selection of experimental conditions and appropriate control groups may play a critical role in parameter identification and detection of differences among groups.
REFERENCES


terminus and first transmembrane segment of a voltage-gated potassium channel. Proceedings of the National Academy of Science USA 95, 2926-2931.


Figure 8.1: A representative signal averaged Lead I recording from an 8-week old C57BL/6 mouse under sevofluorane anesthesia.
Figure 8.2: Cardiac function evaluated via electrocardiography.

*Left Panel:* Six-lead electrocardiogram recorded, under halothane anesthesia, in a 48 week-old female C57BL/6 mouse. Lead I, and Lead III are used to derive Lead II (Lead I + Lead III), aVR (Lead I + Lead II)/2, aVL (Lead I + Lead III)/2, and aVF (Lead II + Lead III)/2.

*Right Panel:* Representative signal-averaged lead I electrocardiograms. Data was obtained from an 8 and 48 week-old female anesthetized with Ketamine/Xylazine (K/X) Halothane (HAL) and Sevoflurane (SEV).
Figure 8.3: Age- and Anesthetic-Dependent Effects on the Electrocardiograms of C57BL/6 mice. Solid bars: 8 Weeks of Age; Striped bars: 48 Weeks of Age. Data is represented as mean ± SEM (n = 10), statistical significance *, p<0.05 compared to 8 week olds; †, p<0.05 compared to halothane (HAL) anesthesia; ‡, p<0.05 compared to sevoflurane (SEV); ‡‡, p<0.05 compared to ketamine/xylazine (K/X) anesthesia.
Figure 8.4: Heart Rate Dependence of Electrocardiographic Intervals.
Closed Symbols: 8 weeks of age; Open Symbols: 48 weeks of age. Circles: Ketamine/Xylazine; Triangles: Halothane; Squares: Sevoflurane.
Figure 8.5: QT interval correction with Bazett’s correction (open circles) and Fridericia correction (closed circles) at both 8 and 48 weeks of age.
Figure 8.6: Number of Animals Required to Detect Significant Differences in Electrocardiographic Variables: Effect of Anesthetic Regimen and Age. Top Row: Power Analysis for 8 week-old Mice. Bottom Row: Power Analysis for 48-week old Mice. (—) Halothane; (····) Sevoflurane; (- - -) Ketamine/Xylazine.
SECTION IV

VASOPROTTECTIVE EFFECTS OF STANDARDIZED GRAPE PRODUCT (USE OF BRACHIAL ARTERY ULTRASOUND TO EVALUATE ENDOTHELIAL FUNCTION IN HUMANS)
CHAPTER 9

VASCULAR ENDOTHELIAL EFFECTS OF A STANDARDIZED GRAPE PRODUCT IN HUMANS
ABSTRACT

Cardiovascular disease is the primary cause of death in the United States. Endothelium derived nitric oxide (NO) is the critical mediator of a physiological response process known as flow mediated vasodilation. Several studies have demonstrated that reduced flow mediated dilation and corresponding endothelial dysfunction is an early event in cardiovascular disease. Furthermore, intake of a high-fat (HF) meal has been shown to cause acute and time dependent impairment in endothelial function. Historical studies have shown that moderate intake of grape products, particularly wine, is associated with decreased cardiovascular risk, but the mechanisms of this effect are not defined. Furthermore, the cardiovascular actions of grape constituents in the absence of ethanol have not been thoroughly investigated. Thus, the primary goal of this study was to test the hypothesis that consumption (acute and chronic) of grape products (particularly in the absence of ethanol) will provide dose- and time-dependent improvement in vascular endothelial performance in normal subjects. Endothelial function was evaluated in normal male subjects (n=5) at 45, 90 and 180 minutes post consumption of GP (36g/100ml: single or multiple dose) or vehicle (sugar control) in the presence or absence of a HF meal. Blood pressure, heart rate, remained unaltered throughout imaging sessions. Endothelial function was significantly improved with an acute consumption of GP at 45 and 90 minutes post consumption of the single dose of grape product. Multiple dosing and chronic intake of GP for 21days improved basal endothelial performance,
assessed by change in the cross-sectional area of the brachial artery (post chronic GP: 703±86 mm$^2$.sec vs. pre-GP: 481±130 mm$^2$.sec; p<0.05). Total plasma antioxidant capacity was also slightly improved with the chronic intake of GP (pre-GP: 949±45 µmoles peroxyl radicals/L vs. post GP: 1080±35 µmoles peroxyl radicals/L; p<0.05). The chronic intake, coupled with an acute dose of GP, protected against HF induced endothelial impairment. Triglyceride levels weakly correlated with HF induced endothelial toxicity (p<0.05), but GP did not stem the HF meal increase in triglycerides. GP had a beneficial effect on endothelial function in normal male subjects and grapes (in the absence of alcohol) may have a beneficial use in cardiovascular disease.
INTRODUCTION

Coronary artery disease (CAD) currently afflicts more than 12 million Americans and results in over 500,000 deaths annually, making CAD the leading cause of death in America. The mechanisms by which CAD is initiated and progresses are multi-factorial, involving a number of different cell types (vascular smooth muscle cells, immune cells, vascular endothelial cells) and co-variates (diet, exercise, genetic, etc). Injury to the vascular endothelium and resultant endothelial dysfunction has been recently demonstrated to a precipitating event in the development of CAD (1). An important component of normal endothelial cell function is the production of nitric oxide (NO) via metabolism of arginine. NO is now recognized as a critical component to a healthy endothelium, providing local modulation of vascular tone and inhibiting blood cell adhesion (2, 3). An impaired capacity of the endothelium dependent and NO mediated relaxant response has been observed in a wide variety of patients with vascular risk factors, including diabetes, smoking history, elevated cholesterol, and familial history of cardiovascular disease (4). Thus it appears that the phenomenon of early endothelial cell dysfunction may be a common and unifying feature of vascular disease development (5).

Despite significant advances in the treatment and prevention of cardiovascular disease, CVD remains the leading killer of men over 45 years and women over 65 years old (6, 7). The classical approach of cardiovascular disease management has been the treatment of symptoms or invasive catheter based therapeutic intervention after diagnosis of an established
condition. This model has provided important medical advances, but high costs and an aging U.S. population are likely to limit its continued success (8). In contrast, recent developments in food science may offer new and rational approaches for prevention and/or management of cardiovascular conditions (6). For many years, historical and epidemiological evidence has suggested the benefits of grape products with respect to cardiovascular disease prevention (9). For example, chronic and moderate consumption of grape products, primarily in the form of wine, has been associated with decreased risk of myocardial infarction, stroke, and reduction in blood cholesterol (10). The data currently available are limited to grape products in the presence of alcohol, and the mechanisms of cardioprotection have not been addressed. Separate from epidemiological studies, some isolated tissue and cell studies from animals suggest that constituents of grapes may enhance vascular function (11-13). Furthermore, two very recent reports have suggested that wine might provide an acute improvement in endothelial function and that a purple grape product may influence endothelial performance in CAD (14, 15).

Collectively, these studies suggest that grape products may have important actions on vascular endothelial performance—this action may help to explain epidemiological data regarding the reduction in cardiovascular disease risk associated with grape products. However, the vascular effects of chronic consumption of grape products in healthy normal subjects, in the absence of ethanol, have not been evaluated. Intake of a high-fat meal has been shown to cause acute and time dependent impairment of endothelial
function (16). Thus, an improvement in endothelial performance \textit{in vivo}, or a reduction of impairment following high-fat meals, may provide an opportunity to delay or prevent deleterious cardiovascular effects. Thus, the primary goal of this study was to test the hypothesis that a standardized product from fresh grapes (acute and chronic consumption), in the absence of ethanol, improves endothelial performance in normal subjects, alone or in combination with a standardized high-fat meal.
METHODS

*Brachial artery ultrasound-measurement of endothelial function in humans*

Noninvasive ultrasound measurements of flow-mediated dilation have been extensively used as a method to assess endothelial functional (17). The physiological principle underlying this test is a phenomenon known as shear-stress induced vasorelaxation (18). This response is known to be endothelium-mediated and governed by its release of nitric oxide (via shear stress induced activation of NOS3) (5, 19). We have an ideal instrument for conducting these measurements in humans (Hewlett Packard SONOS 5500 ultrasound with an 11 MHz vascular imaging probe) and a clinical investigation room dedicated to these human studies. The conditions in the vascular imaging room were maintained at a comfortable and stable temperature. Following an initial stabilization period (20min) in a reclined position, brachial and tibial artery blood pressure (BP) was measured via an automated inflation pressure cuff (Omron Healthcare, Inc.). The Ankle Brachial Index (ABI), a ratio of systolic BP at the ankle versus the brachial artery, was used to evaluate vascular atherosclerotic presence. This index is an established marker of advanced atherosclerosis due to primary lesions that commonly occur in large femoral vasculature. ABI is usually near 1.0 in healthy individuals, advanced claudication can cause values to be near 0.5 (20). Heart rate was monitored electrocardiographically throughout the imaging session. A SONOS 5500
(Hewlett Packard Inc.), equipped with a 11 MHz probe, was used for Doppler and B–mode imaging of the right arm at a position 2–10cm above the antecubital fossa. Brachial artery imaging was maintained at frequency fusion of 5, depth of 3 cm, and the Doppler probe angle was set to 60° after optimal visualization. Compression and gain were adjusted to maximize clarity of the image. Images were captured on a Super VHS tape throughout the session. Basal B–mode and Doppler flow patterns were captured prior to the occlusion. Shear-stress induced vasorelaxation was induced by the release of occlusion of the brachial artery at a position proximal to the imaging point. The vessel was occluded for 5 mins at 200 mmHg using a blood pressure cuff. After cuff release, B–mode and Doppler images were calculated at 15, 30, 60, 90, 120 and 180 seconds post occlusion.

Shown in Figure 1 are representative images of brachial artery diameter and flow velocities at baseline, 15 seconds and 1 minute after releasing the occluder (Upper right panels). Note the significant increase in vessel diameter and the increased flow velocity and area under the velocity curves (an index of volume). Shown in Figure 2 are average responses in healthy control subjects (n=5, each studied on 3 separate days). The intra-subject and inter-subject variability, for change in brachial artery cross-sectional area (BA XS-AREA), was less than 15% (coefficient of variation, SD/meanx100), and inter-subject variability was less than 20%—this non-invasive method was highly reproducible in healthy normal subjects in our hands. The maximal dilation response in our subjects was identical to published reports, as was the time
course of brachial artery diameter changes and flow (21).

**Overall Study Design**

All aspects of the use of human subjects in this study were in accordance with the Ohio State University Biomedical Sciences Institutional Review Board. Healthy male volunteers (n=5) were used in this study. Females were excluded from this study since hormonal cycles have been shown to influence endothelial function (22). The male subjects were screened prior to enrolling the study to ensure that they were free of heart disease, diabetes mellitus, unstable angina, or any other pre-existing condition that would modulate endothelial function. Shown above is a schematic that illustrates the study design, with four components.

The first section was designed to test the effects of a sugar control solution (sugar content matched to dose of grape product). In the second component, we confirmed that a single high-fat meal (sausage egg breakfast sandwich + two hash browns [900cal, 49g total fat, 13g saturated fat, & 245mg cholesterol]) could cause time dependent endothelial function (as per Vogel study) (16). The third component evaluated the acute effects of a single dose
of standardized grape product (36g/100mL). The fourth component evaluated the effects of chronically administered standardized grape product over 21 days (twice daily dosing). Baseline measures of endothelial function on day 22 reflected the endothelial effects of chronic administration of the grape product. Directly following this measurement, an additional single dose of grape product was co-administered with a single high-fat meal. The protective effects of the grape product against a high-fat meal were then evaluated 45 minutes after finishing the grape product and high fat meal, in a procedure identical to the second component, high fat meal alone.

The Standardized Grape Product

The standardized grape product was a freeze-dried preparation of red, green, and blue-black California seeded and seedless grapes (California Table Grape Commission). The powder retains most of the active ingredients in grapes that have been previously shown to have anti-thrombotic, vasorelaxant and antioxidant properties. The dose of grape product employed in these studies has 612 µmoles of flavans, 43 µmoles of anthoncyanins, 3 µmoles of flavanols, and 1 µmole of resveratrol. The dose of grape product was equivalent to 1.25 cups of fresh grapes.

Test day

The subjects were fasted for ten hours prior to assessment. Following an initial stabilization period (20min) in a reclined position, brachial and tibial
artery blood pressure (BP) was measured via an automated inflation pressure
cuff (Omron Healthcare, Inc.). Blood was collected, via a lancet, to measure
baseline (-30min) blood levels of total cholesterol (TC), high-density
lipoproteins (HDL), and triglycerides (TG) (Bioscanner 2000). The Friedwald
equation was used to calculate LDL (LDL = TC-HDL-(TG/5)) (23). A portion of
the blood was collected and stored in capillary tubes (serum separated and
stored at –20°C) for future evaluations of plasma antioxidant status measures.
Blood was also collected and analyzed at 90 and 180 minutes post
administration of the test product. The test product (sugar control, high-fat
meal or grape product) was administered after a baseline (-30 minutes)
measure of endothelial function. Endothelial function measures were repeated
at 45, 90 and 180 minutes post administration of test product.

Total Plasma Antioxidant Capacity Assay

The assay, originally developed by Valkonen and Kussi, fluorometrically
measures the capacity of samples (plasma) to quench free radicals (24). Free
radicals, produced by decomposition of 2,2’-diazobis(2-
amidinopropane)dihydrochloride (AAPH), converts dichlorofluorescein-acetate
(DCFH-DA; a free radical probe), to a highly fluorescent dichlorofluorescein
(DCF). DCF production is measured fluorometrically (excitation 480nm,
emission 526nm) over time (~1hour). Time to onset of fluorescent signal is
used as a measure of antioxidant capacity, as the DCF production
 fluorescence) curve can be shifted to the right with the addition of of trolox
(Figure 3, Panel A). Fluorescence curves can be progressively shifted to the right (increasing onset time) with increasing concentrations of trolox (Figure 3, Panel B). 10µM of trolox (captures 2 moles peroxyl radical per mole of trolox) was used an internal standard when comparing different samples (24). The intra- and inter-assay variability (coefficient of variation, SD/mean x 100%) for this assay were <5% and <10%, respectively.

**Data handling and Statistics**

Doppler flow and B-mode brachial artery images, captured on S-VHS, were used to calculate flow and brachial artery diameter. Cross-sectional (XS) area of the brachial artery was calculated from B-mode images of the brachial artery. Doppler flow images were integrated (velocity time integral = Vti) for the calculation of the brachial artery blood flow (Flow = HR x Vti x XS-area). Brachial artery flow and cross-sectional area, were plotted as percent change from baseline, at various time points collected over the three minutes post-occlusion. Area under the curve measurements were calculated via Graph Pad Prism software (Graph Pad Prism, San Diego, CA) to quantify the endothelial mediated hyperemic response. Multiple comparisons were made using one-way ANOVA (repeated measures) with post-hoc Dunnet’s tests to determine statistical significance against basal responses (Graph Pad Prism). Statistical correlations were evaluated using non-parametric Spearmen’s correlation (Graph Pad Prism). Statistical significance was defined as p<0.05.
RESULTS

Healthy male (non-smokers) subjects were enrolled in this study. The average age of these male subjects was 28±5 years. Blood pressure (BP) and resting heart rates (HR) were within a normal range (Systolic BP: 121±8 mmHg; Diastolic BP: 72±4 mmHg; HR: 58±4 bpm). Lipid levels were also within the normal range (total cholesterol: 154±20 mg/dL; HDL: 55±5 mg/dL; triglycerides: 101±17 mg/dL; LDL: 84±17 mg/dL). The average ABI was 1.2±0.1, consistent with the absence of significant atherosclerosis in these subjects (e.g. in advanced claudification, ABI ~ 0.5) (20).

Brachial artery response following a single dose of grape product

The first section of the study determined the effects of a sugar solution (sugar content and volume matched to grape dose), to ensure that the effects seen from the grape product solution were specific to the grape constituents and not modulated by the effects from the sugar content in the grapes. The effects of a single dose of grape product were then assessed using identical procedures.

Shown in table 1 are the average electrocardiographic (heart rate), hemodynamic (blood pressure and ABI), blood lipid profiles (total cholesterol, HDL, LDL and triglycerides), and total antioxidant capacity for subjects at baseline and following administration of sugar control or grape product. Neither sugar control or the single dose of grape product caused significant
alterations in any of these parameters. Shown in Figure 4 are average shear-stress induced vasorelaxation responses (change in brachial artery cross-sectional area integrated over 3 minute period of analysis) following sugar control or a single dose of grape product. Shear-stress induced vasorelaxation was significantly increased at 45 and 90 minutes following the single dose of grape product, while the sugar control had no effect. Shown in Table 2 are the average hyperemic responses (change in BA blood flow). BA blood flow was not significantly increased (measured by the change in blood flow integrated over 3 minute period of analysis) by either sugar control or the grape product.

Endothelial function was further enhanced with chronic dosing of grape product

The chronic effect of the grape product was evaluated following 21 days of twice-daily dosing. Subjects did not alter their daily diet while on the chronic dosing regimen. Shown in Table 3 are average electrocardiographic (heart rate), hemodynamic (blood pressure and ABI), blood lipid profiles (total cholesterol, HDL, LDL and triglycerides) and prior to initiation of the grape product and after 21 days of grape product consumption. Only a slight increase in total cholesterol levels was observed, with no change in any other of these parameters.

Shown in Figure 5 are average shear-stress induced vasorelaxation responses (change in brachial artery cross-sectional area integrated over 3
minute period of analysis) following a single dose of grape product (“baseline”) versus following 21 days of grape product intake. Shear-stress induced vasorelaxation was further enhanced after 21 days of grape consumption relative to a single dose of grape product (Figure 5, Upper panel, expressed as change in BA cross-sectional area integrated over 3 minute period of analysis). The improvement in stress induced vasorelaxation was accompanied by a significant increase in total antioxidant capacity (Figure 5, Lower panel). Again, BA blood flow was not significantly altered (Table 3).

**Grape product protects against acute endothelial toxicity induced by a single high-fat meal**

Several studies have documented that a single high-fat meal can induce acute endothelial dysfunction (16, 25-27). Therefore, we tested the effect of grape product to attenuate the deleterious effects of a high-fat challenge. Shown in Table 4 are average electrocardiographic (heart rate), hemodynamic (blood pressure and ABI), blood lipid profiles (total cholesterol, HDL, LDL and triglycerides) and total antioxidant capacity values with a high-fat meal alone and in conjunction with grape product. Triglycerides were significantly elevated 3 hours after a single high-fat meal (Table 4). The grape product did not alter the high-fat meal induced increase in triglycerides (Table 4).

Shown in Figure 6 are average shear-stress induced vasorelaxation responses with a high-fat meal alone and in conjunction with grape product
(change in brachial artery cross-sectional area integrated over 3 minute period of analysis). Endothelial function was significantly impaired at 45, 90, and 180 minutes following the high-fat meal alone (Figure 6, Upper panel). However, shear-stress induced vasorelaxation response was restored following co-administration of grape product (Figure 6). Again, there was no significant change in BA blood flow with the high-fat meal or in conjunction with grape product (Table 5). The apparent protection by grape product against endothelial toxicity by a high-fat meal was not explained by inhibition of the increase in circulating triglycerides induced by the high-fat meal (Figure 6, Lower panel).
DISCUSSION

Diet has long been considered an important component in assessing risk for cardiovascular disease (6). Therefore, in this small study we sought to understand the potential contribution of a “medically valuable food” like grapes to endothelial health in normal healthy males. In the last decade, the vascular endothelium has been recognized as a critical participant in the maintenance and regulation of cardiovascular health (28, 29). Endothelial dysfunction is an initiating event in a wide array of acute and chronic cardiovascular disease states (3). However, the mechanisms by which injury occurs are complex and incompletely understood, and preventative approaches for endothelial function are poorly defined, and not currently in routine or convenient use.

Brachial artery ultrasound technology has become an important tool in assessing endothelial health in major cardiovascular risk assessment studies (17). Our study used this technology to evaluate endothelial function, in healthy male subjects, in response to a single dose of standardized grape product and chronic consumption of grape product. We have established a reproducible method for assessing endothelium dependent responses in humans using brachial artery ultrasound. Although our population of healthy normal males under study was relatively small, the highly reproducible baseline values for each individual allowed for statistical comparisons across treatments by comparing each subject to their own baseline on each treatment
day. Since grapes are very high in sugar content we used a corresponding sugar control when evaluating the effects of the grape product.

Agewall et al. recently evaluated the effects of a single dose of red wine on endothelial function in normal subjects and compared this action to the effects of de-alcoholized wine. These investigators observed significant vascular effects for both grape products, using vascular ultrasound methods. Interestingly, the de-alcoholized product was shown to have slight but significant effects on brachial artery endothelial performance during a single intake of 250ml (assessed by flow mediated dilation responses), but the effects of chronic intake were not investigated (14). Similar to Agewall’s study, we found that a single dose of grape product provided an enhancement of shear-stress induced vasorelaxation responses at 45 and 90 minutes post administration. In a separate study, Stein et al. investigated the effects of a purple grape product (Welch’s 100% Concord Grape juice) in patients with angiographic documentation of coronary artery disease (15). These subjects drank approximately 640ml of grape juice daily (in addition to their already established prescription medications), and endothelial performance was assessed using noninvasive brachial artery flow mediated dilation response. Significant improvement in endothelial function was demonstrated after 14 days of juice intake (15). This effect was also observed, in our study, with normal subjects. We found that a 21-day intake of grape product provided a significant enhancement in baseline shear-stress induced vasorelaxation responses in healthy individuals. Interestingly, the total antioxidant capacity
was also increased 21 days following the chronic consumption of the grape product. These data suggest that acute intake of standardized grape product may provide functional benefits to the vascular endothelium even in normal healthy humans, and that more chronic administration holds the potential for added benefit.

A single intake of high fat meal has been shown to cause significant, time-dependent impairments in endothelial function (16). As per Vogel’s study, we found that a standard high fat breakfast meal caused significant endothelial impairment in healthy individuals. This impairment was accompanied by a sustained increase in plasma triglyceride levels. Hypertriglyceridemia has been implicated in contributing to endothelial dysfunction, but the exact mechanisms of high-fat induced endothelial toxicity cannot be attributed to hypertriglyceridemia alone (30). Bae et al suggested that a high-fat meal can cause an increase in leukocyte production of superoxide which might contribute to endothelial dysfunction (26). In a later study Bae et al concluded that the oxidant production under their study conditions was insufficient to produce oxLDL, a well-known endothelial toxicant (25). Interestingly, nitrotyrosine (marker of peroxynitrite formation) levels, have been previously shown to be increased with hypertriglyceridemia, and antioxidants have been shown to have positive effects on controlling the high-fat induced endothelial impairments (30, 31). Consistent with this evidence, we found that standardized grape product provided significant protection against high-fat induced endothelial dysfunction. Similar to other
antioxidant studies, the standardized grape product did not achieve these effects though attenuating the spike in circulating triglycerides (32, 33). Also, this protection may be incompletely explained by the antioxidant properties of the standardized grape product (flavanols, anthocyanidins, stilbenes), as total antioxidant capacity was not significantly altered with a high-fat meal. Resveratrol (a stilbene derived from grape skin) has been shown to up-regulate NOS3 \textit{(in vitro)} in bovine pulmonary artery endothelial cells (BPAEC) under shear stress (34) and has been shown to stabilize NOS3 mRNA, which might explain the improved endothelial function following chronic consumption of the grape product (35). While this study does not indicate a specific mechanism for a grape product protection of high-fat induced endothelial toxicity, it provides strong evidence that grapes may protect the endothelium against high fat induced toxicity, and suggests that further studies of the potential long-term benefits of this approach for the prevention of endothelial injury are warranted.

In summary, acute administration of a standardized grape product significantly increased shear-stress induced vasorelaxation responses in healthy normal males, and this effect was further enhanced during chronic administration. The standardized grape product completely inhibited the acute endothelial dysfunction induced by a single high fat meal, by a mechanism that was independent of circulating triglyceride levels. These data suggest that standardized grape product may have beneficial effects on endothelial health and function in subjects with no evidence of cardiovascular disease, that the
beneficial effects of the grape product were not ethanol-dependent, and that this standardized grape product may have substantial protective effects against the vascular toxicities associated with a high fat diet. Further studies regarding the mechanisms and putative ingredients mediating these protective effects and a further evaluation of the therapeutic potential of this preparation, are clearly warranted.
<table>
<thead>
<tr>
<th>References</th>
<th>Authors</th>
<th>Title</th>
<th>Journal</th>
<th>Volume</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ruschitzka, F.T., Noll, G., and Luscher, T.F.</td>
<td>The endothelium in coronary artery disease</td>
<td>Cardiology</td>
<td>88</td>
<td>Suppl 3:3-19</td>
</tr>
<tr>
<td>2</td>
<td>Boulanger, C.M.</td>
<td>Secondary endothelial dysfunction: hypertension and heart failure</td>
<td>J Mol Cell Cardiol</td>
<td>31</td>
<td>39-49</td>
</tr>
<tr>
<td>3</td>
<td>Shimokawa, H.</td>
<td>Primary endothelial dysfunction: atherosclerosis</td>
<td>J Mol Cell Cardiol</td>
<td>31</td>
<td>23-37</td>
</tr>
<tr>
<td>5</td>
<td>Drexler, H., and Hornig, B.</td>
<td>Endothelial dysfunction in human disease</td>
<td>J Mol Cell Cardiol</td>
<td>31</td>
<td>51-60</td>
</tr>
<tr>
<td>6</td>
<td>Hennekens, C.H.</td>
<td>Increasing burden of cardiovascular disease: current knowledge and future directions for research on risk factors</td>
<td>Circulation</td>
<td>97</td>
<td>1095-1102</td>
</tr>
<tr>
<td>10</td>
<td>Kannel, W.B., and Ellison, R.C.</td>
<td>Alcohol and coronary heart disease: the evidence for a protective effect</td>
<td>Clin Chim Acta</td>
<td>246</td>
<td>59-76</td>
</tr>
</tbody>
</table>


Figure 9.1: Representative images of brachial artery diameter and flow in response to hyperemia.
Figure 9.2: Average responses in brachial artery flow and cross-sectional (xs) area post release of occluder. Data is represented as mean±SEM.
Figure 9.3: DCF fluorescence to measure total plasma antioxidant capacity.

A: Fluorometric measure of oxidant production with and without 10µM Trolox.

B: Oxidant production is slowed with increasing concentrations of Trolox to yield a standard curve of antioxidant capacity. Data is represented as mean±SEM.
Figure 9.4: A single dose of grape product significantly enhanced endothelial function at 45min and 90min post administration. Sugar Control had no significant effect on endothelial function, while a single dose of grape product caused significant improvement in endothelial function at 45 and 90 minutes post dose.

Data is represented as a percentage of basal responses (mean±SEM) for each subject. Statistical significance *, p<0.05, as compared to the raw - 30min brachial artery response to cuff occlusion
Figure 9.5: Responses to a 21day (2X) consumption of grape product.

*Upper Panel*- Endothelial function was significantly improved 21d post chronic grape product consumption.

*Lower Panel*- Total Antioxidant capacity was significantly enhanced 21d post chronic grape product consumption.

Data is represented as mean±SEM. Statistical significance *, p<0.05.
Figure 9.6: Blunting of high-fat induced endothelial dysfunction by a standardized grape product.

*Upper Panel* - Significant impairment in endothelial function at 45, 90 & 180min, but dysfunction is blunted with grape product. Data is represented as a percentage of basal responses (mean±SEM) for each subject. Statistical significance *, p<0.05, as compared to the raw -30min brachial artery response to cuff occlusion.

*Lower Panel* - Grape product did not alter the high-fat induced hypertriglyceridemia. Data is represented as a percentage of basal triglyceride levels for each individual.
<table>
<thead>
<tr>
<th></th>
<th>Sugar Control</th>
<th>Acute Grape Product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-30min</td>
<td>+90min</td>
</tr>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>58±4</td>
<td>58±5</td>
</tr>
<tr>
<td><strong>BP (mmHg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Systolic</strong></td>
<td>121±8</td>
<td>124±10</td>
</tr>
<tr>
<td><strong>Diastolic</strong></td>
<td>72±4</td>
<td>75±6</td>
</tr>
<tr>
<td><strong>ABI</strong></td>
<td>1.2±0.1</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td><strong>Total Cholesterol (mg/dL)</strong></td>
<td>154±20</td>
<td>156±20</td>
</tr>
<tr>
<td><strong>HDL (mg/dL)</strong></td>
<td>55±5</td>
<td>54±6</td>
</tr>
<tr>
<td><strong>LDL (mg/dL)</strong></td>
<td>84±17</td>
<td>85±18</td>
</tr>
<tr>
<td><strong>Triglycerides (mg/dL)</strong></td>
<td>104±12</td>
<td>102±11</td>
</tr>
<tr>
<td><strong>TAC</strong></td>
<td>891±28</td>
<td>953±32</td>
</tr>
</tbody>
</table>

**Table 9.1:** Averaged electrocardiographic, hemodynamic, blood lipid and total antioxidant capacity (TAC (µmoles peroxyl radicals/L)) values at baseline (-30min) and 90min post-test product (sugar control and a single dose of grape product). Data is represented as mean±SEM. [HR: Heart Rate; ABI: Ankle Brachial Index; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein]
**Table 9.2**: Average change in brachial artery blood flow post release of the occluder at baseline (-30min), 45min, 90min and 180min post-test product (sugar control and a single dose of grape product). Data (Area Under Curve generated with the change in BA xs-area over three minutes) is represented as mean ±SEM.

<table>
<thead>
<tr>
<th>Δ BA Flow (mL)</th>
<th>Sugar Control</th>
<th>Acute Grape Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30min</td>
<td>398±47</td>
<td>351±47</td>
</tr>
<tr>
<td>+45min</td>
<td>426±65</td>
<td>511±118</td>
</tr>
<tr>
<td>+90min</td>
<td>451±40</td>
<td>596±173</td>
</tr>
<tr>
<td>+180min</td>
<td>391±68</td>
<td>544±40</td>
</tr>
<tr>
<td></td>
<td>Pre Grape Prdt.</td>
<td>Post (21d) Grape Prdt.</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>58±4</td>
<td>57±4</td>
</tr>
<tr>
<td>BP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>121±7</td>
<td>119±7</td>
</tr>
<tr>
<td>Diastolic</td>
<td>72±4</td>
<td>71±5</td>
</tr>
<tr>
<td>ABI</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>154±20</td>
<td>160±19*</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>52±4</td>
<td>53±6</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>82±16</td>
<td>86±16</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>96±16</td>
<td>104±16</td>
</tr>
<tr>
<td>Δ BA Flow (mL)</td>
<td>475±26</td>
<td>432±45</td>
</tr>
</tbody>
</table>

**Table 9.3:** Averaged electrocardiographic, hemodynamic, blood lipid, and Δ flow values before and after 21 days (2X) of grape product consumption. Data is represented as mean±SEM. Statistical significance *, p<0.05. [HR: Heart Rate; ABI: Ankle Brachial Index; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein]
<table>
<thead>
<tr>
<th></th>
<th>High Fat</th>
<th>High Fat &amp; Grape Product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-30min</td>
<td>+90min</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>57±4</td>
<td>58±3</td>
</tr>
<tr>
<td>BP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>137±6</td>
<td>135±8</td>
</tr>
<tr>
<td>Diastolic</td>
<td>78±6</td>
<td>76±6</td>
</tr>
<tr>
<td>ABI</td>
<td>1.2±0.1</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>163±19</td>
<td>162±19</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>52±6</td>
<td>52±6</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>80±16</td>
<td>69±13</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>88±19</td>
<td>149±26</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAC</td>
<td>1068±38</td>
<td>1084±43</td>
</tr>
</tbody>
</table>

Table 9.4: Averaged electrocardiographic, hemodynamic, blood lipid and total antioxidant capacity (TAC (µmoles peroxyl radicals/L)) values at baseline (-30min), 90min and 180min after a single high fat meal and in conjunction with grape product. Data is represented as mean±SEM. Statistical significance*, p<0.05. [HR: Heart Rate; ABI: Ankle Brachial Index; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein]
Table 9.5: Average changes in brachial artery blood flow at –30min, 45min, 90min and 180min after a single high-fat meal alone and in conjunction with grape product. Data (Area Under Curve generated with the change in BA x-s-area over three minutes) is represented as mean ±SEM.

<table>
<thead>
<tr>
<th>∆ BA Flow (mL)</th>
<th>High Fat</th>
<th>High Fat &amp; Grape Prdt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30min</td>
<td>676±67</td>
<td>432±45</td>
</tr>
<tr>
<td>+45min</td>
<td>652±94</td>
<td>681±178</td>
</tr>
<tr>
<td>+90min</td>
<td>442±86</td>
<td>540±125</td>
</tr>
<tr>
<td>+180min</td>
<td>553±103</td>
<td>585±138</td>
</tr>
</tbody>
</table>
My dissertation research involved several different aspects of cardiovascular disease, and therefore I have divided my dissertation into four sections and herein each section outlines the major conclusions reached from each chapter in the dissertation.

Section I – HIV/AIDS related cardiovascular disease

HIV infection is devastating disease that affects 900,000 people in the United States and 42 million people worldwide (1). Cardiovascular disease is an important complication of HIV infection, affecting a large proportion of these patients. Severe cardiac complications, like congestive heart failure, are primarily seen in AIDS patients, but asymptomatic patients also have detectable and significant signs of LV dysfunction—the connections between these conditions, (i.e. does early pathology mediate later more severe events? Can early intervention delay or prevent AIDS-related cardiomyopathy?), remain to be defined. Prognoses for HIV patients with these events are often significantly worsened. Despite the general recognition that cardiovascular abnormalities develop in these patients and the high costs associated with patient care, the mechanisms involved remain largely undefined. HIV-related cardiovascular disease is unique form of cardiovascular disease and traditional cardiovascular therapy may not be optimal for this population. HIV infection and cardiac complications in humans are often complicated by illicit drug abuse and covariate infections—these co-variates clearly impart adverse effects on disease progression, but the mechanisms involved are very poorly
understood. While the number of patients affected by this complication of HIV/AIDS is likely to continue to grow, a number of promising experimental approaches have been developed to address this problem, including more relevant animal models, a larger appreciation for the broad scope of HIV/AIDS related effects, and the development of larger and better controlled patient populations for clinical and histopathological study. These advances, combined with a greater appreciation of the importance of this condition to the pathology of HIV/AIDS, will hopefully result in the development of therapeutic approaches that are specific to and effective for this unique and growing patient population.

In Chapter 3 we observed that LPBM5 infection, a well-established experimental model for the immunologic complications of HIV-infection, caused early cardiac contractility deficits and later, more severe cardiovascular impairment in mice, with a time course that preceded the development of overt immunodeficiency; the “murine AIDS” model appears appropriate for the mechanistic study of RTV-induced cardiac complications. Although the infectious agent was not HIV itself, the murine AIDS model recapitulates many of the important features of AIDS-related cardiac disease in humans, suggesting that the systemic and/or cardiovascular response to RTV infection may mediate many of these changes, rather than direct effects of the virus itself. Although we observed dramatic changes in immune cell levels and trafficking patterns, a number of structural and biochemical alterations occurred in the cardiac myocytes themselves in RTV-treated
animals. Immune cell interactions incompletely explain the global cardiac muscle changes that occur in this setting, and the cardiac myocyte itself may represent an important site of RTV-related pathogenesis. In Chapter 3, we describe first-time evidence that cardiac RNS formation and oxidative injury may play a significant role in the development of cardiac complications in both the murine AIDS model and in a relevant population of AIDS-related cardiomyopathy patients. Further studies defining the sources and putative intracellular targets of these oxidants will provide important mechanistic insights, and may reveal new therapeutic opportunities for this unique and important cardiovascular disease setting.

A multi-pathogen setting is commonly observed in HIV related cardiomyopathy cases, but few studies have addressed important interactions with respect to retroviral pathogenesis vs. organ dysfunction. In Chapter 4 we have demonstrated that a modest exposure of LPS (e.g. at doses that did not yield significant effects alone in vivo) amplified abnormalities in cardiac structure and function observed in a murine AIDS model. The observed cardiac dysfunction was associated with selective increases in non-focal infiltration of CD68+ cells; these cells were found to be NOS2 positive and correlated to extent of cardiac dysfunction. This amplification interaction was not associated with alterations in retroviral progression or cardiac retroviral content, but an important increase in TLR4 was observed in the combination treatment group only. Evidence of cardiac myocyte induction of TLR4 was also observed in human tissues from AIDS related cardiomyopathy. These
studies, in Chapter 4, demonstrate a high degree of similarity or this reproducible animal model relative to studies in human tissues; furthermore they provide first-time evidence of multi-pathogen enhancements to retrovirus related cardiac complications and implicate innate immunity responses in this setting.

SECTION II – Cocaine related cardiovascular disease

Cocaine abuse remains an epidemic in the U.S. and is associated with high social and medical costs. Estimates of the annual total costs for drug abuse has been estimated to be $98 billion dollars and a majority of these costs are related to cocaine emergency cardiac care as these incidents account for nearly 30% all drug related emergency room (ER) visits, with an annual expenditure of nearly $83 million (2, 3). For example, acute episodes of angina pectoris, cardiac arrhythmias and myocardial infarction have all been commonly reported in cocaine abusers, and occasionally occur in first-time users. Separate from these acute events, chronic conditions including myocarditis, vasculopathy, cardiomyopathy, and congestive heart failure have also been demonstrated in frequent cocaine users (4-7). Despite the high incidence and medical costs, the mechanisms responsible for cocaine-related cardiovascular disease are not well defined. Furthermore traditional therapies commonly employed for acute coronary syndrome have not been demonstrated effective (8-11) and at this time there is no established therapeutic strategy proven safe and effective for this setting. Given the facts
that nearly, 25 million people in the U.S. have used cocaine at least once and more than 5 million people abuse the drug on a regular basis, optimized and cost-effective strategies for cardiac complications are warranted (2).

In Chapter 5, we observed that a single dose of cocaine caused acute electrical abnormalities in mice, analogous to clinical phenomenon observed in humans. Cocaine administration also resulted in protracted cardiac dysfunction (slower conduction capabilities and depressed cardiac output and stroke volume) as measured by electrocardiography and echocardiography. Our murine model has apparent value in investigating cocaine related cardiac toxicity as the mouse exhibits cardiac insufficiencies similar to human cocaine abusers. Furthermore, the mouse has definable cardiotoxic endpoints that allow for the evaluation of novel therapeutics. In addition, we observed extensive dysregulation of NO as evidenced by increased cardiac prevalence of NOS2, reactive nitrogen species and protein-3NT. These initial findings, in mice, suggest that novel therapeutics to control excessive prevalence of cardiac NOS2 and reactive nitrogen species might be useful in managing cocaine related cardiac dysfunction.

Similarly, in Chapter 6, the mouse seems to be an appropriate model to study cocaine related vascular dysfunction especially since a 30mg/kg dose of cocaine causes protracted endothelial dysfunction analogous to the human condition (12). Cocaine related endothelial toxicities involve a concentration and time dependent production of oxidants. Antioxidants may have value ameliorating endothelial dysfunction due to cocaine induced vascular toxicity
and preliminary evidence, in Chapter 6, suggests that cocaine may mediate oxidant production through sigma receptors. Cocaine induced endothelial dysfunction and toxicity in vitro involves both apoptotic and necrotic events that may explain protracted endothelial dysfunction with cocaine use and abuse.

SECTION III – Murine models in cardiovascular disease

Echocardiography offers great potential as a non-invasive tool for cardiac performance measures in mice. However, important considerations regarding anesthetic selection exist for physiologically relevant and statistically reliable performance evaluations. In Chapter 7, we found that significant advantages exist for inhalation anesthesia with halothane (or other similar agents) relative to injectable agents (ketamine/xylazine). These include increased convenience and throughput, maintenance of physiologically relevant hemodynamics, and decreased measurement variability leading to fewer animals required for detection of differences among groups.

In Chapter 8, we found that six-lead measurements provided high quality ECG recordings in lightly anesthetized mice and that the choice of anesthetic played an important role in the observed electrophysiological parameters and in the intra-group variability of these parameter estimates. This effect on variability had important impact on the statistical power and greatly influenced the numbers of animals per group to detect differences. In Chapter 8, we found inhalation anesthetics to be advantageous in that they
provided rapid and convenient onset and offset and an appropriately stable depth of anesthesia for data collections. We also detected significant age effects both with respect to baseline ECG parameters and with respect to optimal approaches for QT interval corrections. These observations demonstrate that noninvasive and high fidelity measurements of ECG's are possible in mice but that careful selection of experimental conditions and appropriate control groups may play a critical role in parameter identification and detection of differences among groups.

SECTION IV – Vasoprotective effects of a standardized grape product (Use of Brachial Artery Ultrasound to evaluate endothelial function in humans)

In Chapter 9, we observed that an acute administration of a standardized grape product significantly increased shear-stress induced vasorelaxation responses in healthy normal males, and this effect was further enhanced during chronic administration. The standardized grape product completely inhibited the acute endothelial dysfunction induced by a single high fat meal, by a mechanism that was independent of circulating triglyceride levels. These data suggest that standardized grape product may have beneficial effects on endothelial health and function in subjects with no evidence of cardiovascular disease, that the beneficial effects of the grape product were not ethanol-dependent, and that this standardized grape product may have substantial protective effects against the vascular toxicities
associated with a high fat diet. Further studies regarding the mechanisms and putative ingredients mediating these protective effects, and a further evaluation of the therapeutic potential of this preparation, are clearly warranted.
REFERENCES


BIBLIOGRAPHY


Aliev G, Bodin P and Burnstock G (1998) Free radical generators cause changes in endothelial and inducible nitric oxide synthases and


*J Am Coll Cardiol* 30:325-333.


*Circulation* 106:1211-1218.

*Clin Cardiol* 18:67-72.

*GMHC Treat Issues* 11:6-12.

*Faseb J* 17:773-775.

Chaves AA, Liu CY, Hallenburg MA, Schanbacher BL and Bauer JA (2000a) LPS immunostimulation enhances retrovirus related cardiac dysfunction during murine AIDS.  
*Circulation* 102:356.

*Faseb Journal* 14:1444.

*Cardiovascular Research*.


Corallo S, Mutinelli MR, Moroni M, Lazzarin A, Celano V, Repossini A and Baroldi G (1988) Echocardiography detects myocardial damage in


Clinical Trial Group Cardiovascular Disease Focus Group. *Clin Infect Dis* **31:**1216-1224.


Lewis W, Haase CP, Raidel SM, Russ RB, Sutliff RL, Hoit BD and Samarel AM (2001) Combined antiretroviral therapy causes cardiomyopathy and


Lipshultz SE, Easley KA, Orav EJ, Kaplan S, Starc TJ, Bricker JT, Lai WW, Moodie DS, Sopko G, McIntosh K and Colan SD (2000b) Absence of cardiac toxicity of zidovudine in infants. Pediatric Pulmonary and


318


immunodeficiency virus infection. Comparison with primary pulmonary hypertension. *Circulation* **89**:2722-2727.


Reilly JM, Cunnion RE, Anderson DW, O'Leary TJ, Simmons JT, Lane HC, Fauci AS, Roberts WC, Virmani R and Parrillo JE (1988) Frequency of myocarditis, left ventricular dysfunction and ventricular tachycardia in


323


APPENDIX A

REACTIVE NITROGEN SPECIES FORMATION AND CARDIAC OXIDATIVE INJURY IN HUMAN HIV-RELATED CARDIOYOPATHY

This appendix represents work done in collaboration with Dr. Michael Mihm (a post doctoral fellow in our lab) to further investigate Human HIV related cardiac complications and corroborate our findings in murine AIDS (Chapter 3 and 4).
ABSTRACT

AIDS-related cardiovascular disease represents an emerging and complex challenge for the cardiology and infectious disease communities. The mechanisms by which cardiac complications develop in these patients may be distinct from non-viral and ischemic etiologies; traditional cardiovascular disease assessments, prognoses, and therapeutic approaches may have limited applicability and/or efficacy. Here we describe an approach for the histopathological study of AIDS related cardiovascular disease in a well-controlled library of cardiac autopsy tissues from AIDS patients, with specific comparisons to healthy control tissues and non-HIV cardiac cases. In these studies we used a relatively new tissue-microarray approach, providing opportunity for high-throughput, objective evaluations of many cases and several cardiac regions simultaneously. Using this approach we tested the hypothesis that HIV/AIDS-related cardiovascular disease is associated with increased cardiac protein oxidation events and investigated potential sources of RNS in this setting. We observed first-time evidence that cardiac RNS formation and resultant protein oxidative injury may play a significant role in the development of HIV/AIDS related cardiac complications, in a relevant, well-controlled human population. Given the high costs and complex drug regimens that are required for this patient population, the development of safe, affordable and selective antioxidant approaches may have unique value in this setting.
INTRODUCTION

The advent of Highly Active Antiretroviral Therapy (HAART) regimens have vastly improved outcomes for HIV patients, significantly increasing AIDS-free living and decreasing the incidence and severity of opportunistic infections. However, as life-expectancies continue to improve for HIV/AIDS patients, a variety of secondary complications to HIV infection have become increasingly apparent, affecting multiple organ systems not directly related to immune function (1, 2). HIV/AIDS-related pulmonary, neurological and cardiovascular diseases have become important contributors to the overall morbidity and mortality of HIV/AIDS. HIV/AIDS-related cardiovascular disease was first described as cardiovascular pathologies (myocarditis, pericardial effusion, left ventricular dilation) at autopsy in AIDS patients with no prior documented evidence of cardiovascular disease (3-5). Additional studies demonstrated left ventricular contractile and conduction abnormalities in HIV/AIDS patients, including reports describing detectable cardiac performance deficits in 40-70% of otherwise asymptomatic HIV infected patients (1, 6). The prevalence of related cardiovascular involvement has been estimated between 28-73%, with 5-20% of patients developing a severe non-ischemic dilated cardiomyopathy that has been termed AIDS-related cardiomyopathy (1, 6). Nearly 50% of pediatric AIDS patients (vertical transmission) also develop chronic cardiovascular disease by age 2, and cardiac failure is a primary cause of mortality in this patient population (7, 8). Overall, this apparently unique form of cardiomyopathy has a poorer prognosis.
relative to non-viral settings of cardiac disease, and the presence of AIDS related cardiovascular disease results in dramatically increased risk of mortality compared to AIDS patients without cardiovascular involvement at a comparable state of disease (CD4+ counts) (9, 10). With an worldwide incidence of HIV+ infection approaching 6,000,000 cases annually, AIDS-related cardiovascular disease represents an emerging medical problem, and may become an increasingly significant etiology of cardiac failure (11, 12).

A number of confounding factors (difficulties obtaining well controlled patient populations, co-existing infections, multi-organ system involvement, a limited number of relevant experimental models available, potential cardiovascular toxicities of anti-retroviral drugs) have complicated the study of AIDS-related cardiovascular disease. As a result, the relationships between AIDS-related cardiovascular disease and more traditional (non-viral) etiologies remain incompletely understood, and no specialized therapy currently exists for this unique patient population. While a variety of mechanisms have been proposed (myocardial infection with HIV, cytokines/inflammatory processes, bacterial/viral myocardial infections and or malignancies, formation of cardiotropic autoantibodies, HAART related toxicities), the putative mechanisms by which AIDS related cardiovascular disease develops remains poorly defined. Furthermore, the relations between focal immune infiltrative events (classical myocarditis) and global left ventricular deficits seen in these patients are incompletely understood. We have recently demonstrated that the “murine AIDS” model (LPBM5 retroviral infection) serves as an appropriate
model for AIDS related cardiovascular complications, and that cardiac formation of reactive nitrogen species, with attendant increases in cardiac protein nitration events, may cause retrovirus-related cardiac insufficiencies. We also obtained some preliminary evidence of this phenomenon in a small sample of HIV/AIDS cardiac tissues (13). Reactive nitrogen species (RNS) are a family of biologically relevant oxidants derived from the interaction of nitrogen based intermediates (e.g. nitric oxide) with reactive oxygen species (superoxide anion, hydroxyl radical, hydrogen peroxide), and are abundantly produced during the inflammatory response in settings of tissue injury/immune activation (14, 15). RNS can have profound cellular effects and toxicities due to the distinct reactivities of RNS relative to their reactive oxygen precursors. These reactivities include the avid capacity to cause nitration of tyrosine residues, both protein bound and free, resulting in the stable formation of 3-nitrotyrosine residues (3NT). Protein-3NT formation has been demonstrated to be a potent structural and functional post-translational protein modification; we and others have shown that increased cardiac protein-3NT formation is associated with multiple acute and chronic settings of cardiovascular disease, and that these increases are often correlated to extent of left ventricular dysfunction (16-20). The role for cardiac protein oxidation in the pathogenesis of AIDS related cardiovascular disease remains undefined.

Despite important advances in using animal models for retrovirus related cardiac investigations, there remains a clear need for detailed studies in human tissue samples. Thus far most of the human reports involve case
reports and/or description of relatively few samples. Furthermore, much of the literature thus far has focused on specific myocardial sites (typically lesion sites) rather than alterations in myocardium per se. Here we describe an approach for the histopathological study of AIDS related cardiovascular disease in a well-controlled library of cardiac autopsy tissues from AIDS patients, with specific comparisons to healthy control tissues and non-HIV cardiac cases. In these studies we used a relatively new tissue-microarray approach, providing opportunity for high-throughput, objective evaluations of many cases and several cardiac regions simultaneously. Using this approach we tested the hypothesis that HIV/AIDS-related cardiovascular disease is associated with increased cardiac protein oxidation events, and investigated potential sources of RNS in this setting.
METHODS

Patient information

Patient histories were reviewed to subclassify autopsy samples into 4 groups: HIV infected patients without evidence of cardiac disease [HIV+/CVD-]; HIV infected with documented evidence of cardiac disease [HIV+/CVD+]; non-HIV infected patients with no evidence of cardiac disease [HIV-/CVD-]; non-HIV infected patients with documented evidence of cardiomyopathy [HIV-/CVD+].

Full thickness anterior left ventricular tissues were obtained as paraffin-embedded autopsy specimens from HIV/AIDS patients and non-HIV infected controls from the National Cancer Institute AIDS Malignancy Bank. All autopsy samples studied were collected within 4 hours of death and age ranges at time of death were 24-63 yrs of age; age at autopsy was not statistically different across groups. All of the samples investigated were collected between 1983 and 1998.

Cardiac involvement in HIV+/CVD+ patients was defined by evidence obtained either clinically and/or at autopsy of the following conditions: myocarditis, pericardial effusion, dilated and/or hypertrophic cardiomyopathy, pericarditis, coronary artery disease, myocardial infarction, and/or congestive heart failure. AIDS patients with confounding risk factors for cardiovascular disease (e.g., smoking, diabetes, known substance abuse) were excluded from analysis. Also excluded were any patients who received HIV protease inhibitors as part of their drug therapy in the months preceding death, as these
agents have been documented as potential contributors to cardiac abnormalities. Detailed clinical characteristics of the populations studied are shown in Table 1.

**Histology and Immunohistochemistry**

Over 260 cardiac autopsy samples were collected into tissue microarrays (TMA) using a Beecher Instruments Tissue Arrayer (see scheme in Figure 1). Full thickness cross-sections of ventricular tissue were similarly oriented and 2.0 mm core punches were collected from the epicardial (outer 1/3 of myocardium), mesocardial (middle 1/3), and endocardial (inner 1/3) regions of the cross-section. Importantly, the regions selected were non-lesion sites, and there were no notable sites of classical myocarditis in any of the full thickness sections. These myocardial cores were then inserted into a composite TMA paraffin block, with 6-10 patients per TMA block). Four-micron sections were evaluated for general histology (Masson’s Trichrome), mast cell presence (Astra Blue stain, Sigma, St. Louis, MO) and eosinophil presence (Vital Red stain, Sigma, St. Louis, MO). Immunohistochemical studies assessed cardiac content and distributions of 3-nitrotyrosine (anti-3NT, Upstate Biotechnology, Lake Placid, NY, 1:400 dilution), nitric oxide synthase II (anti-NOS II, Transduction Labs, Lexington, KY, 1:400 dilution), and tumor necrosis factor-α (anti-TNF-α, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, 1:200 dilution). Additional immunohistochemical studies assessed cardiac presence of markers for neutrophils (anti-myeloperoxidase, Neomarkers, 1:200), and
monocytes/macrophages (anti-CD68+, Neomarkers, 1:400). Immunohistochemical studies were performed as we have previously described. Diaminobenzidine (DAB, 0.06% w/v, DAKO, Carpinteria, CA) was used to provide visualization of immunoreactivity, with methyl green counterstaining.

**Digital image analysis**

Left ventricular photomicrographic images were captured using a Polaroid digital camera (Polaroid) and transferred into research-based digital image analysis software (Image Pro Plus, Media Cybernetics). 5 images of each core punch were captured at 400x original magnification. Extent of immunoreactivity (3NT, NOS II, TNF-α) was determined by measuring optical density of diaminobenzidine signal in each tissue. Images were captured under identical lighting and optical settings, then segmented to eliminate background and nuclear counterstain from analysis. Integrated optical densities were determined for each image as a measure of staining intensity. Intra- and inter-observer variability for this procedure were each less than 2%. In parallel experiments, TMA slides assessed for specific immune infiltrates were captured as described above. Images were then calibrated, and positive cells were segmented and gated based on size, then counted and normalized as a function of left ventricular area.
Statistical Analysis

Differences between treatment groups assessed using two-tailed Student’s t-tests or one-way analyses of variance, with post hoc Newman-Keuls tests to evaluate significant comparisons. p<0.05 described statistical significance.
RESULTS

Detailed clinical characteristics for HIV+/CVD+ patients and controls (HIV+/CVD-, HIV-/CVD-, HIV-/CVD+) included in tissue microarrays are shown in Table 1. Patient age was not statistically different in any group studied, and median age was under 40 years of age. Coronary artery disease was a significant contributing factor in the HIV-/CVD+ group, as 86% of patients had evidence of atherosclerosis at autopsy. However, ischemic heart disease was much less prevalent in the HIV+/CVD+ group, with only 6% of these patients exhibiting coronary artery disease at autopsy. Incidence of inflammatory cardiac disease (myocarditis, pericarditis, pericardial effusion) was increased in the HIV+/CVD+ group compared to the non-infected CVD+ controls. Autopsy reports were also examined for documentation of co-existing pathogens. Although significant increases in fungal, bacterial, and viral infections were observed between the seropositive and seronegative patient groups, a two-fold increase in the incidence of mycobacterium infection was the only significant difference observed between the HIV+/CVD+ and HIV+/CVD- groups, with no significant differences in the incidence of fungal, viral, Gram-positive or Gram-negative infections observed at autopsy.

Shown in Figure 1 is a schematic representation of our approach for creating cardiac tissue microarrays from the 4 patient groups studied. Although initially labor intensive, we successfully collapsed roughly 260 individual full-thickness tissue sites to less than 10 total slides per analytical
run. Cores from endocardial, mesocardial, and epicardial regions were easily identified and collected and provided opportunity to evaluate region specific aspects in each group.

Figure 2 shows region specific prevalence of interstitial fibrosis for the four patient groups studied. Similar to our previous findings in other species, donor control hearts (HIV-/CVD-) exhibited ~5% of total tissue area positive for fibrosis (as detected by trichrome staining and automated digital imaging protocols). In contrast, fibrosis staining was doubled in the HIV+/CVD+ group, and this increase was localized to endocardial regions (p<0.05 vs. HIV-/CVD-). No changes were observed in either HIV+ treatment group (p=NS vs. HIV-/CVD-). Table 2 describes the mean heart weights and heart-to-brain weight ratios for each patient group. Hearts from HIV-/CVD+ patients demonstrated evidence of cardiac remodeling and/or hypertrophy, with a statistically significant increase in mean heart weight relative to HIV-/CVD- controls. Neither HIV+ patient group exhibited significant increases in cardiac weight. Heart-to-brain ratios were also measured to account for potential confounding factors (e.g. cachexia); this parameter was not different in either of the HIV+ patient groups relative to HIV-/CVD- controls.

Although immune cell infiltration has been extensively documented as a cardiac complication of HIV/AIDS, no studies to date have attempted to quantify interstitial immune cell presence in a relevant population. Shown in Figure 3 are representative images from cardiac tissues stained with specific probes for mast cells, neutrophils, and monocyte/macrophages. Cell counts
were determined for each treatment group and expressed per cross-sectional tissue area. Mast cell prevalence was nominal in each treatment group studied (Figure 2, lower left panel, p=NS). Cardiac presence of eosinophils was not detected in any treatment group studied. However, significant increases in CD68+ cell counts were observed in the HIV-/CVD+ and HIV+/CVD- treatment groups compared to HIV-/CVD- controls (Figure 3, lower middle panel). CD68+ cell counts were further increased in the HIV+/CVD+ treatment group (p<0.05 vs. HIV-/CVD+ and HIV+/CVD-). Myeloperoxidase+ cells were dramatically increased from baseline in seronegative patients with cardiovascular disease compared to HIV-/CVD- controls (Figure 3, lower right panel). A slight, but significant increase in MPO+ cells was observed in the HIV+/CVD- group compared to HIV-/CVD-; this increase was magnified in HIV+/CVD+ patients, to levels that were equivalent to the HIV-/CVD+ group. Histological evidence of neutrophil activation/degranulation was observed in some tissue sections from both HIV-/CVD+ and HIV+/CVD+ patients (Figure 3, left panels).

Protein-3NT formation was used as a marker of cardiac RNS formation, and measured by immunohistochemistry. Shown in Figure 4 are representative images of left ventricular cross sections from HIV-/CVD-, HIV+/CVD+, and HIV+/CVD+ patients, where brown staining indicates protein-3NT prevalence. HIV-/CVD- control tissues had low levels of protein-3NT content (Figure 4, Upper Left Panel), which was significantly increased in the HIV-/CVD+ group (Figure 4, Upper Middle Panel), consistent with our previous
reports. Protein-3NT formation was widespread throughout the myocardium, predominated within cardiac myocytes themselves, and was not confined to focal immune cell infiltrates. This increase was greatly potentiated in the HIV+/CVD+ group (Figure 3, Upper Right Panel). Digital image analysis provided quantitative measures of staining intensity for cardiac protein-3NT; both HIV-/CVD+ and HIV+/CVD- demonstrated statistically significant increases in cardiac protein-3NT prevalence compared to HIV-/CVD- controls. These differences were further magnified in HIV+/CVD+ hearts, as the integrated optical density was increased 4-fold over HIV-/CVD+ cardiac protein-3NT levels.

Additional immunohistochemical studies assessed left ventricular myocyte staining for TNF-α and NOSII. HIV-/CVD- control tissues demonstrated low, but detectable levels of cardiac TNF-α (Figure 5). This staining was significantly increased in the HIV-/CVD+ group, consistent with previous reports. Intense staining for TNF-α was also observed in hearts from HIV+/CVD+ patients. Staining was present throughout the myocardium and was present in cardiac myocytes themselves. Relative staining intensities were quantified using digital image analysis, and TNF-α staining intensities in HIV+/CVD+ hearts were significantly higher than HIV-/CVD-, HIV-/CVD+, and HIV+/CVD- groups. Conversely, no increases in cardiac NOSII prevalence were seen in any patient group studied (Figure 6, p=NS).
DISCUSSION

Cardiovascular disease is an increasingly significant complication of HIV/AIDS infection—AIDS patients with cardiovascular involvement have a significantly reduced (5-fold) life expectancy at an equivalent stage of AIDS progression (CD4+ count), and AIDS-related cardiovascular disease has extremely poor outcomes relative to more traditional etiologies of cardiac failure (AIDS-related cardiomyopathy has an adjusted hazard ratio of 5.86 relative to the more common etiology of idiopathic cardiomyopathy) (9, 10). Despite the high incidence of cardiovascular abnormalities and the high costs associated with patient care, the mechanisms involved remain largely undefined. A major obstacle to the mechanistic study of HIV/AIDS-related cardiac complications has been difficulties in collecting adequately large and well-controlled sample populations for rigorous statistical hypothesis testing and the heterogeneity of the patient populations involved (HAART therapy, illicit drug use, nutritional variables, etc) (21). No animal preparation has been established as the most appropriate to model this complicated and multifactorial disease process, and anecdotal reports and case studies provide only limited mechanistic insights. Here we describe the development of a well-controlled library of cardiac autopsy specimens optimized for histopathological studies of HIV/AIDS related cardiac complications. We employed this resource to begin to address the general hypothesis that the mechanisms involved in HIV/AIDS-related cardiac complications may be distinct from more traditional
etiology of cardiomyopathy. Given our recent experimental findings in a murine model of retroviral-induced cardiac dysfunction, we specifically investigated a role for cardiac reactive nitrogen species formation and protein oxidative events in this setting.

Cardiac autopsy specimens were obtained from the NCI AIDS Malignancy Bank and assembled on tissue microarray slides for histopathological analyses. This approach provided numerous advantages, including enhanced reproducibility and increased throughput for conducting histological studies in a large number of autopsy samples. Our patient population was subclassified into HIV-positive patients with documented evidence of cardiovascular disease and appropriate controls: seronegative patients with no cardiac disease, seronegative patients with cardiomyopathy, and seropositive patients with no evidence of cardiac disease. This approach provided the best-controlled study population that has been published to date, with appropriate controls for both general heart pathologies and HIV+ status. Cardiac tissues were sampled only from the anterior myocardium of the left ventricle in areas that had no evidence of focal immune cell infiltration or inflammatory lesions. We focused exclusively on myocardial tissue that was not involved in infiltrative lesioning, in an attempt to describe the status of the working cardiac muscle itself. This approach is distinct relative to recent publications that have concentrated on the contributions of myocardial lesions and myocarditis as the primary etiologies of HIV/AIDS-related cardiac dysfunction. Herein, we describe the effects of HIV/AIDS infection on cardiac
muscle biology, considering the cardiac dysfunction that is observed primarily from a cardiac contractile, not inflammatory, perspective.

Patient records provided initial insights into the etiologies of cardiovascular disease in each patient population. Ischemic heart disease with evidence of cardiac hypertrophy was the predominant etiology of cardiac failure in the HIV-/CVD+ patient group, whereas only 6% of HIV+/CVD+ patients had evidence of coronary artery disease. These data suggest that the precipitating events leading to cardiac dysfunction in these patient populations may not be similar; HIV/AIDS related cardiac disease may require unique perspectives and therapeutic approaches. Furthermore, HIV-/CVD+ hearts developed striking increases in fibrotic deposition, a classical cardiac remodeling response to failing pump performance. These increases were not observed in the HIV+ patient groups, further suggesting that the initiation and progression of cardiac changes in these populations may be distinct.

A pathogenic role for the immune system in acute cardiac injury as well as progressive cardiac and vascular disease is becoming increasingly apparent (22, 23). The interactions between immune cells and cardiac myocytes may have particular relevance in the setting of HIV/AIDS-related cardiac disease. While a number of reports have described the increased prevalence of focal immune cell lesions in hearts from HIV+ patients, the more subtle interactions that may occur with long-term residence of immune cells in the cardiac interstitium have been largely overlooked. We conducted histological studies probing for leukocytes that mediate non-specific immunity,
and that are known to participate in the formation of reactive oxygen and nitrogen species, focusing on areas of the myocardium that did not demonstrate evidence of infiltrative lesions. We found evidence of increased cardiac neutrophil presence in our patient groups with evidence of cardiovascular disease, with equivalent cell counts across both HIV-/CVD+ and HIV+/CVD+ groups. Neutrophils are short-lived as cardiac immune infiltrates and have potent reactive nitrogen species formation capabilities though a variety of nitrogen containing (NO, NO$_2^-$, etc) and reactive oxygen (O$_2^-$, H$_2$O$_2$, OH$,^-$, HOCl, etc) precursors. These data suggest that a steady state infiltration of neutrophils occurs during both settings of cardiovascular disease; this cell type is therefore unlikely to be contributing largely to the increased RNS formation observed. We found more remarkable changes in monocyte/macrophage cell counts, with a 2-3 fold increase in cardiac presence of CD68$^+$ cells in HIV+/CVD+ hearts, compared to their respective controls for cardiac disease (HIV-/CVD+) and viral infection (HIV+/CVD-). Monocyes/macrophages are carriers for HIV, and recent studies in humans have demonstrated that trafficking of HIV-infected CD68$^+$ cells to the heart occurs in patients with AIDS-related cardiomyopathy (24, 25). Macrophages are also high capacity production sites for both reactive oxygen and reactive nitrogen species and TNF-$\alpha$ and may contribute to cardiac oxidative damage in this setting (26-28). The factors involved in the recruitment and/or activation of these immune cells in the heart remain unknown. The selective infiltration of immune cells, and their protracted residence in cardiac muscle interstitium,
has the potential to modulate cardiac function, and these interactions may be as important as focal infiltrative events. Further studies to define the role of immune cell trafficking and activation in cardiac muscle appear warranted in this disease setting.

We, and others have shown that cardiac reactive nitrogen species formation and attendant protein nitration events may be unifying mechanistic events in acute cardiac injury and chronic progressive cardiac decompensation. Nitric oxide (NO) is a key mediator of both immune function and cardiovascular homeostasis, and may be an important pathophysiologic link between immune and cardiovascular system control (29). The reactivities of NO in vivo are highly dependent upon its interactions with other oxidants and its capacity to participate in the production of reactive nitrogen species. Reactive nitrogen species (RNS) are a family of biologically relevant oxidants derived from the interaction of nitrogen based intermediates (most notably NO) with reactive oxygen species (e.g. superoxide anion, hydroxyl radical, hydrogen peroxide) (15). RNS can have profound cellular effects and toxicities due to the distinct reactivities of RNS relative to their reactive oxygen precursors (14). These reactivities include the avid capacity to cause nitration of tyrosine residues, both protein bound and free, resulting in the stable formation of 3-nitrotyrosine residues (30). Protein-3NT formation has been demonstrated to be a potent structural and functional post-translational protein modification, has deleterious effects on cardiac contractility, and has been observed in a panoply of cardiovascular disease states (16-20). Since the
rates of RNS formation are highly dependent on both NO and the reactive oxygen intermediate involved (bimolecular reaction kinetics), increases in either NO formation (via induction of nitric oxide synthases) or reactive oxygen species can promote RNS formation. We have recently shown that a mouse model of acquired immunodeficiency, the “murine AIDS” model, displays time-dependent increases in cardiac RNS formation during retroviral progression, and that the extent of cardiac myocyte protein-3NT formation was inversely correlated to cardiac performance (cardiac output, fractional shortening) in those same mice (13). Here we tested the hypothesis that HIV/AIDS related cardiovascular disease was associated with increases in cardiac RNS formation in humans.

As expected from our previous data, cardiac protein-3NT was increased in HIV-/CVD+ hearts compared to HIV-/CVD- controls—the development of ischemic cardiomyopathy is associated with increased cardiac formation of protein-3NT. Surprisingly, this significant increase was outstripped in seropositive patients with evidence of cardiac disease, as cardiac immunoprevalence for protein-3NT was further increased 4-fold in HIV+/CVD+ hearts compared to HIV-/CVD+ levels. Interestingly, these changes occurred in the absence of cardiac NOS II induction. These findings suggest that cardiac RNS formation and attendant protein nitration may participate in HIV/AIDS-related cardiac pathology, rather than NOS II induction. This is consistent with the bimolecular reaction kinetics of RNS formation and supports the under-appreciated concept that NOS II induction is not obligatory
for promotion of RNS formation and the sustained presence of protein-3NT in vivo. Staining patterns indicate that cardiac myocytes themselves carried a majority of the heart’s protein nitration burden—staining was widespread throughout the myocardium, and not resultant from focal immune cell infiltrates.

The cardiodepressent effects of TNF-α are well-described. Cardiac production of TNF-α can occur in both cardiac myocytes themselves and macrophages with protracted residence in the heart, and is known to stimulate a variety of pathways resulting in reduced cardiac myocyte contractility and survival (26, 27). Stimulation of myocyte TNF-α receptors results in altered cardiac calcium handling (decreased calcium transients), induces cardiac myocyte apoptosis, and promotes the formation of reactive oxygen and reactive nitrogen species [(31, 32). Elevated circulating levels of TNF-α have been shown to be associated with increased severity of heart failure (in a non-HIV+ population) (33). We observed increased cardiac staining for TNF-α in HIV-/CVD+ patients, with a slight, but significantly greater, prevalence in the HIV+/CVD+ group. These changes did not correlate to an increase in cardiac NOS II staining, despite extensive evidence in the literature that TNF-α can acutely stimulate NOS II gene expression and NO formation in a variety of cell types, including cardiac myocytes (34, 35). The chronic effects of steady state TNF-α formation in the heart are poorly defined; these data may suggest that protracted production of TNF-α can result in desensitization of myocardial NOS II induction pathways. These results are not entirely consistent with
recent results published by Barbaro et al, which showed that increased cardiac NOS II staining may be a consistent phenomenon in AIDS patients with cardiac disease (36, 37). As has been shown in experimental studies, important context- and time-dependencies may exist between the induction of NOSII (and other extramitochondrial oxidases) and the formation of protein-3NT in cardiac tissue. TNF-α may be important in both the activation and recruitment of immune cells to the heart, as well as the promotion of oxidative pathways that contribute to RNS formation in this setting. Further studies defining the role of TNF-α pathways (and other pro-inflammatory mediators) in HIV/AIDS-related cardiac injury are clearly warranted, and are ongoing in our laboratory.

Taken together, these studies suggest that important changes are happening on the cardiac myocyte level in patients with HIV/AIDS-related cardiovascular disease. Significant cardiac dysfunction persists despite the absence of overt structural alterations (hypertrophy, fibrosis), suggesting that functional changes predominate at the level of cardiac myocyte contractility, not solely at the level of whole heart morphology as it relates to structural abnormalities or hypocontractile ischemic zones. Despite evidence of increased immune cell residence in myocardial interstitium, we observed protein oxidation and TNF-α throughout the myocardium, predominantly in cardiac myocytes themselves; immune cell interactions may incompletely explain the global cardiac changes that occur in this setting, and the cardiac myocyte itself may represent an important site of HIV/AIDS-related
pathogenesis. These conclusions are only possible and relevant given the human tissue resources we assessed, and investigating myocardial sites distal to focal lesion development.

In summary, AIDS-related cardiovascular disease represents an emerging and complex challenge for the cardiology and infectious disease communities. The mechanisms by which cardiac complications develop in these patients may be distinct from non-viral and ischemic etiologies; traditional cardiovascular disease assessments, prognoses, and therapeutic approaches may have limited applicability and/or efficacy. Here we describe first-time evidence that cardiac RNS formation and resultant protein oxidative injury may play a significant role in the development of HIV/AIDS related cardiac complications in a relevant, well-controlled human population. Given the high costs and complex drug regimens that are required for this patient population, the development of safe, affordable and selective antioxidant approaches may have unique value in this setting.
REFERENCES


### Table A.1: General patient characteristics

General patient characteristics were derived from patient records. Cardiac autopsy specimens were divided into 4 patient groups. Patient age was not significantly different between treatment groups. Type and prevalence of cardiac presentation at autopsy are provided, along with documented evidence of co-existing pathogens (systemic, not just infections of the heart), percentage of patients affected is in parenthesis.
Table A.2: Heart weights and heart/brain ratios

Heart and brain weights were tabulated from autopsy records, and compared between patient groups. Heart to brain ratio was used to normalize for muscle wasting effects that are known to complicate HIV infection. Heart weight was significantly elevated from naïve controls (HIV-/CVD-) in seronegative patients with cardiac disease (HIV-/CVD+). Heart weights were not significantly elevated in either HIV+ treatment group. Heart/brain ratios followed this same pattern, as this ratio was only elevated in the HIV-/CVD+ patient group.

<table>
<thead>
<tr>
<th></th>
<th>HIV-/CVD- (n = 8)</th>
<th>HIV-/CVD+ (n = 7)</th>
<th>HIV+/CVD- (n = 19)</th>
<th>HIV+/CVD+ (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Weight (grams)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>346 ± 61</td>
<td>452 ± 6*</td>
<td>338 ± 64</td>
<td>374 ± 94</td>
</tr>
<tr>
<td>Median</td>
<td>330</td>
<td>450</td>
<td>340</td>
<td>355</td>
</tr>
<tr>
<td>Range</td>
<td>270-460</td>
<td>410-510</td>
<td>230-460</td>
<td>210-450</td>
</tr>
<tr>
<td>Heart/Brain Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.28 ± 0.05</td>
<td>0.36 ± 0.06*</td>
<td>0.25 ± 0.06</td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td>Median</td>
<td>0.27</td>
<td>0.35</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Range</td>
<td>0.23-0.35</td>
<td>0.29-0.43</td>
<td>0.16-0.36</td>
<td>0.15-0.38</td>
</tr>
</tbody>
</table>
**Figure A.1: A tissue microarray approach for human AIDS cardiac autopsy tissues.**

Tissue microarray slides were developed from paraffin embedded blocks of cardiac autopsy specimens, and sectioned for histopathological analysis. Approximately 25 samples were assembled in each block—all specimens were arranged in a 10 slide set, randomized for patient groups such that each slide had samples from each treatment group.
Figure A.2: Cardiac interstitial fibrosis measures. Tissue microarray slides were examined for evidence of fibrosis deposition using Masson’s Trichrome stain and digital imaging procedures. Blue staining indicates collagen presence. Tissues were assessed for cross-sectional area staining positive for collagen and total tissue area, interstitial fibrosis was expressed as % of total tissue area that was stained blue. Representative images shown in upper panels (200x). ENDO, MESO, and EPI denote discrete core punches from endocardial, mesocardial, and epicardial sections of each cardiac cross-section. *, p<0.05 from HIV-/CVD-.
Figure A.3: Cardiac immune cell infiltrates.
Histological and immunohistochemical probes for specific cardiac infiltrates were used to stain tissue microarrays for presence of mast cells, monocytes/macrophages, and neutrophils. Representative images shown in upper panels (400x). Digital imaging and automated cell counting provided quantitative assessments of relative immune cell residence in cardiac tissue in each patient group. *, p<0.05
Figure A.4: Protein-3NT immunohistochemistry.
Tissue microarray slides were examined for cardiac myocyte presence of protein-3NT, using a specific antibody against nitrotyrosine. Digital imaging provided quantitative analysis of relative staining intensities, integrated optical densities (IOD) were determined for each image, and averaged across patient groups. Representative images shown in upper panels (400x), brown staining indicates positive immunoreactivity. ENDO, MESO, and EPI denote discrete core punches from endocardial, mesocardial, and epicardial sections of each cardiac cross-section. *, p<0.05 from HIV-/CVD-; †, p<0.05 from HIV-/CVD+; ‡, p<0.05 from HIV+/CVD-.
Figure A.5: TNF-α immunohistochemistry.
Tissue microarray slides were examined for cardiac myocyte presence of TNF-α, using a specific antibody against TNF-α. Digital imaging provided quantitative analysis of relative staining intensities, integrated optical densities (IOD) were determined for each image, and averaged across patient groups. Representative images shown in top panel (400x), brown staining indicates positive immunoreactivity. ENDO, MESO, and EPI denote discrete core punches from endocardial, mesocardial, and epicardial sections of each cardiac cross-section. *, p<0.05 from HIV-/CVD-; †, p<0.05 from HIV-/CVD+; ‡, p<0.05 from HIV+/CVD-.
Figure A.6: NOS 2 immunohistochemistry.
Tissue microarray slides were examined for cardiac myocyte presence of NOS2, using a specific antibody against NOS2. Digital imaging provided quantitative analysis of relative staining intensities, integrated optical densities (IOD) were determined for each image, and averaged across patient groups. Representative images shown in top panel (400x), brown staining indicates positive immunoreactivity. ENDO, MESO, and EPI denote discrete core punches from endocardial, mesocardial, and epicardial sections of each cardiac cross-section.
APPENDIX B

Transgenic Mice with Cardiac-Specific Expression of Activating Transcription Factor 3, a Stress-Inducible Gene, Have Conduction Abnormalities and Contractile Dysfunction

This appendix reflects work done in collaboration with Dr. Yoshi Okamoto (a fellow in the lab of Dr. Tsonwin Hai), which has been published in the American Journal of Pathology (Am J Pathol 159:639-650), and has been presented here as per the requirements of the journal.

The authors on this appendix are listed below:
ABSTRACT

Activating transcription factor 3 (ATF3) is a member of the CREB/ATF family of transcription factors. Previously, we demonstrated that the expression of the ATF3 gene is induced by many stress signals. In this report, we demonstrate that expression of ATF3 is induced by cardiac ischemia coupled with reperfusion (ischemia-reperfusion) in both cultured cells and an animal model. Transgenic mice expressing ATF3 under the control of the α-myosin heavy chain promoter have atrial enlargement and atrial and ventricular hypertrophy. Microscopic examination showed myocyte degeneration and fibrosis. Functionally, the transgenic heart has reduced contractility and aberrant conduction. Interestingly, expression of sorcin, a gene whose product inhibits the release of calcium from sarcoplasmic reticulum, is increased in these transgenic hearts. Taken together, our results indicate that expression of ATF3, a stress-inducible gene, in the heart leads to altered gene expression and impaired cardiac function.
INTRODUCTION

Heart failure is a complex syndrome that can result from virtually any disorder affecting the myocardium [1], such as ischemic heart disease, hypertension, valvular disease, and primary cardiomyopathy. One common feature of these etiologies is the imposition of an abnormal load on the myocardium. This overload induces complex humoral, mechanical, and neural responses that initially compensate systolic function by increasing heart rate, contractility, and the size of sarcomeres (hypertrophy) [2]. Despite these compensatory mechanisms, however, if the inciting diseases are left untreated, heart failure ensues with dilated cardiomyopathy—the end-stage phenotype of heart failure—regardless of etiology. Therefore, one critical area for heart failure research is to elucidate the responses of cardiomyocytes to the stress of abnormal load.

We have been investigating a stress-inducible gene, activating transcription factor 3 (ATF3). It is a member of the CREB/ATF family of basic region-leucine zipper (bZip) transcription factors [3-8]. Overwhelming evidence from Hai and others indicates that ATF3 is induced by a variety of stress signals in different cell types [4,5]. Previously, we demonstrated that the mRNA level of ATF3 greatly increases in the heart after myocardial ischemia, and ischemia coupled with reperfusion (ischemia-reperfusion), in the kidney after renal ischemia-reperfusion, in the skin after wounding, in the brain after seizure, and in the liver after chemical toxicity and partial hepatectomy (unpublished results) [9,10]. In addition to the above animal experiments, in vitro experiments using
cultured cells also indicate that ATF3 is induced by stress signals, including cytokines [11,12], genotoxic agents such as ionizing radiation [13] and agents known to induce cell death or the JNK/SAPK signaling pathway such as anisomycin [14] and cycloheximide [15]. Therefore, ATF3 is induced in a variety of cell types by many different stress signals, suggesting that it may be a key regulator in cellular stress responses. One common theme of all of the signals that induce ATF3 is that they also induce cellular damage. Therefore, the induction of ATF3 seems to correlate with cellular damage. In this report, we describe our recent studies on the roles of ATF3 in cardiac stress responses. We present evidence indicating that ectopic expression of ATF3 in the heart leads to conduction abnormalities and contractile dysfunction, suggesting that induction of ATF3 by stress signals may play a role in the pathogenesis of stress-associated cardiac diseases.
METHODS

Ischemia-Reperfusion Models

In Vivo Model

Two-month-old male Sprague-Dawley rats were anesthetized, intubated by tracheotomy, and ventilated using a pressure-controlled ventilator. The heart was exposed by the left intercostal approach, and the left coronary artery was ligated with an 18-gauge needle tied against it. Ischemia was confirmed by ST segment elevation in electrocardiography (ECG). After 2 hours of ischemia, the ventricle was reperfused by removal of the needle. At 1 hour after reperfusion, the heart was excised from the surviving animals (~50%) and frozen immediately for in situ hybridization.

In Vitro Model

Cardiomyocytes from Sprague-Dawley rats at 1 to 2 days of age were prepared as described previously [16] with minor modifications. Cells were incubated with buffer containing 20 mmol/L HEPES (pH 6.6), 125 mmol/L NaCl, 4.9 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L NaH₂PO₄, 1.8 mmol/L CaCl₂, 8 mmol/L NaHCO₃, 5 mmol/L NaCN, and 20 mmol/L deoxyglucose for 2 hours to deplete ATP. Cardiomyocytes were then allowed to recover in normal media for 2 hours before the isolation of total RNA.
Generation of the Myosin Heavy Chain Promoter (MyHC)-ATF3 Transgenic Mice

The human ATF3 gene was targeted to the heart using the α-MyHC promoter (from Dr. J. Robbins, University of Cincinnati). Transgenic mice were generated in the FVB/N background, and mice containing the transgene were identified by polymerase chain reaction (PCR) using the upstream primer 5'-GACTTCACATAGAAGCCTAGCC-3' complementary to the α-MyHC region, and the downstream primer 5'-AACCACAACTAGAATGCAGTG-3' complementary to the SV40 polyA region.

In Situ Hybridization and Immunohistochemistry

In situ hybridization and immunohistochemistry were performed as detailed previously [9].

RNA Isolation, Reverse Transcriptase (RT)-PCR, and Dot Blot

Total RNA was isolated using Trizol reagent (Life Technologies, Inc., Rockville, MD). Reverse transcription was performed using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and the resulting cDNA was subjected to PCR. Rat ATF3 mRNA was analyzed by the upstream primer 5'-GCTCTAGAAAAAAAGAGAAGACRGAGTGC-3' and the downstream primer 5'-TCTCCAATGGCTTCAGGGTT-3'. Human ATF3 transgenic mRNA was analyzed by the upstream primer 5'-GAGGTAGCCCCTGAAG-3' complementary to the ATF3 coding region, and the downstream primer complementary to the SV40 polyA region as above. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was analyzed by the upstream primer 5'-
CCGGATCCTGGGAAGCTTGCATCAACGG-3’ and the downstream primer 5’-GGCTCGAGGCGAGTGAGCATGGACTG-3’. Quantitative RNA dot-blot analysis was performed as described previously [17] with modifications. Briefly, RNA was resuspended in diethyl pyrocarbonate-treated water, and denatured by heating to 95°C. Three µg of total RNA per dot was blotted onto nitrocellulose filters using a dot-blot filtration manifold (Bio-Rad, Melville, NY). All samples were analyzed in duplicate. The synthetic oligonucleotides used as transcript-specific probes are as follows: atrial natriuretic factor 5’-AATGTGACCAAGCTGCGTGACACACCACAAGGGCTTAGGATCTTTTGCGATCTGCTCAAG-3’, α-skeletal actin 5’-TGGAGCAAAACAGAATGGCTGGCTTTAATGCTTTCAAG-3’, β-myosin heavy chain (β-MyHC) 5’-GCTTTATTCTGCTTCCACCTAAAGGG-CTGTTGCAAGGCTCAGTGAGGCTTC-3’, myosin light chain 2 ventricular isoform (MLC2v) 5’-CACAGCCCTGGGATGGAGAGTG-GCTGTGGGTCACCTGAGGCTGTGGGTTCAG-3’, sarcoplasmic reticulum calcium channel (SERCA2a) 5’-AGGTGTGTGCTAACAACGCAGATGCA- GCACCGAAGACCCCTTATATTCTGCAAATGG-3’, and GAPDH 5’-GGAACATGTAGACCATGTAGTTGAGGTCAATGAAG-3’. All oligonucleotide probes were 5’ end-labeled with γ[^32P]-ATP using T4 polynucleotide kinase (Promega). The sorcin probe was a random-primed probe derived from an ~300-bp fragment of the mouse sorcin cDNA. The cDNA clone was generated by ligating pBSSK vector with an RT-PCR product derived from mouse mRNA using
the upstream primer 5'-AATGCAGCTGAATGGCTGGAGACAACAC-3' and
downstream primer 5'-CCCAAGCTTTTAGACGTCATGACACACTG-3'. Italics
indicate the PstI and HindIII sites for cloning. Sequence analysis confirmed that
the clone contains a fragment of the mouse sorcin cDNA, and the probe was
found to hybridize to a single band on a Southern blot using mouse genomic DNA
under hybridization conditions identical to those used for dot-blot hybridization.
Quantitation of hybridization signals was accomplished using a Storm 860
PhosphorImaging system and Imagequant software (Molecular Dynamics,
Sunnyvale, CA). The signal intensity of each dot was normalized to that of
GAPDH after correcting for background.

*Histology, Morphometric Analysis, and Electron Microscopy*

Hearts were fixed in 10% buffered formalin for 24 to 48 hours, dehydrated, and
embedded in paraffin. Five-µm sections were stained with hematoxylin and eosin
(H&E) or with Masson's trichrome. For the analysis of ventricular dilation, mice
were anesthetized and heparinized via inferior vena cava; hearts were then
excised, cannulated via aorta, and perfused with cardioplegic solution
(phosphate-buffered saline containing 25 mmol/L KCl and 5% glucose) before
fixation. For morphometric analysis, connective tissues around the heart were
trimmed from the heart after fixation. Atria were separated from ventricles under
the dissection microscope. Organized thrombus, if present, was removed from
the atrium. Atria and ventricles were weighed separately. Electron microscopic
studies of the hearts were carried by the methods described previously [18].
Echocardiography and ECG

Mice were placed under light anesthesia with halothane inhalation (0.5 to 1% halothane in a mixture of 95% O₂ and 5% CO₂) and warmed to maintain body temperature. Two-dimensional and M-mode echocardiographic images were recorded and analyzed by a Sonos 1000 echocardiograph and a 7.5 MHz pediatric ultrasonic probe (Hewlett-Packard Co., Andover, MA) as described previously [19]. ECGs were acquired (sampling rate, 2000 Hz) for 30 seconds with a Biopac MP100 system (Biopac Systems Inc., Santa Barbara, CA) interfaced with a Pentium computer. Data were stored for off-line analysis with Acqknowledge (Biopac Systems, Inc.). All normal sinus rhythm records were signal-averaged before measurement of electrocardiographic intervals. Records with high-degree atrioventricular blocks were analyzed by measuring and averaging consecutive beats. The QTc interval was calculated by dividing the QT interval by the cube root of the R-R interval.

Preparation of Ventricular Myocytes and Measurement of Contractile Function in Vitro

Mice were anesthetized and heparinized via inferior vena cava. Hearts were rapidly excised, cannulated via aorta, and perfused at 37°C with 25 ml of the perfusion buffer: 118 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 0.68 mmol/L glutamine, 11 mmol/L glucose, 25 mmol/L NaHCO₃, 5 mmol/L pyruvic acid, 20 µmol/L EGTA, 1% minimal essential medium amino acids solution (Life Technologies, Inc.), 1% minimal essential medium
nonessential amino acids solution (Life Technologies, Inc.), and 1% minimal essential medium vitamin solution (Life Technologies, Inc.). Hearts were then perfused at 37°C for 15 minutes with the enzyme solution: perfusion buffer with 1 mg/ml collagenase (Worthington, Lakewood, NJ), and 1 mg/ml bovine serum albumin. CaC\textsubscript{12} was added slowly during perfusion to make a final concentration of 0.75 mmol/L. The ventricles were minced and the cells were dissociated in the enzyme solution. Viable cardiomyocytes were obtained by settling in the incubation buffer (perfusion buffer with 25 mmol/L HEPES, 1 mmol/L CaC\textsubscript{12}, 1 µmol/L insulin, 2% bovine serum albumin, and penicillin-streptomycin without NaHCO\textsubscript{3}). Isotonic shortening of individual myocytes in response to electrical field stimulation was analyzed as described previously [20].

Swimming Exercise

Mice at 8 weeks of age were made to swim in a water tank with a surface area of 2200 cm\textsuperscript{2} according to the protocol described by Geisterfer-Lowrance and colleagues [21]. The water temperature was kept at 30 to 32°C throughout the experiment. Two swimming sessions were held daily with a 30-minute interval. The duration of the session was 10 minutes on the first day with an increase of 10 minutes everyday up to 90 minutes each session. Drowning mice were rescued from the water and resumed swimming in the next session. The purpose for rescuing the mice was to avoid death from drowning rather than a cardiac problem. The swimming exercise continued for 22 days.
Statistical Analysis

Statistical analyses include analysis of variance and t-test. A $P$ value of <0.05 was considered statistically significant.
RESULTS AND DISCUSSION

*Induction of ATF3 by Ischemia-Reperfusion both in Vivo and in Vitro*

Although we demonstrated previously that ATF3 is induced in the heart by ischemia-reperfusion [9], in those experiments we examined only a wedge of the left ventricle. To obtain a more complete picture, we examined serial cross sections of the heart derived from rats treated with coronary artery ligation coupled with reperfusion as detailed in the Material and Methods. As shown in Figure 1A, ATF3 was induced in a loop pattern as shown previously; reconstruction of serial sections revealed a cone of ATF3 expression. Control experiments using sham-operated rats showed no induction of ATF3 (data not shown). To determine whether this stress-induced expression of ATF3 can be recapitulated *in vitro*, we isolated cardiomyocytes from newborn rats and treated the cells with a widely used procedure to mimic ischemia-reperfusion *in vitro* [22,23]. Incubation with NaCN and deoxyglucose to deplete ATP was followed by the removal of these metabolic inhibitors. As shown in Figure 1B, the ATF3 mRNA level greatly increased (compare lanes 3 and 4 in Figure 1B) as indicated by reverse transcription coupled with polymerase chain reaction (RT-PCR). The specificity of the band was demonstrated by the lack of signal if reverse transcriptase was deleted in the RT reaction (the minus RT control; Figure 1B, lane 2). The lack of ATF3 signal in untreated cells was not because of a lack of RNA in the sample, because signals for the control mRNA GAPDH were similar between uninduced and induced cells (Figure 1B, lanes 3 and 4, bottom panel).
To investigate the significance of ATF3 induction by cardiac stress, we took a gain-of-function approach and generated transgenic mice expressing ATF3 using the $\alpha$-myosin heavy chain ($\alpha$-MyHC) promoter. The transgenic construct contains the human ATF3 open reading frame and the SV40 polyA signal (Figure 2A). For the convenience of discussion, we will refer to these mice as MyHC-ATF3 mice in the rest of the report. The 5-kb fragment of the MyHC promoter used in this experiment has been demonstrated to drive the expression of transgenes as follows: in atria and in striated muscle surrounding pulmonary veins constitutively starting at embryonic day 10 (e10), and in the ventricles constitutively starting 12 hours before birth [24,25]. Thus far, we have generated five transgenic founders. Postmortem analyses showed that they can be divided into two groups: group I (no. 83, no. 85, and no. 92) with dramatically enlarged atria, and group II (no. 89 and no. 100) with mildly enlarged hearts. Only founders no. 85 and no. 100 gave rise to transgenic progeny before death. RT-PCR analysis using primers specific to ATF3 and the SV40 polyA region showed a specific band of the expected size from RNA isolated from no. 85 (TG1) hearts (Figure 2B). In contrast to the easily detectable signal from no. 85 mice, the signal from no. 100 (TG2) mice was faintly visible. Although the assay was not quantitative, the difference in the signal level was dramatic and reproducible, suggesting that the expression level of the transgene was lower in no. 100 mice than that in no. 85 mice. Consistent with this interpretation, the phenotypes in no. 100 mice were much weaker than that in no.
85 mice (see below). In the rest of this report, we will refer to no. 85 mice as TG1 and no. 100 mice as TG2.

Despite the easy detection of the ATF3 expression in TG1 by RT-PCR, we consistently failed to detect it by in situ hybridization (data not shown), a technique we used to detect the induction of endogenous ATF3 by ischemia-reperfusion. This suggests that the level of transgene expression in TG1 is lower than that of endogenous ATF3 induced by ischemia-reperfusion. This relatively low expression of the transgene argues against the interpretation that the cardiac dysfunction we describe below was because of a nonspecific effect of overexpressing any foreign protein in the heart. The tissue specificity of transgene expression in TG1 was demonstrated by the lack of transgene expression in thymus, liver, spleen, kidney, skeletal muscle, and brain (Figure 2C). The slight expression in the lung is consistent with a previous report that the promoter is active in pulmonary veins [24]. Immunohistochemistry using antibodies against ATF3 confirmed that ATF3 protein is produced in the transgenic heart (Figure 2D).

**Bi-Atrial Enlargement, Cardiac Hypertrophy, and Dilation**

The TG1 mice showed obvious bi-atrial enlargement starting at 3 weeks of age, with a consistently greater enlargement of the right atrium than the left atrium. Figure 3A shows a representative picture. The reason for the difference between the left and right atria is not clear at present. Because bi-atrial enlargement was observed in three founders (no. 83, no. 85, and no. 92), it strongly suggests that this phenotype was because of the expression of the
transgene rather than the sites of integration. Consistent with atrial enlargement, transgenic mice showed a dramatic increase in the heart-to-body weight ratio (data not shown). The majority of the weight increase was because of the increase in atrial weight as shown in Table 1. The increase in ventricular weight was small, but statistically significant. This increase in heart weight is suggestive of cardiac hypertrophy. Therefore, we compared the transgenic hearts with nontransgenic (NTG) hearts by quantitative RNA dot-blot analysis for altered steady-state mRNA levels indicative of a hypertrophic response. As shown in Figure 4, in mice at 15 to 20 weeks of age, α-skeletal actin was up-regulated in the ventricles of both transgenic lines (TG1 versus NTG), 1460 ± 72 versus 100 ± 18, \( P = 0.002 \); TG2 versus NTG, 149 ± 33 versus 100 ± 18, \( P = 0.007 \), and in the atria of TG1 mice (1300 ± 56 versus 100 ± 23, \( P = 0.0051 \)). Atrial natriuretic factor, although not significantly up-regulated in the ventricles of either line, was up-regulated in the aria of TG1 mice (680 ± 87 versus 100 ± 10, \( P = 0.00001 \)). ß-MyHC was significantly up-regulated in the ventricles of TG1 mice (155 ± 51 versus 100 ± 25, \( P = 0.049 \)). Up-regulation of these three markers (α-skeletal actin, atrial natriuretic factor, and ß-MyHC) to varying extent is typical of cardiac hypertrophy in human cardiomyopathy and in various murine models of cardiac hypertrophy. Therefore, these results indicate a significant hypertrophic response in TG1 hearts. Consistent with this notion, TG1 ventricles had significant down-regulation of myosin light chain 2 ventricular isoform (MLC2V) (63 ± 19 versus 100 ± 17, \( P = 0.0116 \), a down-regulation also observed in tissues from hypertrophic human hearts and in hypertrophic and hypertensive primate and
rodent models [26-29]. SERCA2a, a marker of heart failure [30-32] was not significantly different in the ventricles or atria of either line. It is apparent from the literature that different patterns of dysregulation for hypertrophic markers characterize specific murine models; importantly, although some markers may not be up-regulated in specific models, an increase in α-skeletal actin is always observed. Therefore, taken together our dot-blot results indicate a relatively severe hypertrophic response in TG1 ventricles and atria, and a mild hypertrophic response in TG2 ventricles. This is in agreement with the observation of a significant increase in heart-to-body weight ratio in TG1 but not TG2 (Table 1). Because of the large significant increase in both α-skeletal actin and atrial natriuretic factor in TG1 atria, it is possible that the bi-atrial enlargement represents an atrial hypertrophic response.

In summary, these observations—macroscopic enlargement, relative heart weight increase, and altered gene expression—clearly demonstrate cardiac hypertrophy in TG1 mice. However, the hypertrophy was more obvious in the atria than in the ventricles. We speculate that this difference may be because of an earlier expression of the transgene in the atria than in the ventricle, because the MyHC promoter is active in the atria starting on embryonic day 10 but not active in the ventricles until shortly before birth [24,25]. In addition to hypertrophy, the TG1 transgenic mice showed dramatic atrial dilation (data not shown) and right ventricular dilation. Figure 3B shows a representative result from mice at 30 weeks of age. However, the left ventricle did not show obvious dilation. Although the reason for this difference is not clear, one possibility is that right ventricular
dilation is a secondary effect of atrial dysfunction, thus reflecting the different degrees of atrial enlargement described above.

**Myocyte Disarray, Degeneration, and Fibrosis**

Because myocyte disarray is a cellular response that typically accompanies the development of hypertrophy [21], we examined whether the transgenic hearts displayed myocyte disarray. As shown by H&E staining, at 30 weeks of age the TG1 atria were disorganized (Figure 5D). This myocyte disarray was visible as early as 2 weeks of age (Figure 5, compare A and B), but was more obvious in the atria than in the ventricles (data not shown), consistent with the greater atrial phenotypes described above. As evident in Figure 5, B and D, the transgenic hearts also showed karyomegaly, anisokaryosis, and abnormally shaped nuclei, indicative of myocyte degeneration and atrophy [33]. To further compare the structural differences between the TG1 mice and the NTG mice, we examined the myocytes by electron microscopy. Figure 6A shows three degenerating myocytes in the transgenic atria (derived from mice at 25 weeks of age) as diagramed in Figure 6C. Within the myocytes, many abnormal structures were evident, including disoriented myofibrils, degenerating mitochondria, abnormal Z-lines, vacuoles, granules, and degenerating intercalated disks. In addition, electron microscopy analysis showed large cells (consistent with the hypertrophy described above), variation in cell size, inclusion of abnormal material in the nuclei, and accumulations of dense material in the cytoplasm (data not shown). All these are indications of myocyte degeneration, and are in contrast to the normal myocytes from NTG mice, where well-organized myofibrils and Z
lines were evident (Figure 6B). Because another feature of heart failure is fibrosis of the heart wall, we performed Masson’s trichrome staining. As shown in Figure 5F, at 20 weeks of age, the TG1 mice had excess interstitial collagen (blue stain) in the atria, indicating extensive fibrosis. The extent of fibrosis in the ventricles was much lower (data not shown), again consistent with the less severe ventricular phenotypes described above. In summary, microscopic examination indicated that the myocytes were degenerating and the hearts fibrotic in the TG1 transgenic mice. We also performed all of the analyses on TG2 mice and did not find any obvious microscopic abnormalities (data not shown).

**Ventricular Contractile Dysfunction**

The findings described for TG1 are consistent with a nonspecific cardiomyopathy likely to be associated with ventricular dysfunction. To examine the ventricular function, we performed echocardiographic analyses. Consistent with Figure 3A, the echocardiographic images showed enlarged atria; in addition, they showed an apparent rotation of heart in vivo (data not shown). These structural and positional changes blocked the clear resolution of left ventricular wall motion for further analysis. Therefore, we performed an in vitro assay to assess the contractile function of the cardiomyocytes. We isolated ventricular cardiomyocytes from mice at 15 to 20 weeks of age, and measured their isotonic shortening after stimulation in the electrical field. As shown in Figure 7A, myocytes derived from TG1 mice showed a statistically significant reduction in percent cell shortening when compared to myocytes from NTG mice. The reduction was observed either in the absence or presence of the β-agonist
isoproterenol. In addition, the rate of contraction (inotropy) and the rate of relaxation (lusitropy) were significantly slower in TG1 than in NTG mice either in the absence or presence of isoproterenol (Figure 7, B and C). Taken together, these results indicate that the TG1 transgenic myocytes were less contractile and less responsive to β-adrenergic receptor stimulation, a finding consistent with cardiomyopathy.

We also examined the TG2 myocytes by these analyses. Echocardiographic analyses of mice from 6 weeks to 30 weeks of age in a longitudinal study showed statistically significant reductions in left ventricular contractile performance, as evidenced by ~10% reduction in fractional shortening and 15% reduction in stroke volume when compared to NTG mice (Figure 8). Using similar methods, we demonstrated recently that severe left ventricular failure in mice (doxorubicin-induced cardiomyopathy) is associated with ~30 to 35% reductions in fractional shortening and stroke volume relative to corresponding control values [19]. Therefore, the reductions observed in TG2 mice indicate that they had a moderate decrease in left ventricular contractile performance. This moderate decrease is consistent with the mild phenotypes in the TG2 mice described above. M-mode images of the echocardiographic analyses also demonstrated increased left ventricular internal dimension during systole in transgenic animals versus age matched controls (1.83 ± 0.04 mm at 14 weeks and 1.73 ± 0.08 mm at 22 weeks, versus 1.58 ± 0.02 mm control, \( P < 0.05 \)). No change in diastolic dimension was observed. In vitro analysis of the myocytes indicated that they had reduced cell shortening, inotropy, and lusitropy.
(Figure 7). However, the reduction was apparent only at high concentrations of isoproterenol; in the absence of isoproterenol, there was no significant reduction. Because the echocardiographic analysis that showed a statistically significant reduction in fractional shortening was performed in the absence of isoproterenol, this suggests that the in vivo echocardiographic analysis is more sensitive for detecting ventricular dysfunction than the in vitro cell-shortening assay.

Conduction Abnormalities

The gross hypertrophy and structural degeneration of atria in TG1 mice prompted us to examine the mice by electrocardiography. We examined mice at ~ weeks or 20 weeks of age (Table 2). In many of the recordings, the QRS and the T wave were not discrete. Therefore, the QRS duration is not reported. The NTG controls had an age-dependent reduction in the PR interval. This is in contrast to the age-related increase in PR interval reported previously in wild-type 129SvEv inbred mice [34]. The difference may be because of differences in age, strain, or anesthetic regimen. In the young animals (~6 weeks of age), there were no statistically significant differences between transgenic and NTG animals with respect to any ECG intervals. In the older animals (~20 weeks of age), there were significant differences between the transgenic and NTG animals in both heart rate and the QT interval. However, the rate-corrected QT interval (QTc) was not significantly different. In addition, two of the five older transgenic animals demonstrated sinus rhythm with Wenckebach periodicity (data not shown), indicating altered atrioventricular node conduction. In summary, the transgenic
mice have ECG changes consistent with the increased fibrosis and fiber disarray or altered autonomic balance, a potential consequence of the pathology described above.

**Decrease in Cardiac Functional Capacity**

To examine whether the transgenic mice have a diminished cardiac functional capacity, we examined the mice during exercise. We trained six TG1 and six NTG mice at 8 weeks of age to swim as detailed in the Material and Methods. All six NTG mice tolerated the exercise protocol and resumed normal activity immediately after swimming. However, five of the six transgenic mice displayed obvious struggle during swimming and recovered to normal activity slowly afterward. Two transgenic mice were drowning and required rescue in two swimming sessions. One mouse died soon after rescue 12 days into the protocol. Therefore, the transgenic mice had less functional capacity than the NTG mice, consistent with their cardiac contractile dysfunction and conduction abnormalities.

**Altered Expression and a Potential Role of ATF3 in G-Protein Signaling**

Interestingly, expression of sorcin, a gene whose product inhibits the release of calcium from sarcoplasmic reticulum [35-37], was increased in these transgenic hearts. We examined sorcin, because it was identified in a DNA microarray screen in cultured cells ectopically expressing ATF3 (A. E. Allen-Jennings, L. Gang, K. L. Gardner, and T. Hai, unpublished results). As shown by quantitative dot-blot analysis, sorcin mRNA was significantly higher in TG1 than NTG atria (Figure 4). Because calcium release from sarcoplasmic reticulum plays
a pivotal role in cardiac function, the increased expression of sorcin, an inhibitor of this process, may contribute to the cardiac dysfunction observed in the transgenic mice. Recently, Redfern and colleagues [38] generated transgenic mice expressing a modified G_i-coupled receptor (Ro1) in the heart in an inducible manner. They showed elegantly that expression of Ro1 causes ventricular conduction delay and a lethal cardiomyopathy [38]. Intriguingly, ATF3 was identified in their study as a gene up-regulated in the transgenic hearts by a DNA array analysis, suggesting a role for ATF3 in G-protein signaling [38].

The Role of ATF3 in Cardiac Stress Response

As described in the Introduction, ATF3 is a member of the CREB/ATF family of transcription factors. Because these transcription factors are involved in the regulation of a variety of genes, they have been used as a paradigm for studying regulation of gene expression by many investigators. CREB, a widely studied member of this family, was implicated to play a role in the heart. When a dominant-negative form of CREB was expressed in the heart, mice carrying this transgene developed four-chamber dilated cardiomyopathy [39,40]. Because the dominant-negative form of CREB interferes with the normal function of CREB, these results indicate that CREB is important for some normal functions of the heart. Our work suggests that another member of this family, ATF3, may also play a role in the heart, although most likely a role different from that of CREB. Because ATF3 is not detectable in the heart under nonstressed conditions, we suspect that it does not play a role in cardiac function under normal conditions.
However, it plays a role in cardiac stress response, because it is induced in the heart by stressors such as myocardial ischemia-reperfusion.

The phenotypes we observed in the MyHC-ATF3 transgenic mice suggest that ATF3 is a detrimental stress-inducible gene. This notion is consistent with our preliminary results suggesting that stress signals may induce ATF3 by activating the JNK/SAPK and p38 stress kinases (J. Chen and T. Hai, unpublished results). Activation of these stress kinases in cardiomyocytes has been implicated to lead to detrimental effects [41-43]. If ATF3 is indeed a downstream target gene for the JNK/SAPK and p38 signaling pathways, it may be one of the mediators for the stress pathways to elicit detrimental effects. In summary, our results are consistent with the interpretation that expression of ATF3 in the heart leads to cardiac dysfunction. Because ATF3 is a stress-inducible gene, our results may help to understand the roles of gene regulation in stress-associated cardiac diseases.
REFERENCES


### Table B.1: Morphometric Analysis of Mice

Atrial weight (AW) or Ventricular weight (VW) from NTG, TG1, TG2 mice at 6 weeks (6W) or 35 weeks (35W) of age was normalized to body weight (BW). Values are mean SE.

*P<0.005 versus NTG

<table>
<thead>
<tr>
<th></th>
<th>NTG</th>
<th>TG1</th>
<th>TG2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AW/BW</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6W</td>
<td>0.477±0.050 (n=9)</td>
<td>2.105±0.562† (n=7)</td>
<td>0.543±0.039 (n=7)</td>
</tr>
<tr>
<td>35W</td>
<td>0.442±0.045 (n=6)</td>
<td>2.434±0.423† (n=8)</td>
<td>0.428±0.05 (n=7)</td>
</tr>
<tr>
<td><strong>VW/BW</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6W</td>
<td>3.838±0.242 (n=9)</td>
<td>4.421±0.293* (n=7)</td>
<td>3.872±0.149 (n=7)</td>
</tr>
<tr>
<td>35W</td>
<td>3.410±0.373 (n=6)</td>
<td>4.145±0.221* (n=8)</td>
<td>3.611±0.365 (n=7)</td>
</tr>
</tbody>
</table>
Table B.2: Electrophysiological Analysis of Mice

ECG intervals for NTG, TG1, and TG2 at indicated age are tabulated. Values are mean±SE

<table>
<thead>
<tr>
<th>Age</th>
<th>6 weeks</th>
<th>20 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>NTG (n=3)</td>
<td>TG1 (n=6)</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>525±76</td>
<td>466±81</td>
</tr>
<tr>
<td>PR (msec)</td>
<td>34.8±4.2</td>
<td>32.2±3.6</td>
</tr>
<tr>
<td>QT (msec)</td>
<td>64.3±5.6</td>
<td>66.2±6.7</td>
</tr>
<tr>
<td>QTc (msec)</td>
<td>132±17.5</td>
<td>132±19.5</td>
</tr>
</tbody>
</table>

*, Reduced compared to 6-week-old NTG mice (P<0.05).
†, Increased compared to all other groups (P<0.05).
‡, Reduced compared to all other groups (P<0.05).
FIGURES

A

[Diagram of heart sections from rats subjected to coronary artery ligation followed by reperfusion analyzed by in situ hybridization.]

B

<table>
<thead>
<tr>
<th>RT</th>
<th>I/R</th>
<th>ATF3</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure B.1: ATF3 is induced by myocardial ischemia-reperfusion. 
A: Heart sections from rats subjected to coronary artery ligation followed by reperfusion were analyzed by in situ hybridization. B: Cardiomyocytes isolated from newborn rats were incubated with cyanide and deoxyglucose followed by the removal of these metabolic inhibitors. ATF3 and GAPDH mRNA levels were analyzed by RT-PCR. I/R, ischemia-reperfusion; RT, reverse transcriptase.
Figure B.2: MyHC-ATF3 transgenic mice express the transgene in the heart. A: A schematic of the MyHC-ATF3 construct. Open boxes indicate noncoding exons of the α-MyHC gene. HA, hemagglutinin tag. B: Atrial and ventricular total RNAs were isolated from NTG, no. 85 heterozygous (TG1), no. 100 heterozygous (TG2), and no. 100 homozygous (TG2hm) mice. ATF3 and GAPDH mRNA levels were assayed by RT-PCR. C: Indicated tissues from TG1 mice were analyzed as in B. D: Atrial and ventricular sections derived from TG1 mice were analyzed by immunohistochemistry using antibodies against ATF3. A, atrium; V, ventricle. Scale bar, 50 µm.
Figure B.3: TG1 transgenic mice have enlarged atria and dilated right ventricles.
A: Hearts were removed from NTG and TG1 mice at 20 weeks of age and photographed. B: Transverse sections of NTG and TG1 mice at 30 weeks of age were stained by H&E. RL, right ventricle; LV, left ventricle. Scale bar, 1 mm.
Figure B.4: MyHC-ATF3 transgenic mice have increased gene expression indicative of hypertrophy.
Total atrial and ventricular RNAs were isolated from NTG, TG1, and TG2 mice at 15 to 20 weeks of age, and analyzed by dot blot for the indicated mRNA. All assays were performed in duplicate, and individual signal intensities were normalized against GAPDH signals. Relative mRNA levels were calculated by arbitrarily defining the normalized signals from NTG hearts as 1. Data represent mean ± SD from multiple samples (NTG: 4 atria, 6 ventricles; TG1: 4 atria, 4 ventricles; TG2: 5 atria, 8 ventricles). *, P < 0.05 versus NTG.
Figure B.5: Histological analyses indicate myocyte disarray and fibrosis in TG1 hearts.  

A–D: Atrial sections from NTG and TG1 mice at 2 weeks of age (A and B), or 30 weeks of age (C and D) were stained by H&E. E and F: Atrial sections from NTG and TG1 mice at 20 weeks of age were stained by Mason trichrome. Scale bar, 20 µm.
Figure B.6: Ultrastructural analyses indicate myocyte degeneration in TG1 hearts.
A and B: Atria from TG1 and NTG mice at 25 weeks of age were analyzed by electron microscopy [original magnifications: x10,000 (A) and x7000 (B)].
C: A schematic diagram delineates three myocytes shown in A. abZ, abnormal Z-line; ct, connective tissue; dmf, disoriented myofibril; dmt, degenerative mitochondria; did, degenerating intercalated disks; dmy, degenerating myocytes; g, granule; mt, mitochondria; v, vacuole; Z, Z-line.
Figure B.7: Cardiomyocytes derived from MyHC-ATF3 transgenic ventricles have decreased contractile function.

Ventricular cardiomyocytes were isolated from NTG, TG1, or TG2 mice at 15 to 20 weeks of age. Their percentage of cell shortening, lusitropy, and inotropy were measured after stimulation in the electrical field in the absence or presence of isoproterenol. Percent of cell shortening = (L_0 - L_i)/L_0, where L_0 is the length of resting cells and L_i is the length of the electrically stimulated cells. There was no statistically significant difference in the resting cell length between TG and NTG cells: NTG 104 ± 3 µm (n = 26), TG1 101 ± 5 µm (n = 24), TG2 109 ± 4 µm (n = 28). Data represent mean ± SD from 11 to 28 cells derived from five to seven mice. *, P < 0.05 versus NTG; **, P < 0.005 versus NTG; ***, P < 0.0005 versus NTG.
Figure B.8: TG2 transgenic mice have time-dependent cardiac performance deficits.
NTG and TG2 mice were examined by echocardiographic analyses in a longitudinal manner from 6 weeks to 30 weeks of age. Data represent means ± SE for transgenic mice (n = 8 to 12) and NTG mice (n = 10 to 15). Dotted lines represent 95% confidence intervals for age-matched NTG mice that did not show any significant age dependencies. *, P < 0.05 TG versus NTG.