OXIDATIVE STRESS AND XANTHINE OXIDASE IN ACUTE AND CHRONIC CARDIAC VOLUME OVERLOAD IN RATS

by

JAMES D. GLADDEN

LOUIS J. DELL’ITALIA, COMMITTEE CHAIR
MARCAS M. BAMMAN
VICTOR DARLEY-USMAR
JEFFREY A. ENGLER
LORI L. MCMAHON

A DISSERTATION
Submitted to the graduate faculty of The University of Alabama at Birmingham,
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA
2011
OXIDATIVE STRESS AND XANTHINE OXIDASE IN ACUTE AND CHRONIC CARDIAC VOLUME OVERLOAD IN RATS

JAMES D. GLADDEN

CELLULAR AND MOLECULAR PHYSIOLOGY

ABSTRACT

Mechanisms of left ventricular dysfunction in cardiac volume overload (VO) are not well understood and there is no medical therapy. Cardiac VO is marked by eccentric remodeling and contractile dysfunction ultimately resulting in cardiac failure. Oxidative stress is implicated in the pathophysiology of heart failure and recent evidence suggests xanthine oxidase (XO) plays a role in VO.

To study VO, we used a rat model of aortocaval fistula (ACF). ACF results in early diastolic stress on the left ventricle (LV) and recapitulates the progressive nature of heart failure with contractile function being initially maintained and then depressed by 6 weeks. To determine the role of XO in the setting of VO, we utilized an established XO inhibitor, allopurinol, in the ACF animal model. The following questions have been addressed in this dissertation: 1) What are the early events in VO that set forth a cycle of progressive remodeling and dysfunction? 2) Does XO play a role in these events? 3) Is XO a valid therapeutic target in cardiac VO both in the acute and chronic setting? In testing these concepts, we have used a combined in vivo and in vitro approach to determine the cardiac response to VO with end points including; cardiac function, cardiac remodeling, bioenergetics, and cardiac efficiency.

Our data support a causative role for XO in both the acute phase of VO and in the transition to cardiac failure. These findings establish an interplay between XO activation
and bioenergetic dysfunction that may provide a new therapeutic target to prevent progression to heart failure in VO.

Keywords: volume overload, oxidative stress, stretch, mitochondria, allopurinol, heart failure
DEDICATION

The work in this dissertation is dedicated to my family, my wife Bailey, and my son Henry. The inspiration and motivation they provide has given me the ability to face any challenge.
ACKNOWLEDGEMENTS

I acknowledge the many people who have been instrumental in the completion of this work. This work would not be possible without the contributions of Mr. Eddie Bradley, Mrs. Pam Powell, Dr. Mustafa Ahmed, Dr. June Zheng, Dr. Kevin Wei.

I appreciate the effort and guidance from my graduate committee. Drs. Dale Parks, Jeffrey Engler, Lori McMahon, Marcas Bamman, Victor Darley-Usmar have each served a key role in my training. I also thank Dr. Robin Lorenz, Dr. Louis Justement, Dr. Dale Benos, Dr. Kevin Kirk, Dr. Peter Smith, Mrs. Paula Willey, Mrs. Mindy Robbins, the Department of Physiology and Biophysics, and the Medical Scientist Training Program for their support.

Dr. Louis Dell’Italia has guided me through this process and provided both life and career lessons at every turn. I will always carry the critical thinking, ethics, and love for science he has taught me by example during the last four years.
# 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>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Heart Failure</td>
<td>1</td>
</tr>
<tr>
<td>Cardiac Structure and Anatomy</td>
<td>2</td>
</tr>
<tr>
<td>Cardiomyocyte Contraction</td>
<td>4</td>
</tr>
<tr>
<td>LV Function and LV Functional Analysis</td>
<td>7</td>
</tr>
<tr>
<td>Cardiac Function</td>
<td>7</td>
</tr>
<tr>
<td>Assessment of LV Function</td>
<td>9</td>
</tr>
<tr>
<td>Volume Overload versus Pressure Overload Heart Failure</td>
<td>13</td>
</tr>
<tr>
<td>Volume Overload in Valvular Heart Disease</td>
<td>17</td>
</tr>
<tr>
<td>Mitral Regurgitation</td>
<td>18</td>
</tr>
<tr>
<td>Oxidative Stress in VO HF</td>
<td>19</td>
</tr>
<tr>
<td>Sources of Oxidative Stress</td>
<td>23</td>
</tr>
<tr>
<td>NADPH Oxidase</td>
<td>23</td>
</tr>
<tr>
<td>Mitochondrial Sources of ROS</td>
<td>24</td>
</tr>
<tr>
<td>Xanthine Oxidase</td>
<td>26</td>
</tr>
<tr>
<td>Xanthine Oxidase Inhibition</td>
<td>31</td>
</tr>
<tr>
<td>XO Inhibition in Animal Models and Patients with HF</td>
<td>31</td>
</tr>
<tr>
<td>Oxidative Stress and Bioenergetic Dysfunction in VO</td>
<td>33</td>
</tr>
<tr>
<td>Mitochondria, XO, and Oxidative Stress in VO</td>
<td>34</td>
</tr>
<tr>
<td>Mitochondrial Function and Assessment</td>
<td>35</td>
</tr>
<tr>
<td>Hypothesis and Summary</td>
<td>42</td>
</tr>
<tr>
<td>NOVEL INSIGHTS INTO INTERACTIONS BETWEEN</td>
<td></td>
</tr>
<tr>
<td>MITOCHONDRIA AND XANTHINE OXIDASE IN ACUTE CARDIAC</td>
<td></td>
</tr>
<tr>
<td>VOLUME OVERLOAD</td>
<td>44</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHRONIC INHIBITION OF XANTHINE OXIDOREDUCTASE IMPROVES</td>
<td></td>
</tr>
<tr>
<td>LEFT VENTRICULAR FUNCTION AND REMODELING IN THE VOLUME OVERLOADED</td>
<td>84</td>
</tr>
<tr>
<td>RAT HEART</td>
<td></td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>115</td>
</tr>
<tr>
<td>Introduction</td>
<td>115</td>
</tr>
<tr>
<td>Interactions Between Mitochondria, XO, and LV Function in Acute Cardiac</td>
<td></td>
</tr>
<tr>
<td>VO</td>
<td>119</td>
</tr>
<tr>
<td>Cardiac VO</td>
<td></td>
</tr>
<tr>
<td>XO Activation and Distribution in LV Tissue in Acute VO</td>
<td>119</td>
</tr>
<tr>
<td>Role of XO Inhibition on LV Function in Acute VO</td>
<td>122</td>
</tr>
<tr>
<td>Bioenergetic Dysfunction and XO in Acute VO</td>
<td>125</td>
</tr>
<tr>
<td>Protein Nitration in Acute VO</td>
<td></td>
</tr>
<tr>
<td>Mechanical Stretch Induces XO Activity by a Mitochondrial ROS</td>
<td></td>
</tr>
<tr>
<td>Dependent Pathway</td>
<td>127</td>
</tr>
<tr>
<td>Inhibition of XO Improves LV Function and Remodeling in Chronic VO</td>
<td>131</td>
</tr>
<tr>
<td>Effect of Allopurinol on the LV Temporal Response</td>
<td></td>
</tr>
<tr>
<td>to Chronic VO</td>
<td>131</td>
</tr>
<tr>
<td>Allopurinol Improves Contractile Efficiency in Chronic VO</td>
<td>133</td>
</tr>
<tr>
<td>Sarcomeric Protein Expression in Chronic VO</td>
<td>137</td>
</tr>
<tr>
<td>Future Studies</td>
<td>138</td>
</tr>
<tr>
<td>Direct Bioenergetic Measurement in Chronic VO with Allopurin</td>
<td>138</td>
</tr>
<tr>
<td>Determine if MitoQ Therapy is Beneficial in VO</td>
<td>139</td>
</tr>
<tr>
<td>Determine if Ca(^{2+}) Handling Proteins are Post-Translationally</td>
<td></td>
</tr>
<tr>
<td>Modified in VO</td>
<td>140</td>
</tr>
<tr>
<td>Examine the Role of Mitochondria-Sarcoplasmic Reticulum</td>
<td></td>
</tr>
<tr>
<td>Crosstalk in VO</td>
<td>140</td>
</tr>
<tr>
<td>Determine the Point of No Return for XO Inhibition in VO</td>
<td>141</td>
</tr>
<tr>
<td>Conclusions</td>
<td>142</td>
</tr>
<tr>
<td>GENERAL LIST OF REFERENCES</td>
<td>144</td>
</tr>
<tr>
<td>APPENDIX: INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE</td>
<td></td>
</tr>
<tr>
<td>APPROVAL FORM</td>
<td>165</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOVEL INSIGHTS INTO INTERACTIONS BETWEEN MITOCHONDRIA AND XANTHINE OXIDASE IN ACUTE CARDIAC VOLUME OVERLOAD</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Morphometric Data in Sham and ACF rats ..............................................................82</td>
</tr>
<tr>
<td>2</td>
<td>Echocardiographic and Hemodynamic Data in Sham and ACF rats .........................83</td>
</tr>
<tr>
<td>CHRONIC INHIBITION OF XANTHINE OXIDOREDUCTASE IMPROVES LEFT VENTRICULAR FUNCTION AND REMODELING IN THE VOLUME OVERLOADED RAT HEART</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>LV Temporal Response to Chronic ACF .................................................................113</td>
</tr>
<tr>
<td>2</td>
<td>LV Hemodynamic and Functional Parameters in Chronic 8 Week ACF .................114</td>
</tr>
<tr>
<td>3</td>
<td>Morphometric Data on 8 Weeks of ACF .................................................................114</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cardiac Structure</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Cardiomyocyte Ca(^{2+}) Handling</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>LV Echocardiography</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>LV Pressure Volume Analysis</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>Ventricular Remodeling in Pressure and Volume Overload</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>Creation of the Aortocaval Fistula</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>Oxidative Stress in Cardiac Volume Overload</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>Xanthine Oxidase Expression in Normal and VO Patients</td>
<td>22</td>
</tr>
<tr>
<td>9</td>
<td>MitoQ Structure and Function</td>
<td>27</td>
</tr>
<tr>
<td>10</td>
<td>Structure of XOR</td>
<td>29</td>
</tr>
<tr>
<td>11</td>
<td>XOR and the Purine Degradation Pathway</td>
<td>30</td>
</tr>
<tr>
<td>12</td>
<td>Mitochondrial Oxidative Phosphorylation</td>
<td>37</td>
</tr>
<tr>
<td>13</td>
<td>Measurement of Isolated Mitochondrial Function</td>
<td>40</td>
</tr>
<tr>
<td>14</td>
<td>Bioenergetic Measurements in the Seahorse Extracellular Flux Analyzer</td>
<td>41</td>
</tr>
</tbody>
</table>

NOVEL INSIGHTS INTO INTERACTIONS BETWEEN MITOCHONDRIA AND XANTHINE OXIDASE IN ACUTE CARDIAC VOLUME OVERLOAD

1 Allopurinol treatment in ACF and its effects on diastolic cardiac function ....... 73

ix
**LIST OF FIGURES (continued)**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>LV End-systolic pressure-volume relationship in ACF and the effects of allopurinol ................................................................. 74</td>
</tr>
<tr>
<td>3</td>
<td>Distribution of XO/XDH in cardiomyocytes in sham and 24 hr ACF left ventricles ........................................................................................................... 75</td>
</tr>
<tr>
<td>4</td>
<td>XO activity is predominantly increased in cardiomyocytes in 24 hr ACF .......... 76</td>
</tr>
<tr>
<td>5</td>
<td>Protein levels of NADPH oxidase, eNOS, iNOS, and protein nitration in LV myocardium ........................................................................................................ 77</td>
</tr>
<tr>
<td>6</td>
<td>Respiration of isolated subsarcolemmal mitochondria (SSM) in 25 hour ACF and the effects of allopurinol ................................................................. 78</td>
</tr>
<tr>
<td>7</td>
<td>Stretch induces an increase in XO activity by a mitochondrial-derived ROS dependent pathway in adult rat cardiomyocytes ........................................ 79</td>
</tr>
<tr>
<td>8</td>
<td>Mechanical stretch is associated with myofibrillar structural abnormalities and mitochondrial swelling in isolated adult rat cardiomyocytes ................. 80</td>
</tr>
<tr>
<td>9</td>
<td>Increased xanthine oxidase activity leads to mitochondrial dysfunction in the VO heart ........................................................................................................ 81</td>
</tr>
</tbody>
</table>

**CHRONIC INHIBITION OF XANTHINE OXIDOREDUCTASE IMPROVES LEFT VENTRICULAR FUNCTION AND REMODELING IN THE VOLUME OVERLOADED RAT HEART**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Allopurinol improves cardiac remodeling in response to chronic volume overload ........................................................................................................ 108</td>
</tr>
<tr>
<td>2</td>
<td>Allopurinol improves LV systolic function in response to chronic volume overload of 8 weeks of ACF .......................................................................................... 109</td>
</tr>
<tr>
<td>3</td>
<td>Allopurinol increases contractile efficiency in chronic volume overload .......... 110</td>
</tr>
<tr>
<td>4</td>
<td>Maximum oxygen consumption and Emax in response to chronic volume overload ................................................................................................................. 111</td>
</tr>
<tr>
<td>5</td>
<td>Ca$^{2+}$ regulation proteins in chronic volume overload ................................................................................................................................. 112</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>XO as a Central Factor in VO</td>
<td>118</td>
</tr>
<tr>
<td>2</td>
<td>Bioenergetic and Diastolic Function</td>
<td>124</td>
</tr>
<tr>
<td>3</td>
<td>Link between VO, XO, and the Mitochondria</td>
<td>126</td>
</tr>
<tr>
<td>4</td>
<td>XO Distribution is Localized to Areas of Damage in Response to Stretch</td>
<td>129</td>
</tr>
<tr>
<td>5</td>
<td>Cardiac Contractile Efficiency in Chronic VO</td>
<td>135</td>
</tr>
</tbody>
</table>
INTRODUCTION

Heart Failure

Cardiovascular disease (CVD) leads all other categories in terms of morbidity and mortality. Currently over 60 million people in the US suffer with at least one type of CVD\(^1\). CVD has been the number one cause of death every year in the United States every year since the 1920’s\(^1\). This unrelenting cause of mortality does not hold regard for race or gender and statistics for men and women are both equally grim. Estimates for the costs related to CVD in 2011 are over $360 billion dollars\(^1\).

There is a wide range of causes of heart failure (HF) and the natural history of HF can vary. However, most major cardiac insults end in HF. Currently, 5 million people suffer from HF and 600,000 new cases develop each year. Approximately 1 out of every 2 HF patients will die within 5 years of onset\(^1\). Therefore, HF represents a major public health concern.

The study of comprises several major research areas and is the focus of this dissertation. Understanding the cause and initiation of the pathological cardiac changes that set a course to development of failure are important for directing therapies towards a preventative approach. Next, understanding how the heart compensates for the increased work/stress load it faces during failure will provide insight into the deleterious changes that eventually overwhelm the heart. Finally, knowledge of the events that are involved
in the transition from compensated to overt end-stage failure may provide treatments aimed at preventing/delaying this transition thereby improving patient outcomes.

Cardiac Structure and Anatomy

The heart is a muscular pump responsible for maintaining blood flow to transport oxygen, carbon dioxide, nutrients, waste products, and other important biological substances to and from all tissues and organs of the body. The mammalian heart (Figure 1) is divided anatomically into 4 chambers (2 ventricles and 2 atria) and functionally into 2 circuits (right side-pulmonary, left side-systemic). The right and left atria and ventricle are separated by the interatrial and interventricular septum, respectively. The direction of blood flow in the heart is governed by a set of one-way valves. The tricuspid valve (right side) and mitral valve (left side) separate the atria and ventricle. These valves allow blood to move from atria to ventricle during diastole and are closed during systole. During ventricular contraction or systole, the pulmonic valve (right side) and aortic valve (left side) allow blood to flow to the lungs and systemic circulation, respectively. To accomplish the goal of moving blood, the heart must generate pressure by contracting to cause forward fluid movement and must relax to allow the chambers to refill for the next cycle. This rhythmic and cyclical beating is governed primarily by an intricate set of nervous and conduction tissue purposely distributed throughout the heart.

The functional contractile unit of the heart is the cardiomyocyte. Depending on the species, cardiomyocytes range from 50-150um in length and 10-20um in diameter.\(^2\) They are joined to each other by intercalated discs at each end and can attach to more than one neighbor. The cardiomyocyte utilizes energy to generate a linear
Figure 1. Cardiac Structure. The heart is organized into 4 major chambers (2 atria and 2 ventricles). The left side of the heart provides blood flow to the systemic circulation and the right side delivers to the pulmonary circulation. The direction of blood flow during each cardiac cycle is governed by a set of one-way valves. The ventricles are separated by the intraventricular septum and the atria by the interatrial septum. Blood flows from the atria to the ventricle during relaxation or diastole (directional arrows). During contraction the left ventricle ejects blood across the aortic valve into the aorta. The right ventricle ejects across the pulmonic valve into the pulmonary artery and then to the lungs. This anatomical arrangement forms a closed circuit of blood flow that, under normal conditions, delivers oxygen and nutrients to tissues and then removes their waste for excretion.
force by physically shortening itself. These cells are highly metabolic and while only accounting for one third of the total cell number in the heart they account for two thirds of its total mass. The cardiomyocyte contains proteins which comprise the structural apparatus for contraction, including actin and myosin, and an large amount of mitochondria for energy production. While the individual cardiomyocyte only contracts in a linear fashion, their three dimensional arrangement into twisted sheets allow for the electrically coordinated heart beat to produce a wringing motion resulting in blood ejection.\(^2\)

The cardiomyocyte requires an large amount of energy to generate the forces required to produce blood flow. This energy is primarily provided by the mitochondrial network which produces over 90% of cellular ATP.\(^3\) The mitochondria consists of roughly 30% of the volume of the myocardium and this value varies only slightly from mouse to man.\(^4\) In the cardiomyocyte two populations of mitochondria are present and are distinguished by their location and cristae morphology.\(^5, 6\) The intermyofibrillar mitochondria (IFM) reside between the myofibrils and exhibit tubular cristae architecture.\(^3\) The subsarcolemmal mitochondria (SSM) lie beneath the sarcomeres and their cristae are mostly lamelliform.\(^7\) The mitochondria are located in high density around areas which consume large amounts of energy, namely the sarcomeric proteins responsible for cardiomyocyte contraction.

**Cardiomyocyte Contraction**

Initiation of cardiomyocyte contraction depends on intracellular Ca\(^{2+}\) and is highly regulated (Figure 2). Cardiomyocyte membrane depolarization causes the opening
of the L-type Ca\(^{2+}\) channel and entry of a small amount of Ca\(^{2+}\). The ryanodine receptor (RyR) lies in close proximity to the L-type Ca\(^{2+}\) channel and is a Ca\(^{2+}\) release channel sensitive to Ca\(^{2+}\). Once open, the sarcoplasmic reticulum (SR) bound RyR releases large amounts of Ca\(^{2+}\) which drastically increases the intracellular Ca\(^{2+}\) concentration causing cardiomyocyte contraction. The majority of intracellular Ca\(^{2+}\) that causes contraction is released from the SR and is taken back up by a specific pump, sarcoplasmic reticulum Ca\(^{2+}\) -ATPase (SERCA2), during diastole. Recently, the modulation of SERCA2 activity has become important field of study in heart failure. Decreased SERCA2 activity is generally considered pathologic and has been implicated in dilated cardiomyopathy and congestive heart failure. Aberrant Ca\(^{2+}\) handling from SERCA2 leads to decreased reuptake during diastole and subsequent decreased release in systole which negatively impacts contractility. Importantly, SERCA2 Ca\(^{2+}\) reuptake is negatively regulated by phospholamban (PLN) and sarcolipin (SLN) by decreasing its affinity for Ca\(^{2+}\). PLN has been extensively studied and overexpression of PLN is associated with decreased contractile performance in vivo. SLN is the more recently discovered regulatory protein for SERCA2 activity. Emerging evidence suggests Ca\(^{2+}\) handling may be a new target in the setting of advanced HF and work in dissertation suggests VO may cause alterations in sarcomeric Ca\(^{2+}\) proteins.
Figure 2. Cardiomyocyte Ca\textsuperscript{2+} Handling. Upon depolarization modest amounts of Ca\textsuperscript{2+} enters through the L-Type Ca\textsuperscript{2+} channel. This causes a Ca\textsuperscript{2+}-induced-Ca\textsuperscript{2+}-release from the ryanodine (RyR) channel. The cytoplasmic Ca\textsuperscript{2+} concentration is greatly increased and causes myofilament contraction. During relaxation Ca\textsuperscript{2+} is removed from the cytoplasm primarily by sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2). SERCA2’s affinity for Ca\textsuperscript{2+} and therefore ability to reuptake Ca\textsuperscript{2+} is negatively modified by both phospholamban (PLN) and sarcolipin (SLN).
LV Function and LV Functional Analysis

Cardiac Function

The cardiac cycle can be conveniently divided into four phases. First, during the filling phase, the heart relaxes and fills with blood during what is termed diastole. Diastolic relaxation is an energy dependent process that utilizes energy or ATP to break actin-myosin crossbridges. The load the heart experiences during this phase is termed preload. Conventional determination of preload is done at the end of diastolic relaxation and is reflected by the LV volume (LV end-diastolic volume) and pressure (LV end-diastolic pressure). Major determinates of preload include total blood volume, systemic venous return, physiological structural abnormalities, and intrinsic ventricular properties such as compliance. Second, during isovolumic contraction, the heart begins to contract in response to a coordinated electrical stimulation. In the case of left side circulation, pressure builds during this phase until the aortic valve opens due to the pressure gradient. Third, during the ejection phase, blood is ejected until the ventricular pressure is no longer sufficient to keep the aortic or pulmonic valve open. The pressure that the heart is working against during this phase is termed afterload and is reflected by the mean arterial pressure (MAP). Afterload is primarily affected by the arterial resistance in the systemic circulation which is determined at the level of the arteriole. Finally, during the isovolumic relaxation phase, the heart relaxes until the ventricular pressure falls below that in the atria and the mitral and tricuspid valve open to allow filling during diastole.
Changes in either afterload or preload can affect the function of the heart. In the case of increased preload, increased stretching forces are directed into the myocardial wall.\textsuperscript{25, 26} The elastic properties of the ventricular wall cause a reactionary increase in force generation which is evidenced by a greater amount of ejected blood or stroke volume (SV).\textsuperscript{27} This relationship between preload and cardiac function is termed the Frank-Starling relationship.\textsuperscript{28} This phenomenon importantly allows an intrinsic mechanical beat-by-beat regulation of the cardiac cycle to accommodate volumetric changes without changing the heart rate. This relationship can be observed in the dimensions of the ventricular chamber at end systole.\textsuperscript{29} As preload increases, the dimension of the ventricle at end-diastole increases. However, due to the Frank-Starling relationship the ventricle can contract down to the same dimension at the end of systole thereby increasing SV. This is also reflected by the total amount of blood the heart pumps per minute which is termed cardiac output (CO) and is determined by the equation $\text{CO}=\text{SV} \times \text{heart rate}$. While the SV can change in a compensatory direction in response to preload changes, this relationship cannot increase indefinitely. Indeed, the Frank-Starling relationship can reach a plateau or even decline in the setting of excessive stretch. Conversely, increases in afterload typically cause a decrease in CO. This is due to increased amount of work which is required to eject a similar volume against a higher pressure gradient. While increased afterload is associated with higher developed ventricular pressures, it is also associated with decreased velocity of contraction and therefore SV is decreased.

While the Frank-Starling mechanism regulates beat-by-beat changes in cardiac loads other long-term global mechanism are present to govern blood flow to tissues.
Baroreceptors and chemoreceptors in the carotid arteries and in the aortic arch can detect changes in MAP in the resolution of seconds. MAP is determined by \[\text{MAP} = \text{CO} \times \text{total peripheral resistance (TPR)}\]. In response to changes in MAP, the parasympathetic/sympathetic nervous system react accordingly to alter the three major compensatory mechanisms: 1) cardiac contractility, 2) heart rate, 3) vascular tone. These mechanisms work to change CO and TPR to restore a normal MAP. Long-term maintenance of CO and MAP are regulated by many neurohormonal pathways such as the renin-angiotensin system.

**Assessment of LV Function**

It is possible to interrogate the ventricle for its functional state using a number of technical modalities. Echocardiography utilizes ultrasound waves which are directed towards the heart at high frequency and then the reflection of the wave is collected by a probe. This digital reflection signal can then be reconstructed into an image which displays the structural properties of the heart in real-time. This technique allows for measurements of important ventricular parameters including end-systolic/diastolic dimension and volume, ejection fraction (EF), and velocity of contraction (VCFr). Importantly, if a geometrical shape of the LV chamber is assumed then the LV volume can be computed. Much work has been invested into good estimates of LV volumes based on the dimension and the equation utilized throughout this work is the Teicholz formula.\(^{30,31}\)

\[
LV Volume = \left[ \frac{7}{2.4 + LVID} \right] \times (LVID)^3
\]

Where \(LVID\) = LV internal dimension at any time point.
Echocardiography probes can be set to two major acquisition modalities (Figure 3). B-mode allows scanning of a 2-D slice of the heart in which an entire cross section from the base to the apex can be visualized. M-mode focuses the probe in a single position and allows high frequency observation of the two heart walls. The placement of the probe influences the measurements and is typically placed in reference to the papillary muscles. M-mode allows for real-time high-frequency (1000 Hz) data collection of LV chamber dimension, LV wall thickness, and with application of the Teichholz formula, LV volumes at all points throughout the cardiac cycle. The introduction of a pressure catheter into the LV allows for the measurement of LV pressure during the cardiac cycle. Recently, advances in experimental technologies have allowed for the simultaneous collection of LV pressure and echocardiography data which can be combined to generate a pressure-volume (PV) loop. Importantly, many LV functional parameters have been validated using the collection of PV-loops at hemodynamic baseline and at altered cardiac preload/afterload. Since these measurements are taken at various loads, it is thought that they are less influenced by the load at which an individual measurement is taken making them more “load independent”. Therefore, these PV-loop derived parameters provide an experimental route to study the health of the ventricle in the setting of altered preloads (Figure 4).
Figure 3. LV Echocardiography. Echocardiography is a useful tool to non-invasively measure cardiac function in humans and rodents. A long axis view of the LV at end systole and end diastole is presented (upper two panels). The dimension of the ventricle is marked in each upper panel (white arrows). The lower panel demonstrates m-mode echocardiography in which a single slice of the LV is imaged at a high frequency, allowing a higher resolution. The LV endocardial wall is traced and allows for real-time calculations of ventricular function.
Figure 4. LV Pressure Volume Analysis. Simultaneous LV pressure catheterization and echocardiography can be combined to generate a LV pressure volume loop (upper left). This plot demonstrates the shape of a normal pressure volume loop with end-diastole and end-systole labeled. Time is not represented by an axis but progresses counter-clock wise within the loop. Venous return to the heart can be stopped by an inferior vena caval occlusion and a family of pressure volume loops can be generated in the same heart (upper right). Time progresses from loop to loop in the right to left direction. A line fitted to each end systolic point defines the end-systolic pressure volume relationship (ESPVR). An exponential line is fitted to the end diastolic points to define the end-diastolic pressure volume relationship (EDPVR). The area of the pressure volume loop (lower panel) defines LV stroke work. The area bounded by the ESPVR, EDPVR and the pressure volume loop defines LV potential energy.
Volume Overload versus Pressure Overload Heart Failure

HF is defined as the “inability of the heart to pump blood … at a sufficient rate to meet the metabolic demands of the body”.¹ HF is a progressive disease resulting from an initial insult that may be gradual or rapid in onset and may be caused by genetic or environmental factors. The initial insult that causes HF can typically be categorized as either a pressure overload (PO) or volume overload (VO). Clinical examples of VO include aortic valve regurgitation (AR); mitral valve regurgitation (MR); and occasionally arteriovenous shunts of a traumatic or therapeutic nature. Clinical examples of PO are typically aortic valve stenosis or hypertension. The heart responds to PO and VO by cardiac hypertrophy (Figure 5), albeit in completely different manners.⁴¹, ⁴² VO causes an eccentric hypertrophy with a large increase in ventricular chamber diameter compared to changes in ventricular wall thickness (wt).⁴³ In contrast, PO results in a concentric hypertrophy with large increase in wt compared to chamber diameter. Further, these varying responses are associated with a unique set of gene expression profiles.⁴⁴-⁴⁶ It is thought that changes increased wall stress, which is increased at end-systole in PO versus at end-dia­stole in VO, drive the pathological changes underlining each disease. However, while this division of HF into PO and VO is convenient conceptually, it is rare for these insults to occur in isolation and the natural history for either case is development of a dilated failing heart.⁴⁷-⁵¹

The study of HF experimentally is commonly divided into three phases: 1) the initial acute phase immediately following the insult; 2) the stable or compensated phase; and 3) failure or decompensated phase.¹ Models of PO in animals are achieved by a mechanical banding of the aortic arch or systemic afterload alterations such as salt loading.⁵²-⁵⁴ VO can be modeled effectively in large animals by surgical interruption of
the mitral or aortic valves and in large or small animals utilizing an aortocaval fistula (ACF) (Figure 6). The ACF in rats is a well characterized and validated animal model of VO exhibiting all three phases of cardiac failure. ACF results in a biventricular VO with high out, no retrograde flow, and normal to decreased afterload. ACF models the increased stress on the heart from high-flow ateriovenous shunts used for hemodialysis which has been shown to contribute to LV chamber dilation in HF patients. ACF also models the ventricular stresses associated with HF resulting from valvular disease, patent ductus arteriosus, hyperthyroidism, and pregnancy. ACF generates progressive ventricular hypertrophy and dilation. Initial studies demonstrated an acute increase in LV ventricular end-diastolic pressure (LVEDP) in response to ACF. This was concurrent with an acute decrease in systolic function and 90% of the animals had died of HF by 21 wks. Other studies demonstrated an early decrease in MAP that occurred as a result of total peripheral resistance despite an increase in CO. These animals also demonstrate changes seen in VO patients including; respiratory distress, peripheral edema, and lethargy. Recently, we demonstrated the acute phase (24 hr) of ACF is associated with increased levels of oxidative stress concurrent with depressed bioenergetic function. Further, studies of gene expression in these animals revealed marked downregulation of genes related to bioenergetic function during the transition from the compensated phase (5 wk) to the decompensated phase (15 wk). Interestingly, a reversible model of ACF, closure of the shunt after 4wk demonstrated only a partial reversion of hypertrophy and a complete recovery of hemodynamic performance. This finding suggests that early pathological changes in these animals persist after the insult is removed and that therapies could be directed at protecting the myocardium and preventing early damage.
Figure 5. Ventricular Remodeling in Pressure and Volume Overload. Gross sections of right and left ventricles are displayed. This short axis section demonstrates differential LV hypertrophy in response to pressure overload (top panel) and volume overload (bottom panel). Pressure overload results in a concentric hypertrophy pattern with decreased chamber diameter and LV wall thickening. Volume overload displays an eccentric remodeling pattern with LV wall thinning in relation to LV chamber dilation.
Figure 6. Creation of the Aortocaval Fistula. Volume overload is induced in the rat by creation of an aortocaval fistula (ACF). A hypodermic needle is inserted in the descending aorta and passed over to the adjacent inferior vena cava (IVC). This creates a fistula between the two vessels. The needle is removed and the hole is covered with cyanoacrylate glue to prevent leakage. A portion of the arterial blood is shunted to the IVC where it mixes with venous blood. This process increases the venous return to both the right and left ventricles and effectively models cardiac volume overload.
Volume Overload in Valvular Heart Disease

Valvular heart diseases are highly prevalent and have been increasingly recognized as an important public health problem.\textsuperscript{64} HF due to VO is largely valvular in origin. The two valves that cause VO of the LV are the aortic valve and the mitral valve or a combination thereof. In both cases, an incompetency of the valve to completely close leads to a regurgitation of blood volume during the phase of the cardiac cycle at which it should be closed. This effectively shifts blood volume from the systemic or pulmonary circuits into the LV.

Aortic regurgitation (AR) results from an incomplete closure of the aortic valve during diastole.\textsuperscript{65} This can be caused by any pathology that either damages the valve or geometrically disallows the valve to close. AR is considered a normal pressure form of VO due to the unchanged afterload.\textsuperscript{66} There is an increased volume of blood that the heart must eject to maintain forward stroke volume (normal forward stroke volume + the regurgitated volume). This increased total ejection volume has to be delivered to the high pressure aorta. The underlying pathology and the resulting forces on the valve can cause the valve cusps to thicken, thin, or become calcified. AR causes the heart to hypertrophy with replication of sarcomeres in series and fiber elongation.\textsuperscript{43} This is largely thought to be due to diastolic wall stress being increased largely due to an elevated diastolic pressure and chamber enlargement.\textsuperscript{67} Histological evaluation demonstrates degeneration of the cardiomyocyte with focal myofibrillar lysis in association with areas of fibrosis. The changes in the ultrastructure of the cardiomyocyte are thought to underlie the contractile dysfunction these patients exhibit. Importantly, as the valve and its supporting structure
change over time the regurgitant volume can increase, thus perpetuating the cycle of VO and valvular/cardiac remodeling.

**Mitral Regurgitation**

Moderate or severe MR is the most frequent form of valvular disease in the US. An estimated 2–2.5 million people were affected in the year 2000, a number expected to double by 2030 due to population growth and aging.\(^{48, 68}\) Isolated MR from myxomatous degeneration of the mitral valve represents a low pressure VO because excess volume is ejected into the low pressure left atrium. This is in contrast to AR, where excess volume is ejected into the high pressure aorta. Thus, the decrease in forward cardiac output in MR is compensated by an increase in LV stroke volume that is mediate by augmentation of LV preload (end-diastolic volume) and an increase in adrenergic drive. These compensatory mechanisms preserve LV ejection fraction (EF), even in the face of increasing LV end-systolic dimension/volume and LV end-systolic wall stress over time. Because of the favorable effects of increased preload and sympathetic drive on LV shortening, the cut-off for surgical intervention is an LV EF of 60% and/or an LV end-systolic dimension > 40 mm.\(^{69, 70}\) However, even when LV EF is > 60% prior to surgery, LV EF can decrease after surgery, causing increased morbidity and mortality.\(^{48, 71}\)

The mechanisms involved in the transition to irreversible cardiomyocyte damage in chronic isolated MR remain elusive. This is compounded by the fact that symptoms of shortness of breath may be very subtle and that LV function and geometry may change in the absence of symptoms. Thus, the success of adherence to guidelines requires very close surveillance, which has most likely contributed to the recent reports that patients
with isolated MR are not receiving timely surgery, even with advances of surgical repair and minimally invasive surgery.\textsuperscript{72} It is also of interest that waiting > 2 months after development of symptoms or achievement of accepted guidelines (LV EF<60\% or LV end-systolic dimension > 40 mm) results in a worse outcome, thereby demonstrating the narrow time window for development of irreversible cardiomyocyte damage.\textsuperscript{73} All of these concerns have resulted in a controversy regarding recommendation for early surgery in asymptomatic MR patients.\textsuperscript{49,74-76}

Medication to prolong the time to surgery is also lacking. The low pressure volume overload of isolated MR is associated with a loss of collagen,\textsuperscript{44,77} a decrease in protein synthesis,\textsuperscript{78} and an eccentric form of LV remodeling with LV wall thinning (Figure 1).\textsuperscript{44,77} These myocardial changes are not improved by the antifibrotic and antihypertrophic effects of renin-angiotensin system (RAS) blockade, which explains the failure of RAS blockade or vasodilators in attenuating the LV remodeling in this condition.\textsuperscript{69,70} β\textsubscript{1}-receptor blockade has met with success in improving LV and cardiomyocyte function in dogs with experimentally-induced MR,\textsuperscript{79-81} but does not attenuate LV dilatation and has not been studied in a randomized clinical trial. Thus, a better understanding of the biochemical and molecular changes in this unique hemodynamic stress is necessary to identify new drug targets that attenuate LV dilatation and improve LV function.

**Oxidative Stress in VO HF**

Over the past 20 years evidence has been steadily increasing for a role of oxidative stress in the pathophysiology of HF.\textsuperscript{82} Evidence for oxidative stress in HF
patients includes biomarkers which are increased in both the systemic circulation and pericardial space. The pathological effects of oxidative stress are considered to come from reactive oxygen and nitrogen species (ROS/RNS) dependent damage to proteins, DNA, lipids, and recently alterations of intracellular signaling pathways (Figure 7). ROS are characterized by their high reactivity and include free radical and non-free radical species. Free radical molecules have ≥1 unpaired electrons such as, superoxide (O$_2^-$) and hydroxyl (OH$^-$). Non-free radical ROS such has hydrogen peroxide (H$_2$O$_2$) are species that are capable of generating free radicals without containing unpaired electrons. ROS occur in many natural metabolic pathways but become pathological when they overwhelm the innate antioxidant systems that scavenge and degrade ROS. A collection of superoxide dismutase enzymes convert O$_2^-$ to H$_2$O$_2$ which is broken down by glutathione peroxidase and catalase to H$_2$O. Nonenzymatic antioxidants such as vitamins E, C, beta carotene, ubiquinone, lipotic acid and urate also contribute to removal of ROS. The balance between ROS and their breakdown defines the redox state of the cell and an imbalance with excess ROS defines “oxidative stress”. A multitude of studies have demonstrated even early in MR there is evidence of myocardial dysfunction. Several studies have reported decreased force generation of LV muscle strips, derangement of LV calcium handling proteins, and increased LV cytokines in patients with MR despite LV EF > 60%—all of which can be associated with and/or attributed to increased oxidative stress. We have recently reported increased xanthine oxidase (XO) (Figure 8) and other markers of oxidative stress in patients with VO of MR at the time of mitral valve repair. There is marked deposition of lipofuscin, a
Figure 7. Oxidative Stress in Cardiac Volume Overload. The increased hemodynamic stress in volume overload causes oxidative stress which can alter cell signaling and damage proteins and DNA. These pathological changes contribute to cardiac dysfunction in volume overload.
Figure 8. Xanthine Oxidase Expression in Normal and VO Patients. LV biopsies were taken at time of reparative surgery in patients with chronic VO of MR. Normal tissues were acquired from autopsy samples from patients without cardiac pathology. Samples were fixed and paraffin embedded. Staining with a XO antibody demonstrated XO (green) in a striated pattern with Z-bands in normal human cardiomyocytes (A). XO in cardiomyocytes from isolated MR showing a punctuate perinuclear (blue DAPI staining) (lower white arrow) distribution and associated with myofibrillar degeneration demonstrated by open spaces (upper right arrow) (B).
non-degradable material primarily composed of oxidatively modified protein and lipid degradation residues. Lipofuscin accumulation is usually seen in the senile heart and is considered to be an end product of excessive oxidative stress that overwhelms protective mechanisms. Lipofuscin accumulation has been shown to have deleterious effects on cellular function including triggering of mitochondrial pro-apoptotic pathways in cardiomyocytes and fibroblasts and its accumulation in the heart is irreversible. In addition, staining of nitrotyrosine—a marker of oxidative and nitrative damage—is increased in areas of lipofuscin accumulation in the MR hearts. Along with increased oxidative stress, there was extensive evidence of myofibrillar degeneration, which has also been identified in the rat and dog with isolated VO. Finally, there was LV systolic dysfunction six-month post-mitral valve repair manifested by decreased LV EF and LV strain rates. These results suggest a strong association between oxidative stress and underlying cardiomyocyte abnormalities as a potential cause of LV dysfunction in a clinical setting of cardiac VO.

Sources of Oxidative Stress

**NADPH Oxidase**

NADPH oxidases are enzymes that consist of a catalytic NOX subunit and 4 cytosolic regulatory subunits, p40phox, p47phox, p67phox and the membrane bound p22phox subunit, as well as, Rac GTP-binding protein. The NADPH oxidases exist as 5 Nox isoforms numbered 1 through 5 and their expression varies in each tissue. Nox2 and Nox4 are the primary isoforms expressed in the heart. NADPH oxidase generates superoxide when it transfers electrons from NADPH across the cellular membrane and
react with oxygen. NADPH oxidase activity is increased in both PO\textsuperscript{98} and HF.\textsuperscript{99, 100} Importantly, NADPH oxidases have been shown to activate XO and induce nitric oxide synthase (NOS) uncoupling.\textsuperscript{101} These studies suggest along with a direct contribution to ROS, NADPH oxidase can modulate or amplify ROS generation from other sources.

\textit{Mitochondrial Sources of ROS}

The mitochondria produce ROS as a byproduct of normal metabolic processes.\textsuperscript{102} Electrons are produced or leaked from the electron transport chain at Complexes I, III, IV and can combine with other species to produce ROS.\textsuperscript{103-105} Mitochondria are both a source and target for ROS suggesting a complex interaction with other ROS producing enzymes and possibilities for feed-forward cycles of damage. In addition, HF is associated with pathological changes in mitochondrial bioenergetics.\textsuperscript{106-109} In disease, ROS generated from mitochondria can damage or modify proteins, lipids, and DNA leading to exacerbation of the pathology. During oxidative phosphorylation, Complex IV transfers electrons to O\textsubscript{2} and H\textsubscript{2}O is produced. However, a portion of O\textsubscript{2} in the mitochondria is only partially reduced yielding superoxide thereby contributing to ROS.

Many sites in the mitochondria can generate or contribute to ROS production. It is generally thought that complexes I and III play the biggest role.\textsuperscript{110} Complex I (NADH-ubiquinone oxidoreductase) is a large multi-subunit protein bound to the inner membrane and is exposed to both the matrix and intermembrane space. It accepts electrons from NADH and transfers them to the coenzyme Q reduction site.\textsuperscript{111, 112} These electrons are then transferred to ubiquinone forming ubiquinol. However, Complex I may also transfer electrons to molecular oxygen creating superoxide. The precise mechanisms that govern
when Complex I may contribute to superoxide production are not fully known. However, studies have implicated the NADH:NAD+ ratio, mitochondrial membrane potential, and pH differences across the inner mitochondrial membrane in associated with ROS produced at Complex I.\textsuperscript{102} ROS generation at Complex III (ubiquinone-cytochrome c oxidase) has also been extensively studied. Complex III oxides ubiquinol in the inner membrane space and transfers electrons reducing cytochrome c. As in the case of Complex I, Complex III can aberrantly transfer electrons to molecular oxygen creating superoxide.\textsuperscript{113, 114} However, the amount of ROS generated by Complex III is thought to be much less than Complex I. Many studies involving ROS generation at Complex III are faulted by use of non-physiological concentrations of succinate falsely elevating ROS generation through reverse electron transport at Complex I.\textsuperscript{115, 116} In addition to Complex I and III, significant ROS production has been attributed to the tricarboxylic acid cycle (TCA) enzyme alpha-ketoglutarate and monoamine oxidases in the outer mitochondrial membrane.\textsuperscript{102}

Superoxide and hydrogen peroxide can negatively impact cytoskeletal structure and function, as well as multiple metabolic processes in the cardiomyocyte, either independently or after reaction with nitric oxide.\textsuperscript{117, 118} In addition to cardiomyocyte myofilaments, one of the key targets for the actions of ROS/RNS in the cell is the mitochondrion, because of its high concentration of reactive proteins and lipids that are in close proximity to multiple enzymes containing redox active iron and copper.\textsuperscript{119, 120}

The study of mitochondrial ROS contribution to pathology is a vibrant field and several specific inhibitors have been studied. One of the most studied mitochondrially targeted antioxidant is 10-(6'-ubiquinonyl)-decyltriphenylphosphonium (MitoQ).\textsuperscript{121-124}
MitoQ is a derivative of ubiquinol which is conjugated to a lipophilic triphenylphosphonium (TTP) (Figure 9). TTP is a cation that is attracted to the high membrane potential of the mitochondria resulting in a 100-1000 fold accumulation. Ubiquinone in vivo is primarily reduced and ubiquinol is acts as both an antioxidant and electron carrier. In its quinol form, MitoQ is absorbed to the inner mitochondrial membrane and acts an antioxidant being oxidized to the quinone form. Importantly, it is continually recycled to the active quinol antioxidant form by Complex II. Further, MitoQ has been show to be rapidly taken up by cells in culture driven by the plasma membrane potential which is followed by accumulation of MitoQ in the mitochondria.

A portion of work in this dissertation focuses on the role of mitochondrial ROS in activation of other oxidative enzymes. To study mitochondrial ROS in the cardiomyocyte, MitoQ was used in vitro in isolated adult cardiomyocytes under the stressor of mechanical stretch.

**Xanthine Oxidase**

Myofibrillar degeneration can occur as a result of increased intracellular oxidative stress and the finding of increased cardiomyocyte XO in the MR hearts is of particular interest. Xanthine oxidoreductase (XOR), when transformed from its parent enzyme xanthine dehydrogenase (XDH) into its oxidase form XO, generates superoxide and hydrogen peroxide upon conversion of xanthine to hypoxanthine and hypoxanthine to uric acid. XOR exists as XDH and XO, both of which metabolize purines to form uric acid. XOR is a molybdopterin-containing flavoprotein and is comprised of two identical 145kDa subunits. The crystal structure of both XDH and XO have been
Figure 9. MitoQ Structure and Function. MitoQ has an antioxidant quinone moiety that is linked to a lipophilic triphenylphosphonium (TTP) that targets it to the mitochondria. MitoQ rapidly equilibrates across the plasma membrane and accumulates in the mitochondria. Once the quinol form of MitoQ acts as an antioxidant, it is recycled to the quinone form by Complex II in the mitochondria. These properties allow MitoQ to effectively serve as a mitochondrially targeted antioxidant.
extensively studied and recently determined.\textsuperscript{137-142} Each 145k kDa subunit is comprised of an N-terminal 20 kDa iron containing domain, a 40 kDa FAD domain, and a C-terminal 85 kDa molybdopterin redox center (Figure 10).\textsuperscript{137}

In its oxidase form, XOR produces superoxide and hydrogen peroxide when oxygen is used as an electron acceptor during purine metabolism. Xanthine hydroxylation occurs in the molybdopterin center. XOR is translated at the level of the ribosome into XDH. Conversion of XDH to XO can occur by either sulphydryl oxidation or proteolytic cleavage (Figure 11). While there is recent evidence that XO protein expression may be partially controlled at the transcriptional level, the regulation of XO activation by either of these pathways is not fully elucidated.\textsuperscript{143-145} It has been established that XDH to XO conversion via thiol oxidation can be dependent on ROS, specifically from NADPH derived\textsuperscript{101} and mitochondrially derived species\textsuperscript{146}. Further, many stresses can induce XO activity including; ischemia-reperfusion\textsuperscript{147}, rapid cardiac pacing\textsuperscript{148,149}, and mechanical stretch\textsuperscript{150}. Mechanical stretch is of particular importance in cardiac diseases associated with dilation. As the heart dilates or end diastolic wall stress is increased, the primary force the heart wall experiences is stretch.\textsuperscript{26} Indeed, cardiomyocytes exposed to cyclic stretch demonstrate increased ROS.\textsuperscript{151} These findings suggest ROS and XO play a role in VO HF. The work in this dissertation focuses on XO as a causative factor in the progression of VO to overt HF.
Figure 10. Structure of XOR. XOR consists of two identical subunits each weighing 140 kDa. Each subunit has a 20 kDa N-terminal domain containing two iron-sulfur centers (2Fe/2S), a central 40 kDa FAD binding domain, and a C-terminal domain with both a molybdenum cofactor site (MoCo) and the substrate binding site. These domains are separated by two hinge regions.
Figure 11. XOR and the Purine Degradation Pathway. The starting point of the purine degradation pathway is ATP. ATP is metabolized through a multistep process involving many enzymes to form hypoxanthine. Hypoxanthine is metabolized by either XO or XDH to form xanthine. Xanthine is then also metabolized by either XDH or XO to form uric acid. XOR is transcribed into XDH which can then be converted permanently via proteolysis or transiently via sulfhydryl oxidation to XO. XDH utilizes NAD+ as an electron acceptor while XO uses O₂ during purine metabolism. When O₂ accepts an electron, the superoxide (O₂⁻) free radical is generated thereby contributing to oxidative stress.
Xanthine Oxidase inhibition

The first XO inhibitor, allopurinol, began development in the 1940’s and was approved by the Food and Drug Administration in 1966 for treatment of the hyperuricemia in gout and to this day, remains the primary treatment. Allopurinol is a hypoxanthine analog that is metabolized to the active metabolite oxypurinol which is a noncompetitive inhibitor of XO. Allopurinol is rapidly absorbed with peak plasma concentrations within 1 hour with oral administration. Allopurinol has a half life of 2-3 hours in plasma and its metabolite oxypurinol has a much long half-life of ~24 hours. The side-effect profile of both allopurinol and oxypurinol is mild and consists of gastrointestinal distress and hypersensitivity reactions. There are new efforts to develop alternative XO inhibitors that have higher bioavailability and longer lasting decreases in hyperuricemia. Febuxostat and Y-700 are two such compounds with higher bioavailability and are currently under examination at the clinical trial level.

The work in this dissertation utilizes allopurinol to inhibit XO in the setting of VO HF. The choice of allopurinol was based on cost, availability, and most importantly, the wide breadth of knowledge concerning allopurinol’s use in humans and animals. This allows any findings to be directly translated into patient care applications with minimal concerns on efficacy and safety.

XO Inhibition in Animal Models and Patients with HF

XO inhibition has been shown to be beneficial in multiple animal models of HF. These studies span multiple species and causes of HF including ischemia/reperfusion,
rapid pacing, pressure overload, and post-myocardial infarction. In humans, LV XO is upregulated in patients with dilated cardiomyopathy and intracoronary infusion of allopurinol improves LV contractile performance without increasing myocardial oxygen consumption (MVO₂). XO inhibition improves endothelial function and LV EF in patients with HF and in patients with diabetes. Allopurinol augments LV contractility during exercise and following dobutamine infusion in dogs with pacing-induced HF. 

In vitro studies have demonstrated that XO depresses myofilament sensitivity to calcium and that it co-localizes with NOS-1 in the sarcoplasmic reticulum in the mouse cardiomyocyte. This co-localization can regulate excitation-contraction coupling and myofilament oxidative damage. Allopurinol also prevents maladaptive alterations in Ca²⁺ cycling proteins and preserves the phospolamban-to-SERCA ratio and improved β-adrenergic hyporesponsiveness. These effects at the level of cardiomyocyte contractile proteins are consistent with the finding of XOR/XO along the z-line of the normal human cardiomyocyte from our studies.

Despite all of these positive studies of XO inhibition in human and animal models of HF, oxypurinol has recently been shown to have no beneficial effect in patients with established Class III or IV heart failure in the OPT-CHF Trial. However, improvement does occur in treated patients who have greater reductions in serum uric acid. More importantly, the major difference between preventing progression to HF in VO, as proposed here, and the OPT-CHF Trial is that OPT-CHF patients have advanced HF. This is critical because the bioenergetic defects that we propose are due to oxidative damage to the mitochondrion in VO development are already so advanced in OPT-CHF patients that they are beyond therapeutic restoration. Of interest, early XO inhibition
prevents HF in a rabbit model of coronary occlusion, while late XO inhibition, once heart failure is established, offers no significant protective effects.\textsuperscript{166} Whether XO plays a role in adverse LV remodeling and functional impairment resulting from myocardial stretch in isolated VO has not previously been investigated.

**Oxidative Stress and Bioenergetic Dysfunction in VO**

Recent studies have demonstrated that excessive mechanical stretch in the lung increases XO activity and plays a prominent role in acute lung injury.\textsuperscript{150} Why should VO cause an increase in oxidative stress? A variety of factors, e.g. strenuous exercise, muscle injury during ischemia/reperfusion, certain diseases and aging, increase ROS production to the extent that they are not buffered by endogenous antioxidants.\textsuperscript{167} Mechanical stretching of cardiomyocytes causes an amplitude-dependent increase in ROS production, which is associated with specific phenotypic effects.\textsuperscript{151} Specifically, low levels of ROS production, observed in cardiomyocytes stretched at low amplitude, correlate with fetal gene expression and hypertrophy, while high amplitude stretch induces higher ROS production and apoptosis.\textsuperscript{168} We have shown increased LV oxidative stress in patients and dogs with isolated MR\textsuperscript{71} and hypothesize that VO causes oxidative stress and cardiomyocyte mitochondrial dysfunction, resulting in a progressive increase in LV end-systolic volume, LV wall thinning, and decrease in LV function.

During heart failure, the myocardium decompensates because there is an imbalance between energy production and use, leading to a state of energy deficit.\textsuperscript{169} Volume overload produces an increase in pressure-volume area. This predicts higher MVO\textsubscript{2},\textsuperscript{170} and has been reported to increase 2-fold in patients with volume overload.\textsuperscript{171}
This places great stress upon the mitochondria to maintain LV compensation but presumably can be accomplished because mitochondria make up at least 20% of the dry weight of the cardiomyocyte. However, when mitochondria are damaged by ROS the individual components of the respiratory chain complexes make additional ROS, thus amplifying the initial insult and decreasing mitochondrial reserve capacity as outlined in. In support of this concept, oxidative phosphorylation is impaired in papillary muscles of VO patients undergoing mitral valve replacement for isolated MR. In addition, we find increased oxidative stress and clusters of small mitochondria, indicative of a bioenergetic defect, in patients with isolated MR.

**Mitochondria, XO, and Oxidative Stress in VO**

The biochemistry of ROS/RNS can be direct, as activation of metalloproteins (MMPs), or indirect by proteins modifications through mechanisms such as nitration or secondary lipid products. These reactions can occur at several sites in the cell, including the mitochondrion, and are the routes through which ROS/RNS have the potential to specifically modify proteins. These modifications provide a mechanism for the modulation of signal transduction pathways and when uncontrolled, cause dysfunction in metabolic or cell signaling. Changes in the production of ROS/RNS play an important role in the etiology of mitochondrial dysfunction. In addition, the mitochondrion is capable of generating superoxide, especially through Complex I. The interaction of superoxide with nitric oxide can generate RNS leading to a progressive loss of mitochondrial function through the post-translational modification of mitochondrial proteins and DNA.
The efficiency of mitochondrial energy and oxidant production is dependent upon a number of factors including the local concentrations of both ROS/RNS, mitochondrial antioxidants, cytokines, electron transport efficiency, metabolic reducing equivalent availability (NADH and FADH$_2$), uncoupling protein activities, and overall organelle integrity (damage to membranes, DNA, and proteins). An important aspect of the enzymology of XO is that it uses the products of ATP catabolism as substrates for the generation of ROS. In the context of the increased energy demands of VO, this establishes a “perfect storm” of pathological interplay between purine metabolism and energy production by the mitochondrion. Therefore, the increased MVO$_2$ results in even more substrates for XO in VO, and mitochondria become progressively more damaged and less efficient. Importantly, XO protein is increased in the LV of patients with isolated MR, despite LV EF >60% and no coronary disease.$^{71}$ The findings of increased XO and oxidative stress in patients with well preserved LV systolic function makes a strong case for a major role of XO as a source of ROS in the cytosol which can contribute to decreased mitochondrial and LV function. The work in this dissertation explores the relationship between XO and bioenergetic function and its association with maladaptive LV remodeling/function in the setting of cardiac VO.

*Mitochondrial Function and Assessment*

The state of bioenergetic function determines the ability of a cell to meet the demands it faces from an energetic standpoint. As the major source of ATP production, the mitochondria play a central role in the state of energy production and regulation in the cardiomyocyte. The heart demands an enormous amount of energy to produce effective
contraction and is heavily dependent on healthy mitochondria. The mitochondria is a cellular organelle consisting of an outer and inner membrane surrounding a matrix. The inner membrane forms folds called cristae which serve to increase surface area within the matrix. The mitochondria produce approximately 90% of the energy used in the cardiomyocyte. The mitochondria is involved in many pathways that produce energy in the cardiomyocyte including; pyruvate oxidation, the citric acid cycle (TCA), β-oxidation of fatty acids, and the final shared pathway of oxidative phosphorylation. The ultimate goal of these pathways in the mitochondria is to produce a chemiosmotic gradient to drive the conversion of ADP to ATP. Oxidative phosphorylation (Figure 12) is localized to a series of complexes (I-V) within the inner mitochondrial membrane. These complexes transfer electrons in series and the energy released is used to transfer hydrogen atoms out of the matrix into the inner membrane space thereby creating a chemiosmotic gradient. This gradient is then used at complex V or ATP synthase to produce ATP from ADP. Complex I oxidizes NADH from the TCA cycle and transfers 2 electrons to ubiquinone this causes 4 protons to be shuttled from the matrix to the intermembrane space. Complex II oxidizes succinate from the TCA cycle generating fumarate and ubiquinone. Complex III is fed ubiquinone from either Complex I or II and reduces cytochrome c and is coupled to proton movement to the intermembrane space. Cytochrome c is then oxidized by Complex IV where electrons are used to reduce O₂ to H₂O and 4 protons are moved from the matrix to intermembrane space. The proton gradient created is now used by Complex V to drive the phosphorylation of ADP producing ADP.
Figure 12. Mitochondrial Oxidative Phosphorylation. Complex I oxidizes NADH (from TCA cycle) and shuttles electrons to the CoQ site, where they are used to reduce ubiquinol to ubiquinone. Complex II oxidizes succinate (from TCA cycle) and transfers the electrons to CoQ. The ubiquinone produced by either Complex I or II can then feed into Complex III which reduces cytochrome c (Cyt c). Reduced Cyt c is then reoxidized by Complex IV, which uses electrons to reduce O$_2$ to H$_2$O. This electron (e$^-$) transfer (red line) is coupled to pumping protons (H$^+$) from the matrix to the intermembrane space to produce a chemiosmotic gradient. This gradient is then used by Complex V to drive the phosphorylation of ADP to produce ATP.
Due to the increase demand of energy in HF and the central role mitochondria play in energy production it is important to experimentally address the state of mitochondrial and bioenergetic function of the cardiomyocyte. It is possible to determine the oxygen consumption of isolated mitochondria using a clark-type oxygen sensing electrode (Figure 13).\textsuperscript{61,183} The isolation of mitochondria is achieved using a differential centrifugation approach. In the case of the cardiomyocyte, there are two major populations of mitochondria; the SSM and the IFM. It is possible to isolate both populations independently. After the mitochondria have been isolated from LV tissue they are subjected to various substrates in a particular order to effectively interrogate portions of the electron transport chain. Addition of glutamate and malate evaluate the integrity of the TCA cycle and electron transport chain.\textsuperscript{61, 184} State 2 mitochondrial respiration is studied with the addition of the glutamate/malate as substrates in the absence of added ADP. Respiratory state 3 rate of oxygen consumption is then measured in the presence of excess ADP.\textsuperscript{61, 183} While these measurements are sensitive and give insights into the cardiomyocyte’s bioenergetic state, they are not without their limitations. Limiting factors of this method include; an arduous isolation process, lack of innate cellular environment, O\textsubscript{2} consumption by the probe itself.

Recent technological advances have enabled bioenergetic measurements of intact live cardiomyocytes. The XF24 extracellular flux analyzer (Figure 14) can measure the two major energy-producing pathways of the cell simultaneously.\textsuperscript{185, 186} Both mitochondrial respiration (O\textsubscript{2} consumption) and glycolysis (extracellular acidification) can be studied in a high-throughput real-time manner. This is achieved with a specially designed microplate and cartridge system that is lowered over a monolayer of cultured
cells creating a microchamber. Importantly, the system can be returned to baseline by raising the probe, thus preventing the cells from becoming hypoxic. This allows for multiple measurements over time. The system also allows for injection of specific inhibitors to interrogate the electron transport chain. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) is a mitochondrial protonophore that uncouples oxidative phosphorylation and maximizes O2 consumption. Addition of FCCP is used to derive the maximal oxygen consumption rate or MaxOCR. Finally, non-mitochondrial O2 consumption is measured by addition of two inhibitors. Antimycin A inhibits the oxidation of ubiquinol in the electron transport chain and rotenone disrupts the formation of the chemiosmotic gradient and rotenone interferes with electron transport at Complex I. Work in this dissertation utilizes this technology to gain insight into the cardiomyocyte’s bioenergetic response to VO.
Figure 13. Measurement of Isolated Mitochondrial Function. Isolated cardiomyocyte mitochondria are suspended in buffer in a temperature controlled chamber. An O₂ sensitive electrode measures voltage changes resulting from a decrease in O₂ concentration in the buffer. Addition of glutamate and malate results in a moderate increase in O₂ consumption rate representing state 2 mitochondrial function. Addition of saturating levels of ADP greatly increase O₂ consumption rate at ATP-synthase and represent state 3 mitochondrial function.
Figure 14. Bioenergetic Measurements in the Seahorse Extracellular Flux Analyzer. Cardiomyocytes are plated in a special cartridge/plate system. A probe is lowered creating a temporary microchamber. Specialized probes then measure transient O₂ and pH changes. Basal O₂ consumption is determined before any treatments. Max O₂ consumption is then determined by injecting FCCP, a mitochondrial uncoupler. Lastly, antimycin A and rotenone are injected to fully inhibit the mitochondrial electron transport chain, thereby determining the non-mitochondrial O₂ consumption rate.
Hypothesis and Summary

Our studies utilized an ACF model in rats to generate a state of cardiac VO which was studied at different time points. These studies were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Our preliminary data indicated that acute ACF was associated with LV and bioenergetic dysfunction. XO activity was increased in LV tissue from 24 hr ACF and in isolated cardiomyocytes exposed to cyclical mechanical stretch.

These data led to the overall hypothesis tested in the current research: VO results in increased ROS derived from XO which dictate LV remodeling, dilatation and progressive cardiac dysfunction by negatively impacting bioenergetic function and contractile efficiency of the myocardium. This hypothesis was critically evaluated with a combined in vitro and in vivo approach.

Initially, we performed an extensive characterization of acute VO using 24 hrs ACF by utilizing simultaneous echocardiography and high-fidelity pressure catheterization and analysis of PV loop data. The well characterized XO inhibitor, allopurinol, was utilized in these animals to determine the role of XO derived ROS on cardiac function in response to VO in vivo. These studies were coupled with analysis of bioenergetic function of isolated cardiac mitochondria and examination of protein modifications resulting from excess oxidative stress.

To establish a cause and effect role between the myocardial stretch in VO and XO activation we mechanically stretched isolated adult cardiomyocytes in vitro and measured XO activity by high performance liquid chromatography. These studies were
supplemented by transmission electron microscopy of stretched cardiomyocytes with and without allopurinol or the mitochondrial ROS inhibitor MitoQ to determine the ultrastructural changes of the cardiomyocyte and its components under this stress.

To determine if XO is involved in the progressive decline in cardiac function and transition to HF associated with chronic VO, allopurinol was utilized in 8 week ACF animals. Biweekly serial echocardiography characterized the temporal response of the LV to VO with and without allopurinol. *In vivo* assessment of functional, morphometric, and hemodynamic parameters was performed as in the setting of acute 24 hour ACF. These studies were performed in conjunction with biochemical analysis of LV tissue to determine alterations in Ca$^{2+}$ handling proteins to determine their involvement in cardiac contractile efficiency.

Isolated adult cardiomyocytes from chronic VO hearts were studied *in vitro* using a technologically advanced approach employing the Seahorse XF24 extracellular flux analyzer to determine their bioenergetic capacity. This along with LV PV loop analysis was used to characterize LV contractile efficiency in response to chronic VO with and without allopurinol treatment.
NOVEL INSIGHTS INTO INTERACTIONS BETWEEN MITOCHONDRIA AND XANTHINE OXIDASE IN ACUTE CARDIAC VOLUME OVERLOAD

by

JAMES D. GLADDEN, BLAKE ZELICKSON, CHIH-CHANG WEI, ELENA ULASOVA, JUNYING ZHENG, MUSTAFA AHMED, YUANWEN CHEN, MARCAS BAMMAN, SCOTT BALLINGER, VICTOR DARLEY-USMAR, LOUIS J. DELL’ITALIA

Submitted to Free Radical Biology and Medicine

Format adapted for dissertation
Abstract

Xanthine oxidoreductase (XOR) is increased in the left ventricle (LV) of humans with volume overload (VO) and mitochondrial inhibition of the respiratory chain occurs in animal models of VO. Since mitochondria are both a source and target of reactive oxygen and nitrogen species, we hypothesized that activation of XOR and mitochondrial dysfunction are interdependent. To test this we used the aortocaval fistula (ACF) rat model of VO and a simulation of the stretch response in isolated adult cardiomyocytes with and without the inhibitor of XOR, allopurinol, or the mitochondrially targeted antioxidant MitoQ. XO activity was increased in cardiomyocytes from ACF vs. sham rats (24h) without an increase in XO protein. A two-fold increase in LV end-diastolic pressure/wall stress and a decrease in LV systolic elastance with ACF were improved with allopurinol (100 mg/kg) started at ACF induction. Subsarcolemmal state 3 mitochondrial respiration was significantly decreased in ACF and normalized by allopurinol. Cardiomyocytes subjected to 3 hour cyclical stretch resulted in an increase in XO activity and mitochondrial swelling, which was prevented by allopurinol or MitoQ pretreatment. These studies establish an early interplay between cardiomyocyte XO activation and bioenergetic dysfunction that may provide a new target that prevents progression to heart failure in VO.
Introduction

Volume overload (VO) increases diastolic load and results in a progressive eccentric left ventricular (LV) remodeling and systolic dysfunction leading to cardiac failure (1). Currently, there is no medical therapy that halts the progression to heart failure in an isolated VO of aortic or mitral regurgitation (2). We have recently shown that patients with isolated mitral regurgitation have decreased LV systolic function 6 months post-mitral valve repair, despite LV ejection fraction (EF) > 60% prior to surgery (3). Biopsies taken at the time of mitral valve repair demonstrate significant myofibrillar loss with increased xanthine oxidoreductase (XOR), protein nitration, and lipofuscin accumulation in cardiomyocytes, consistent with increased formation of reactive oxygen and nitrogen species (ROS/RNS). Correspondingly, there is also evidence of aggregates of small mitochondria in cardiomyocytes, which is generally considered a response to bioenergetic deficit in cells.

XOR exists as xanthine dehydrogenase and xanthine oxidase (XO), both of which metabolize purines to form uric acid (4). In its oxidase form, XOR produces superoxide and hydrogen peroxide when oxygen is used as an electron acceptor during purine metabolism. Superoxide and hydrogen peroxide can negatively impact multiple metabolic processes in the cardiomyocyte either independently or after reaction with nitric oxide (NO) (5,6). One key target for the actions of ROS/RNS in the cell is the mitochondrion because of its high concentration of reactive proteins and lipids in close proximity to multiple enzymes containing redox active iron and copper (7,8).

Not surprisingly, increasing evidence implicates mitochondrial dysfunction in a broad range of cardiovascular pathologies, many of which are associated with increased
ROS/RNS (9-11). Mitochondria are a major source of ROS within the cardiomyocyte, mostly due to the univalent reduction of oxygen to superoxide at complexes I, and III (12,13) \(^{189,190}\). Importantly, exposure of mitochondria to ROS/RNS can lead to mtDNA damage that compromise protein synthesis and directly modify mitochondrial proteins (8,12). This in turn can lead to increased mitochondrial ROS production and a vicious cycle can then be established between the generation of ROS from non-mitochondrial sources, bioenergetic dysfunction, and oxidative damage to the organelle. Based upon these concepts we hypothesize that XO activation in VO is intimately involved in the mitochondrial dysfunction associated with this form of hemodynamic stress.

To test these concepts we have used a model of VO by inducing an aortocaval fistula (ACF) in rats. Acutely, ACF causes a three-fold increase in LV end-diastolic pressure and a significant increase in LV chamber diameter, consistent with an increase in LV preload (14). The increased work load associated with ACF is manifested by an increase in the pressure volume area (PVA), suggesting increased myocardial oxygen demand (MVO\(_2\)) and ATP consumption (15) and is associated with a decrease in subsarcolemmal mitochondrial function (16). Thus, in this setting, it is likely that a decrease in the bioenergetic capacity of the mitochondria plays a significant role in the myocardium’s ability to meet increased hemodynamic and oxygen demand.

Previous studies have demonstrated beneficial effects of XO inhibition with allopurinol or oxypurinol in multiple animal models of myocardial disease, such as ischemia (17,18), diabetic cardiomyopathy (19), pacing-induced tachycardia in the dog (20), and in the spontaneously hypertensive rat (21). Volume overload increases the physical stress on the heart due to the elevated diastolic load that increases the stretching
force on the heart wall, especially during early diastolic filling (22). Interestingly, acute stretch in the lung increases XO activity in endothelial cells and mediates oxidative tissue damage (23). Further, cardiomyocytes display increased ROS in response to mechanical stretch (24). However, the effect of isolated VO or its potential impact resulting from stretch has not previously been studied in human or animal models and its effects on bioenergetic and cardiac function are unknown. Taken together, these findings suggest that the stretching force associated with VO may induce XO activity resulting in increased oxidative stress in cardiomyocytes.

While the mechanism of XO activation in heart failure is not well understood, it is plausible that mitochondrial-induced oxidative stress can lead to the activation of XO by posttranslational modification of the enzyme. XO is generated from its parent enzyme XDH either by irreversible proteolytic cleavage or transient thiol oxidation within the XOR protein (25,26). In patients with heart failure, ROS have been shown to increase sulfhydryl oxidation and have been shown to increase XO activity (27). To test the hypothesis that oxidative stress in the mitochondrion and XO activation are linked in VO, we have utilized the mitochondrially-targeted antioxidant mitoubiquinone (Mito Q) (28-30). Mito Q has been shown to protect mitochondrial function in a number of cardiovascular models including ischemia-reperfusion, endotoxemia, and cardiac hypertrophy (31-33). In the present study, we demonstrate that allopurinol attenuates the mitochondrial dysfunction associated with VO and that cyclic stretch activates XO through a mechanism dependent on mitochondrial ROS.
Materials and Methods

Animal Preparation

Sprague-Dawley rats (200-250g) at 12 weeks of age were subjected to sham and ACF surgery as previously described in our laboratory (14,16) with and without XO inhibitor allopurinol (100 mg/kg, Sigma), which was started at the time of sham surgery or ACF induction. Separate sets of sham and ACF rats were sacrificed 24 h after surgery for studies of isolated cardiomyocytes (N=5 per group) and heart mitochondria (N=6 per group). Another group of sham and ACF rats were studied for in vivo hemodynamic and echocardiographic measurements prior to sacrifice and this tissue was used for protein analysis. To examine the effects of cardiomyocyte stretch on XO activity, 12 week old Sprague-Dawley rats (200-250g) were sacrificed and isolated cardiomyocytes were obtained for in vitro stretch studies. This study was approved by the University of Alabama at Birmingham Animal Resource Program.

Hemodynamics and Echocardiography

Echocardiography and hemodynamics were performed prior to sacrifice using the Visualsonics imaging system (Vivo 770, Toronto, Canada) combined with simultaneous high-fidelity LV pressure catheter recordings (Millar Inst. Houston, TX). With the rat under isoflurane anesthesia, a high-fidelity LV pressure catheter was advanced into the LV cavity via a right carotid cut-down. Simultaneous LV pressure and echocardiographic dimensions (wall thickness and chamber diameter) were obtained using software included in the Visualsonics system. LV volume was calculated from traced m-mode LV dimensions using the Teicholz formula.
\[ V = \frac{7}{(2.4 + \text{LVID})} \cdot [\text{LVID}]^3 \]

Where \( V \) = volume, \( \text{LVID} \) = LV internal dimension.

LV wall stress was calculated from traced m-mode LV dimensions and simultaneous LV pressure data using the equation below.

\[ \text{LV } \sigma = \frac{[\text{LVP} \cdot r]}{[2 \cdot \text{LVwt}]} \]

Where \( \text{LV } \sigma \) = LV wall stress, \( \text{LVP} \) = LV pressure, \( r \) = LV chamber radius, \( \text{LVwt} \) = LV wall thickness.

The LV pressure-volume data were analyzed for LV PVA and stroke work using the Labscribe2 (iWorx System Dover, NH) software package.

**Isolation of Heart Subsarcolemmal Mitochondria and Activity Measurements.**

Heart subsarcolemmal mitochondria (SSM) were isolated from LV tissue (70mg) as previously described in our laboratory\( (16,34) \). The pellet resulting from centrifugation of LV homogenate at 1000xg for 5 minutes (4°C) was discarded. The supernatant was centrifuged at 6000xg for 10min and the resulting pellet was washed twice in isolation buffer and used for respiration measurements.

Oxygen consumption was measured using a Clark-type electrode (Hansatech Instruments, Norfolk, UK). State 2 mitochondrial respiration was initiated upon addition of glutamate/malate (5mmol/L) as substrates. Respiratory state 3 rate of oxygen consumption (nmol \( \text{O}_2 \) min\(^{-1}\)·mg protein\(^{-1}\)) was measured in the presence of 1.5 mmol/L ADP and State 4 determined after utilization of 15 µmol/L adenosine diphosphate (ADP).
Isolation of LV Myocytes

Cardiomyocytes were isolated from Sham and ACF rats, as described in our laboratory (14,16). Briefly, hearts were perfused with perfusion buffer (120 mmol/L NaCl, 15 mmol/L KCl, 0.5 mmol/L KH$_2$PO$_4$, 5 mmol/L NaHCO$_3$, 10 mmol/L HEPES, and 5 mmol/L glucose, at pH 7.0) for 5 min and digested with perfusion buffer containing 2% collagenase II (Invitrogen, Carlsbad, CA) for 30 min at 37°C. The right ventricle, atria and apex were removed before the perfused-heart was minced. The digestion was filtered, washed and cells were pelleted. Only samples with purity and viability (rod-shaped) > 95% or 80%, respectively, were used.

Application of Stretch to Isolated Adult Rat Cardiomyocytes

Cells (2 x 10$^6$/well) were allowed to adhere to laminin coated Flexcell plates (Flexcell International Corp., Hillsborough, NC, USA) in DMEM medium containing 10% FBS, 2 mM glutamine, 10 U/mL penicillin, and 100 mg/mL streptomycin for 2 hours before use. Cells were subjected to cyclical strain (60 cycles/min, 3h) on the Flexcell Strain apparatus (model FX-4000; Flexcell International, Hillsborough, NC, USA) at a level of distension sufficient to promote an increment of approximately 5% in surface area at the point of maximal distension on the culture surface. A group of cells stretched for 3 hours were also subjected to either the mitochondrial ROS inhibitor Mito Q (10 nM or 50 nM) or 250 µM allopurinol. Control cells were prepared on identical culture plates but were not exposed to stretch.
**Immunohistochemistry in LV Myocardium**

Rat hearts were immersion-fixed in 10% neutral buffered formalin and paraffin-embedded. 5µm sections were mounted on slides, deparaffinized in xylene and rehydrated in a graded series of ethanol. After blocking with 1x PBS/1% Casein, overnight incubation at 4°C with either XO antibody (1:1000) or nitrotyrosine antibody (1:1000) Upstate Biotechnology, Inc Lake Placid, NY. Alexa Fluor conjugated secondary antibodies (Molecular Probes, Eugene, OR; 1:500 each) were applied to visualize XO (green) and nitrotyrosine (red) in the tissue. Nuclei were stained (blue) with DAPI (1.5µg/ml; Vector Laboratories, Burlingame, CA). Image acquisition (100x objective, 4000x video-screen magnification) was performed on a Leica DM6000 epifluorescence microscope with SimplePCI software (Compix, Inc., Cranberry Township, PA). Images were adjusted appropriately to remove background fluorescence.

**Western Blot**

Tissue lysate (30 µg protein) was separated on 4-12% Bis-Tris gradient gel (Invitrogen), transferred to a PVDF membrane, then incubated with antibody to either XO (1:1000) Santa Cruz Biotechnology, Santa Cruz, CA, NAPDH oxidase p47 phox subunit (1:1000) Cell Signaling Technology, Inc. Danvers, MA, iNOS (1:1500) Santa Cruz Biotechnology, Santa Cruz, CA, eNOS (1:750) BD Biosciences, Franklin Lakes, NJ, β-tubulin (1:2000) Sigma-Aldrich, St. Louis, MO, overnight at 4°C followed by incubation with HRP conjugated secondary antibodies. Membranes were incubated with Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to X-ray film. Densitometry analysis was performed on XOR expression levels which were normalized.
to tubulin in Sham vs ACF. NADPH oxidase, eNOS, and iNOS expression were normalized to total protein determined by densitometric analysis from ponceau stained membranes in all groups. Membranes were stained with ponceau stain for 2 mins and then destained in phosphate buffered saline to remove background.

*Measurement of XO Activity in LV Tissue and Isolated Cardiomyocytes*

XO activity was measured using high performance liquid chromatography (HPLC) with electrochemical detection (ESA Couarray). LV free wall tissue or isolated cardiomyocytes were homogenized in RIPA buffer. Before measuring enzymatic activity, endogenous urate was removed by eluting the sample on a Sephadex G-25 column. Xanthine (75µM) was then added, and XO activity assessed by monitoring urate production. The specificity of this detection method for urate production by XO was verified by inhibition of urate formation following allopurinol addition in duplicate samples. Activity was normalized to post column protein concentration determined by bicinchoninic acid (BCA) protein assay for myocardial tissue samples and isolated cells.

*Transmission Electron Microscopy of Adult Rat Cardiomyocytes*

Cells were fixed in 25% glutaraldehyde overnight. Cells were then suspended in phosphate buffered saline and carefully removed from the flexcell membrane using a cell scraper. Cells were pelleted and mounted for transmission electron microscopy which was performed by EmLabs Inc. Birmingham, Al.
Statistics

Data are expressed as mean ± SEM. XO activity data was compared using Student’s t-test for control/stretched isolated cardiomyocytes and Sham/ACF LV tissue homogenates and isolated cardiomyocytes. A two-way ANOVA with student-Newman-Keuls post hoc test was used for all other comparisons among sham, ACF, Sham + allopurinol and ACF + allopurinol. P< 0.05 was considered statistically significant.

Results

Morphometric and Hemodynamic Effects of Allopurinol on Aortocaval Fistula.

ACF increases venous return to the heart resulting in a left ventricular VO and diastolic dysfunction evidenced by increased LVEDP and LVED wall stress (σ). At 24 hours after induction of ACF, body weight, heart weight$body weight$ ratio, or heart rate did not differ among Sham, ACF, Sham + allopurinol, and ACF + allopurinol rats (Table 1). Mean arterial pressure (MAP) was decreased with ACF and was unaffected by allopurinol (Table 1). LV end-diastolic dimension (LVEDD) and LVED volume were increased in ACF vs sham rats and were unaffected by treatment with allopurinol (Table 2). LVED pressure and LVED σ increased in ACF vs. sham rats and were normalized in ACF rats treated with allopurinol (Figure 1). LV ejection fraction did not change with ACF and was unaffected by allopurinol (Table 2).

LV Pressure-Volume Analysis

LV high-fidelity pressure was matched to simultaneous echocardiographic dimensions to generate LV pressure-volume loops. Data collected during a 3-5 sec
Inferior vena cava occlusion yielded a set of LV pressure-volume loops at multiple LV preloads (Figure 2). Prior to occlusion, the LV pressure-volume area was increased 20% in both ACF and ACF + allopurinol vs. sham rats (P<0.05 in both cases), indicative of increased LV work load and oxygen demand. Analysis of the LV end-systolic pressure-volume relationship (ESPVR), a relatively load independent measure of LV contractile function, demonstrated a decrease in LV contractility in the ACF group vs. Sham rats (0.36±0.07 vs 1.48±0.2 mmHg/µl, P<0.05), which was significantly improved in ACF + allopurinol rats (0.71±0.07 mmHg/µl) vs ACF rats (P<0.05).

**XO Activity is Increased in ACF Myocardial Tissue and Isolated Myocytes**

Immunohistochemistry from sham and control ACF rats demonstrated extensive XO/XDH distribution in endothelial cells and interstitial cells (Figure 3C) and along Z lines in cardiomyocytes (Figure 3A and B), which is consistent with our previous report in the human heart (3). To determine if XO is activated during ACF, XO activity was measured in both LV homogenates and isolated cardiomyocytes from ACF rats (Figure 4A and B). Cardiomyocyte XO specific activity was approximately 10% of the XO activity in whole LV tissue homogenates after normalization to sample protein content (Figure 4A and B). ACF LV tissue homogenates demonstrated a modest 15% increase in XO activity compared to Sham LV homogenates. Interestingly, XO activity from isolated ACF cardiomyocytes was increased 300% compared to sham cardiomyocytes. This suggests that ACF causes greater XO activation in cardiomyocytes even though other cellular locations of XO such as the endothelium and interstitial cells may contain higher basal levels of the enzyme.
Allopurinol Decreases Protein Nitrination in ACF LV Myocardium.

Western blot analysis of iNOS expression demonstrated no significant change in ACF vs Sham (Figure 5A). However, treatment with allopurinol was associated with increased iNOS expression in both Sham and ACF vs. untreated Sham and ACF rats (P<0.05 in both cases). Expression of the NADPH oxidase subunit p47 phox and eNOS did not significantly differ among all groups (Figure 5A, 5B). Immunohistochemical analysis for nitrotyrosine demonstrated increased staining in ACF left ventricles compared to all other groups (Figure 5D vs 5C, 5D, and 5E). It is of interest that in the ACF group (Figure 5D) the staining was aligned along the Z-lines, suggesting increased nitration with elevated XO.

Effects of ACF on Mitochondrial Function

Oxygen consumption was determined in isolated subsarcolemmal mitochondria (SSM) from all experimental groups (Figure 6). As we reported previously (16), State 3 mitochondrial oxygen respiration was decreased in ACF vs. sham rats (Figure 6). In the current study, allopurinol normalized State 3 mitochondrial respiration in the ACF + allopurinol vs. ACF rats. Allopurinol had no effect on State 2 or State 4 mitochondrial respiration but significantly decreased State 3 respiration in Shams. The reasons for this change are not clear but could be due to effects of allopurinol on the purine salvage pathway leading to a decreased activation of mitochondrial respiration or biogenesis.

Effects of Cardiomyocyte Stretch on XO Activity and Mitochondrial Morphology
VO causes increased myocardial stretch and since this has been reported to increase ROS formation (22), we hypothesized that this could contribute to XO activation. To test this hypothesis, isolated adult rat LV myocytes were plated on laminin-coated Flexcell plates and stretched at 1 Hz at 5% sinusoidal strain for 3 hours. Protein homogenates from stretched vs. unstretched cardiomyocytes demonstrated an increase in XO activity at 3 hrs after stretch (Figure 7). Pre-treatment with Mito Q in unstretched cells and in cells subjected to 3 hours stretch demonstrated no increase in XO activity with both 10 nM or 50 nM doses of Mito Q. Taken together, these results suggest a cause and effect relationship between isolated stretch and increased cardiomyocyte XO activation that is induced by mitochondrial-derived ROS.

**Effect of Mechanical Stretch on Myofibrillar and Mitochondrial Structure**

Transmission electron microscopy of stretched cardiomyocytes revealed a marked decrease in myofibrillar density as well as structural disruption of the Z-line (Figure 8). In addition, there was evidence of mitochondrial swelling and loss of electron density of cristae in stretched cells vs. unstretched cardiomyocytes. Z-line structural integrity and mitochondrial morphology were preserved by pre-treatment with allopurinol or Mito Q.
Discussion

Inflammation and oxidative stress produce ROS/RNS through a number of enzymatic systems including XO. It is well known that mitochondria are both targets and sources of oxidative stress, which we have shown in VO results in inhibition of the respiratory chain. This is important since VO produces an increase in pressure-volume area (PVA) as shown in Figure 2, which is known to require higher myocardial oxygen consumption (MVO₂) and ATP consumption (15). We reasoned that this combination of increased energy demand and mitochondrial dysfunction increases the susceptibility of the VO heart to failure. Indeed, we have recently established a novel interaction between bioenergetics and activation of MMPs in the cardiomyocyte of the VO heart (16). This is particularly interesting in the context of XO since its substrates, xanthine and hypoxanthine, are elevated under increased bioenergetic demand and bioenergetic dysfunction. In the current study, we report that VO causes an increase in XO activity in LV tissue and cardiomyocytes without changes in total XO protein, consistent with an oxidative post-translational activation of XO (Figure 4).

Since the activation of XO through this mechanism involves the oxidation of thiols, we reasoned that mitochondrial derived oxidants are an early event that could lead to the activation of XO. To test this hypothesis, MitoQ is used to inhibit mitochondrially-derived ROS in the stretched cardiomyocytes. Indeed, MitoQ prevents both stretch induced-XO activation and mitochondrial swelling and disorganization (Figure 8). These results suggest that mitochondria, which comprise 30% of the cardiomyocyte by volume, may be an important source of ROS and play a regulatory role in XO activation in the VO cardiomyocyte.
The current study also demonstrates loss of myofibrillar integrity in isolated cardiomyocytes subjected to cyclic stretch. We have previously shown that 24 hours of ACF results in increased TNF-α levels (35) and ROS formation and matrix metalloproteinase (MMP) activation (16) within cardiomyocytes. Cytokines, XO, and ROS have been shown to cause MMP activation (36-38) and there is increasing evidence that cardiomyocyte MMP activation is responsible for myosin and troponin degradation during cardiac ischemia reperfusion injury (39,40). In addition, transgenic mice expressing active MMP-2 driven by the α-myosin heavy chain promoter exhibit breakdown of Z-band registration, lysis of myofilaments, and disruption of sarcomere and mitochondrial architecture (41). It is of interest that we have recently demonstrated extensive cardiomyocyte myofibrillar loss in association with increased oxidative stress in the myocardium of VO patients with chronic isolated mitral regurgitation (3). Thus, it is tempting to speculate that mitochondrially derived ROS and XO-mediated MMP activation may play a causative role in the myofibrillar degeneration that has now been identified in the rat (16), dog (42,43), and human (3) with isolated VO.

Increased cardiomyocyte XO activity with acute ACF is also associated with decreased State 3 maximal bioenergetic capacity of isolated subsarcolemmal mitochondria, which is normalized by allopurinol (Figure 6). Mechanical stretch is associated with increased cardiomyocyte XO activity and abnormal mitochondrial structure that is prevented by allopurinol and MitoQ. NADPH oxidase and uncoupled NOS activation have also been identified in cardiomyocyte stretch (44,45). The current study demonstrates XO activation in a heart failure model may be a direct response to physical stretch. Further, our in vitro studies also implicate the mitochondria as a source
of ROS by demonstrating that XO activation and mitochondrial and cytoskeletal derangements with stretch can be prevented by MitoQ. Therefore, it is tempting to speculate that XO activation is related to ROS production from mitochondrial structural alterations and that allopurinol and MitoQ may have synergistic effects in vivo. These data do not exclude a role for NADPH oxidase and iNOS in contributing to the VO-dependent response to stretch but they do suggest that they are required to interact with both mitochondria and XO to contribute to the pathology.

The in vivo and in vitro findings of the current study suggest that increased XO activity and mitochondrial oxidative stress are central factors in bioenergetic dysfunction in the face of the increased ATP requirements and MVO₂ of VO. Studies by Hare and coworkers have shown that acute administration of allopurinol decreases the MVO₂ while improving contractile function in patients with dilated cardiomyopathy (46) and in dogs with pacing tachycardia induced heart failure (47), suggesting improved myocardial efficiency. We further speculate that increased levels of ADP and AMP, particularly in the setting of mitochondrial dysfunction, that are degraded to XO substrates hypoxanthine and xanthine, can set up a self-perpetuating cycle by which activated XO produces ROS that damage mitochondria, that in turn causes further ROS production and XO activation (Figure 9). In support of this argument, it is of interest that the acute stretch of VO causes relatively greater XO activation in isolated cardiomyocytes (300%) than in the LV tissue homogenate (15%).

Importantly, ACF XO activity is increased in the setting of unchanged NADPH oxidase and iNOS/eNOS expression. Allopurinol treatment increases iNOS expression in both the Sham and ACF (Figure 5). While the reasons for this finding are not completely
clear there is extensive evidence of crosstalk between the XO-NOS pathways (48). Since allopurinol administration is associated with improved bioenergetics, in this study and in previous work (46,47), it is possible that enzymes responsible for enhancing purine salvage may act as the intermediary between XO and iNOS. For example, AMP-activated protein Kinase (AMPK) activity is increased in response to energy demand and can directly inhibit iNOS expression (49). It is possible that allopurinol administration alters the AMP/ATP ratio and decreases AMPK activity therefore resulting in an increase in iNOS expression. Interestingly, nitrotyrosine staining was increased in ACF and normalized with allopurinol suggesting XO-derived ROS independently increase nitrative modifications without changes in NOS expression. These findings suggest increased XO activity is a major contributor to cardiac dysfunction in this setting.

LV ejection fraction is preserved after 24 hours of ACF. However, the LV ESPVR, which provides a load independent index of LV contractility, is depressed in the acute 24 hour ACF, and allopurinol improves both LV contractility and diastolic function (Table 2). It is of interest that increased cardiac ADP levels have been linked to diastolic dysfunction by outcompeting ATP at the actin-myosin crossbridge site and subsequently impairing the relaxation process by delayed ADP dissociation, which is the rate limiting step in cross bridge cycling (50). Indeed, artificially altered ADP levels have been shown to directly correlate with increased LVEDP in the rat heart (51). The beneficial effect on LVED σ is particularly important because wall stress is the driving force for LV hypertrophy in VO (1). Because LV mitochondrial and diastolic function are simultaneously normalized by allopurinol, it is tempting to speculate that allopurinol
attenuates ROS-dependent mitochondrial damage and improves diastolic function by improving maximal ADP-stimulated respiratory capacity and ADP/ATP ratio.

Whether allopurinol improves LV function in acute VO through its effect on mitochondrial respiration cannot be conclusively demonstrated in the current study, since we only measured the respiration of sub-sarcolemmal mitochondria respiration and not intermyofibrillar mitochondrial respiration. In addition, *in vitro* studies demonstrate that XO depresses myofilament sensitivity to calcium and that it co-localizes with nitric oxide synthase-1 in the sarcoplasmic reticulum in the mouse cardiomyocyte, which can regulate excitation-contraction coupling as well as myofilament oxidative damage (52,53). Specifically, XO inhibition restores ryanodine receptor nitrosylation, reverses diastolic sarcoplasmic reticulum calcium leak, and improves cardiomyocyte contractility in the spontaneously hypertensive heart failure rat (54) and improves the maladaptive changes in calcium cycling proteins associated with LV failure in the pacing tachycardia dog model (55). In the current study, XO protein staining by immunohistochemistry is most concentrated at the Z-line in the cardiomyocyte, which is similar to the findings in the human cardiomyocyte (3). Further, *in vitro* stretch of cardiomyocytes results in loss of myofibrillar structural integrity of the Z-line concurrent with increase XO activity, both of which are prevented with allopurinol or Mito Q. Thus, we cannot rule out an alternative calcium/myofilament mediated mechanism by which allopurinol improves LV contractile performance in this acute VO.

In summary, we have shown that acute VO increases XO activity in heart tissue and isolated cardiomyocytes and that defects in subsarcolemmal mitochondrial respiration and LV dysfunction with VO are reversed by allopurinol. Further, cyclic
stretch of cardiomyocytes increases XO activity producing mitochondrial structural defects that are attenuated by allopurinol or MitoQ. Taken together, these studies indicate that XO activation from stretch induced oxidative stress may be central to both bioenergetic and LV dysfunction in acute VO.

Acknowledgements

This study is supported by NHLBI Grants RO1 HL54816 (LJD) and Specialized Center of Clinically Orientated Research in Cardiac Dysfunction P50HL077100 (LJD). BRZ was supported by National Institute of Health grants T 32 HL007918.
References


Figure 1. Allopurinol treatment in ACF and its effect on diastolic cardiac function. Sham and ACF rats were studied with and without allopurinol (Allop. 100 mg/kg) initiated at the time of ACF induction. Simultaneous echocardiography and LV high-fidelity pressure catheterization were obtained at 24 hours. LV end diastolic (LVED) pressure and wall stress is increased with ACF and normalized with allopurinol *=P<0.05 vs. sham, sham + Allop., and ACF + Allop.
Figure 2. LV End-systolic pressure-volume relationship in ACF and the effects of allopurinol. Representative examples of LV pressure-volume loops generated by a transient inferior vena cava occlusion in Sham (upper left), ACF (upper right), Sham+allopurinol (Allop. lower left), and ACF+allopurinol (lower right). The slope of the LV end-systolic pressure volume relationship is decreased in ACF compared to Sham and significantly improved in ACF+allopurinol (see Table 2, LV ESPVR).
Figure 3. Distribution of XO/XDH cardiomyocytes in sham and 24 hr ACF left ventricles. Immunohistohemistry demonstrates XO/XDH in cardiomyocytes in coordination with Z-lines (arrows), in sham (A) and 24 hour ACF LVs (B) the box in the top right of each panel is a higher magnification view of the same image. In addition, panel B demonstrates XO/XDH in interstitial cells (fibroblasts and endothelial cells) in which nuclei stained with DAPI (blue) take on a more green appearance. In C, cross section of an arteriole in a sham LV demonstrates XO/XDH staining in endothelial cells (arrow) and in smooth muscle cells. The immunoadsorbed antibody (D) demonstrates the blue staining of nuclei with DAPI and lack of XO/XDH staining supporting the specificity of the XO/XDH antibody. White bar represents 20 µm (note different magnifications).
Figure 4. XO activity is predominantly increased cardiomyocytes in 24 hr ACF.
XO activity was measured in LV homogenates (A) and isolated cardiomyocytes (B) after 24 hours of ACF. *P<0.05 vs. sham (n = 4) per group. Protein levels were assessed using an XO/XDH antibody in LV tissue homogenates (sham, n=5 ACF, n=4) (C, densitometry analysis in E) and isolated cardiomyocyte homogenates (n=3) (D, densitometry analysis in F).
Figure 5. Protein levels of NADPH oxidase, eNOS, iNOS, and protein nitration in LV myocardium. Expression of iNOS and the NADPH subunit p47 phox was measured in LV tissue homogenates by western blot (A) and densitometry analysis was performed (B) n=3 per group. Immunohistochemistry (n=3 per group) demonstrates increased nitrotyrosine (Red) staining in ACF LV tissue (D) compared to Sham (C), Sham+allopl. (E) and ACF+allopl. (F). Nitrotyrosine appeared to be associated with the Z-line in the ACF in contrast to a more diffuse pattern in all other groups. The inset box (top right box for each panel) is an immunoabsorbed negative for each animal. Nuclei were stained with DAPI (blue) and white bar represents 20 µm. *=P<0.05 vs Sham, §=P<0.05 vs ACF.
Figure 6. Respiration of isolated subsarcolemmal mitochondria (SSM) in 24 hour ACF and the effects of allopurinol. State 2 and 4 SSM respiration did not differ among all groups. State 3 SSM respiration, a reflection of the mitochondria’s maximal ability to consume oxygen, was decreased in ACF vs. shams and significantly improved in ACF + allopurinol, suggesting XO-mediated bioenergetic dysfunction in ACF. n=6 per group.
Figure 7. Stretch induces an increase in XO activity by a mitochondrial-derived ROS dependent pathway in adult rat cardiomyocytes. Adult rat cardiomyocytes were cultured in Flexcell culture plates. XO activity was measured in unstretched cells and in cells stretched at 5% for 3 hours. XO activity was also measured after 3 hours of stretch in cells pre-treated with Mito Q, added at the concentrations shown and started 30 min prior to the initiation of stretch (n=5) #=P<0.05 vs. untreated unstretched, *=P<0.05 vs. untreated 3 hour stretch.
Figure 8. Mechanical stretch is associated with myofibrillar structural abnormalities and mitochondrial swelling in isolated adult rat cardiomyocytes. Upper panels demonstrate unstretched cardiomyocyte control, control + allopurinol (250 µM), control + Mito Q (50 nM) at 4,500x and 17,000x. Lower panels demonstrated cardiomyocytes subjected to 3 hours of stretch and treated with either 250 µM allopurinol or 50 nM Mito Q. In the 3 hour stretch cardiomyocytes there is marked structural breakdown of myofilaments and Z-line (black arrows) and mitochondrial swelling and loss of electron density (white arrows) in stretched cells compared to controls. Z-line structural integrity and mitochondrial morphology are preserved by allopurinol or Mito Q pre-treatment.
Figure 9. Increased xanthine oxidase activity leads to mitochondrial dysfunction in the VO heart. The increase in pressure volume area (PVA) in VO leads to increased usage of ATP for energy, resulting in increased levels of ADP and AMP. ADP and AMP are then degraded into hypoxanthine (HX) via purine catabolism. XO reacts with HX forming superoxide (O$_2^-$) as a byproduct, which damages mitochondria, leading to bioenergetic dysfunction. O$_2^-$ damages respiratory chain complexes either directly or through interactions with NO, generating peroxynitrite which elicits diverse deleterious effects on the mitochondrion. Collectively, this causes increased electron leak to form more ROS and decreased ATP production. An increase in ROS/RNS and decrease in ATP production feed back into the cycle causing further mitochondrial damage resulting in LV dysfunction.
Table 1 Morphometric Data in Sham and ACF rats.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>ACF</th>
<th>Sham+Allop.</th>
<th>ACF+Allop.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>205±5</td>
<td>203±7</td>
<td>207±7</td>
<td>197±5</td>
</tr>
<tr>
<td><strong>Heart Rate (bpm)</strong></td>
<td>374±10</td>
<td>368±6</td>
<td>360±7</td>
<td>362±11</td>
</tr>
<tr>
<td><strong>MAP (mmHg)</strong></td>
<td>96±3</td>
<td>62±3*</td>
<td>97±7</td>
<td>70±3*</td>
</tr>
<tr>
<td><strong>Heart Weight (g)</strong></td>
<td>0.74±0.02</td>
<td>0.79±0.02</td>
<td>0.75±0.02</td>
<td>0.72±0.02</td>
</tr>
<tr>
<td><strong>LV Weight (g)</strong></td>
<td>0.51±0.012</td>
<td>0.55±0.01</td>
<td>0.52±0.02</td>
<td>0.49±0.01</td>
</tr>
<tr>
<td><strong>Lung Weight (g)</strong></td>
<td>1.16±0.02</td>
<td>1.18±0.03</td>
<td>1.15±0.03</td>
<td>1.07±0.03</td>
</tr>
<tr>
<td><strong>Heart/Body Weight</strong></td>
<td>0.0036±0.000083</td>
<td>0.004±0.0001</td>
<td>0.0036±0.0001</td>
<td>0.004±0.00007</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>7</td>
<td>8</td>
<td>6</td>
<td>8</td>
</tr>
</tbody>
</table>

*= P<0.5 vs Sham, data = mean ± SE
Table 2 Echocardiographic and hemodynamic data in sham and ACF rats.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>ACF</th>
<th>Sham+Allopurinol</th>
<th>ACF+Allopurinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVESD (mm)</td>
<td>3.54±0.3</td>
<td>3.1±0.3</td>
<td>3.1±0.42</td>
<td>3.5±0.2</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>6.4±0.2</td>
<td>6.7±0.3*</td>
<td>6.4±0.3</td>
<td>7±0.1*</td>
</tr>
<tr>
<td>LVESV (µL)</td>
<td>90±9</td>
<td>92±17</td>
<td>69±14</td>
<td>95±13</td>
</tr>
<tr>
<td>LVEDV (µL)</td>
<td>326±17</td>
<td>389±26</td>
<td>308±22</td>
<td>364±18</td>
</tr>
<tr>
<td>EF (%)</td>
<td>73±2</td>
<td>77±3</td>
<td>77±4</td>
<td>74±3</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>2.8±0.1</td>
<td>3±0.2</td>
<td>3±0.2</td>
<td>2.7±0.1</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>1.8±0.1</td>
<td>1.8±0.2</td>
<td>1.8±0.1</td>
<td>1.5±0.03</td>
</tr>
<tr>
<td>LVES σ</td>
<td>43±7</td>
<td>23±4*</td>
<td>34±11</td>
<td>32±4*</td>
</tr>
<tr>
<td>LVED σ</td>
<td>2.5±0.2</td>
<td>8±1.1*</td>
<td>4±1.5</td>
<td>5±0.6* §</td>
</tr>
<tr>
<td>LV ESPVR</td>
<td>1.48±0.2</td>
<td>0.36±0.07*</td>
<td>1.62±0.26</td>
<td>0.71±0.07* §</td>
</tr>
</tbody>
</table>

N 7 8 6 8

LV end-systolic dimension (LVESD), LV end-diastolic dimension (LVEDD), LVES volume (LVESV), LVEDV, LV Ejection Fraction (EF), LVES Posterior wall thickness (LVPWs), LVED Posterior wall thickness (LVPWd) LVES wall stress (σ), LV ES Pressure-Volume Relationship*= P<0.05 vs Sham, § = P<0.05 vs ACF, data = mean ± SE
CHRONIC INHIBITION OF XANTHINE OXIDOREDUCTASE IMPROVES LEFT VENTRICULAR FUNCTION AND REMODELING IN THE VOLUME OVERLOADED RAT HEART

by

JAMES D. GLADDEN, BLAKE ZELICKSON, COLIN WIDENER, CHIH-CHANG WEI, MUSTAFA AHMED, VICTOR DARLEY-USMAR, LOUIS J. DELL’ITALIA

In preparation for American Journal of Physiology: Heart and Circulatory Physiology

Format adapted for dissertation
Abstract

There is no effective medical therapy to prevent progressive cardiac remodeling and failure in chronic volume overload (VO) states. We previously demonstrated upregulation of xanthine oxidase (XO) in the myocardium of VO mitral regurgitation (MR) patients and acute inhibition of XO in volume overloaded aortocaval fistula (ACF) in rats preserves cardiac and bioenergetic function. However, whether chronic XO inhibition will delay disease progression is unknown. In the current investigation we test the hypothesis that inhibition of XO would prevent progressive remodeling and preserve cardiac function in chronic VO in the rat. Age-Weight matched Sprague-Dawley rats were randomized to either Sham or ACF ± allopurinol, (n=>7 per group). Allopurinol (100 mg/kg/day) was mixed in with standard chow. Echocardiography was performed at 2, 4, 6, and 8-weeks post induction of ACF. Isolated cardiomyocytes were isolated from each group at 8 weeks and oxygen respiration studies were conducted in the Seahorse extracellular flux analyzer. Left ventricular (LV) end-diastolic dimensions were significantly increased in ACF groups compared to Sham confirming significant volume overload. LV end-systolic dimension was increased in untreated ACF vs. Sham, however this increase was attenuated in ACF+allopurinol (p<0.05 vs. ACF). LV ejection fraction, fractional shortening, velocity of circumferential shortening, maximal elastance, and contractile efficiency were all significantly depressed in the untreated ACF vs. sham (p<0.05 in all cases) however they did not differ significantly from sham in the ACF+allopurinol group. Allopurinol treatment attenuates adverse LV remodeling and preserves LV function in the chronic VO of ACF in rats. Inhibition of XO may provide a novel strategy for treatment of chronic VO states such as MR in humans.
Introduction

Volume overload (VO) increases diastolic load and results in progressive eccentric left ventricular (LV) remodeling and systolic dysfunction leading to cardiac failure.(15) Currently, there is no medical therapy to slow or stop this progression.(7) Recently, our laboratory has implicated oxidative stress in patients with VO caused by isolated chronic mitral regurgitation.(1) Specifically, xanthine oxidase (XO) was elevated in myocardial biopsies from these patients concurrent with evidence of oxidative damage. XO produces reactive oxygen species (ROS) when oxygen is used as an electron acceptor during purine metabolism.(28) ROS can negatively impact multiple metabolic processes in the cardiomyocyte by altering mitochondrial function and other sensitive targets.(5, 36)

VO can be induced in rodent models via aortocaval fistula (ACF).(14, 39) Acutely, ACF causes a three-fold increase in LV end diastolic pressure and a significant increase in chamber diameter, consistent with an increase in LV preload. In the chronic setting, ACF causes an eccentric remodeling pattern with greater relative changes in chamber diameter than in wall thickness with increases in both LV end systolic dimension and LV end diastolic dimension and is associated with contractile dysfunction.(33) Acute and chronic VO cause an increased myocardial workload resulting in increased myocardial oxygen demand (MVO₂) and ATP consumption.(27) In this setting, alterations in the contractile efficiency of the myocardium would play a significant role in the myocardium’s ability to meet increased hemodynamic and oxygen demand.
XO expression is increased in heart failure(1), and animal/human studies demonstrate XO inhibition with allopurinol improves LV function in the failing heart.(9, 12, 34) Recently, we demonstrated acute ACF at 24 hours is associated with depressed LV systolic/diastolic and bioenergetic function both of which are improved with XO inhibition with allopurinol. However, whether XO inhibition can protect LV function in the setting of chronic volume overload has not been studied. Further, inhibition of XO has been shown to increase contractile efficiency in patients with dilated cardiomyopathy.(9) This suggests an association between the ability of the stressed myocardium to efficiently meet increased energetic demands and the maintenance of contractile performance.

Emerging evidence implicates bioenergetics and contractile efficiency in a broad range of cardiovascular pathologies, many of which are associated with increased ROS. Importantly, ROS can cause mitochondrial DNA damage that compromises protein synthesis and directly modifies mitochondrial proteins.(5, 16) Indeed, acute ACF is associated with mitochondrial dysfunction that is prevented by allopurinol. In chronic ACF, gene array analysis suggests bioenergetic involvement with decreased gene expression related to fatty acid, amino acid, and glucose metabolism.(10, 13) In addition, chronic ACF is associated with decreased contractile efficiency which is cause by decreased cardiomyocyte sensitivity to Ca^{2+}.(37) Further, ROS generated from XO can oxidize sarcomeric proteins and negatively affect cardiomyocyte Ca^{2+} sensitivity.(11) Ca^{2+} handling is highly regulated and can affect both myocardial efficiency and contractile function.(4) Recent work has demonstrated aberrant Ca^{2+} handling due to the regulation of sarcoplasmic reticulum Ca2+ ATPase-2 (SERCA2) by phospholamban
(PLN) and sarcolipin (SLN). 

Recent technological advances have made it possible to study cellular bioenergetics in intact cells by utilization of the Seahorse Extracellular flux analyzer. 

In the current study, we hypothesize that inhibition of xanthine oxidase in chronic VO will improve cardiac contractile efficiency and protect cardiac function.

While XO inhibition in patients and animal models of acute VO improves cardiac function and efficiency, chronic XO inhibition in this setting is unstudied. To test the hypothesis that chronic XO inhibition protects cardiac function and remodeling we treated ACF rats with allopurinol for 8 weeks. In the present study, we demonstrate that allopurinol preserves cardiac function and efficiency in a chronic model of VO and is associated with decreased maximal oxygen consumption at the level of the cardiomyocyte. Cardiac dysfunction in chronic VO was associated with changes in sarcomeric Ca\(^{2+}\) handling proteins which were prevented with allopurinol. This study suggests allopurinol protects the metabolic efficiency of the cardiomyocyte and prevents cardiac dysfunction in chronic VO.

Methods

**Animal Preparation**

Sprague-Dawley rats (200-250g) at 12 weeks of age were subjected to either to sham or ACF, as described previously, with and without XO inhibitor allopurinol (100 mg/kg, Sigma) for 8 weeks. Allopurinol was started at time of surgery and delivered in standard rat chow. Separate sets of sham and ACF rats were sacrificed 8 weeks after surgery for studies of isolated cardiomyocytes (N=6 per group) and heart mitochondria (N=6 per group). Another set of sham and ACF rats were studied for in
in vivo hemodynamic and echo measurements prior to sacrifice and this tissue was used for protein analysis (N=5 per group). This study was approved by the University of Alabama at Birmingham Animal Resource Program.

**Hemodynamics and Echocardiography**

Echocardiography and hemodynamics were performed prior to sacrifice using the Visualsonics imaging system (Vivo 770, Toronto, Canada) combined with simultaneous high-fidelity LV pressure catheter recordings (Millar Inst. Houston, TX). With the rat under isoflurane anesthesia, a high fidelity LV pressure catheter was advanced into the LV cavity via a right carotid cut-down. LV pressure and echocardiography dimensions (wall thickness and chamber diameter) were obtained simultaneously using software included in the Visualsonics system. LV volume was calculated from traced m-mode LV dimensions using the Teicholz formula.

\[ V = \frac{7}{(2.4 + \text{LVID})} \cdot (\text{LVID})^3 \]

Where \( V \) = volume, \( \text{LVID} \) = LV internal dimension.

LV wall stress was calculated from traced m-mode LV dimensions and simultaneous LV pressure data using the equation below.

\[ \text{LV} \sigma = \frac{\text{LVP} \cdot r}{2 \cdot \text{LVwt}} \]

Where \( \text{LV} \sigma \) = LV wall stress, \( \text{LVP} \) = LV pressure, \( r \) = LV chamber radius, \( \text{LVwt} \) = LV wall thickness. These LV pressure-volume data were analyzed for LV PVA and stroke work using the Labscribe2 (iWorx System Dover, NH) software package.
Isolation of LV Cardiomyocytes

Cardiomyocytes were isolated from Sham and ACF rats, as described previously in our laboratory(39). Briefly, hearts were perfused with perfusion buffer (120 mmol/L NaCl, 15 mmol/L KCl, 0.5 mmol/L KH$_2$PO$_4$, 5 mmol/L NaHCO$_3$, 10 mmol/L HEPES, and 5 mmol/L glucose, at pH 7.0) for 5 min and digested with perfusion buffer containing 2% collagenase II (Invitrogen, Carlsbad, CA) for 30 min at 37°C. The right ventricle, atria and apex were removed before the perfused-heart was minced. The digestion was filtered, washed, and cells were pelleted. Only samples with purity and viability (rod-shaped) > 95% or 80%, respectively, were used.

Cellular Bioenergetics

To determine the effects of volume overload and treatment with allopurinol on cellular bioenergetics, the Seahorse Bioscience extracellular flux analyzer (XF24) was used to measure the O$_2$ consumption of adult cardiomyocytes in culture. Real-time, non-invasive measurements of O$_2$ consumption were determined using fluorescent probes adhered to disposable assay cartridges. These measurements were used to correlate O$_2$ consumption rate (OCR) to mitochondrial function. Primary adult mouse cardiomyocytes were attached to specialized V28 plates (Seahorse Bioscience, Billerica, MA) coated with laminin at 7500 cells/well. The cells were then allowed to attach for 2 hours, after which time the culture media was changed to unbuffered DMEM supplemented with 1% FBS and 4 mM l-glutamine for the XF24 assays.

Parameters of mitochondrial function were measured utilizing the ability of the XF24 to inject compounds into the wells through an assay as described previously.
Briefly, basal oxygen consumption of the cardiomyocytes was determined by measuring the OCR of the cells over time without any treatment. Next, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 1 µM) was injected to stimulate the maximal OCR of the cells. Finally, antimycin A (10 µM) and rotenone (1 µM) were injected simultaneously to completely inhibit the mitochondrial electron transport chain, thus yielding the non-mitochondrial OCR of the myocytes. The amount of cardiomyocytes plated per well and the concentrations of these compounds in these experiments were determined by titrating for their maximal effect (data not shown). The OCR of each well was normalized to the total protein in that well, as measured by the DC protein assay (BioRad, Hercules, CA).

*Immunohistochemistry in LV Myocardium*

Rat hearts were immersion-fixed in 10% neutral buffered formalin and paraffin-embedded. 5µm sections were mounted on slides, deparaffinized in xylene and rehydrated in a graded series of ethanol. After blocking with 1x PBS/1% Casein, overnight incubation at 4°C with sarcolipin antibody (1:300) Santa Cruz Biotechnology, Santa Cruz, CA. Alexa Fluor conjugated secondary antibodies (Molecular Probes, Eugene, OR; 1:500 each) were applied to visualize SLN (red). Nuclei were stained (blue) with DAPI (1.5µg/ml; Vector Laboratories, Burlingame, CA). Image acquisition (100x objective, 4000x video-screen magnification) was performed on a Leica DM6000 epifluorescence microscope with SimplePCI software (Compix, Inc., Cranberry Township, PA). Images were adjusted appropriately to remove background fluorescence.
**Western Blot**

LV tissue lysate (30 μg protein) was separated on 4-12% Bis-Tris gradient gel (Invitrogen), transferred to a PVDF membrane, then incubated with antibody to either SERCA2 (1:1000), PLN (1:1000) Santa Cruz Biotechnology, Santa Cruz, CA, or β-tubulin (1:2000) Sigma-Aldrich, St. Louis, MO, overnight at 4°C followed by incubation with HRP conjugated secondary antibodies. Membranes were incubated with Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to X-ray film.

**Statistics**

Data are expressed as mean ± SEM. A two-way ANOVA with student-Newman-Keuls post-hoc test was used for all other comparisons among sham, ACF, Sham+allopurinol and ACF+allopurinol. p < 0.05 was considered statistically significant.

**Results**

*Allopurinol Prevents Progressive LV Dilation and Dysfunction*

Serial echocardiography was performed at 2, 4, 6, and 8 weeks in each group. Heart rate was not changed between any groups among all time points (Table 1&2). LV end diastolic dimension (LVEDD) was significantly increased by 4 weeks in both ACF groups compared to Sham and remained so at 6 and 8 weeks. LV end systolic dimension (LVESD) also significantly increased by 4 weeks of ACF vs Sham and remained elevated at 6 and 8 weeks (Table 1&2). This increase was prevented with allopurinol treatment (ACF+allopurinol) at all time points. The indices of LV systolic function, LV fractional shortening (LVFS) and velocity of circumferential shortening (VCFr), in the ACF fell
below sham levels by 6 weeks with LVEF being decreased by 8 weeks (Table 1&2). LVEF, LVFS, and VCFr were all significantly increased in ACF+allopurinol vs ACF by 4 weeks which persisted at 6 and 8 weeks. These data suggest allopurinol prevents the progressive systolic remodeling and systolic dysfunction associated with chronic VO.

*Morphometric and Hemodynamic Effects of Allopurinol on 8 Weeks of ACF*

Body weight was lower in both groups treated with allopurinol. Heart weight, LV weight, RV weight, heart weight/body weight ratio, and lung weight were increased in both ACF groups compared to their respective sham (Table 3). Mean arterial pressure (MAP) was increased with allopurinol treatment in Sham+allopurinol vs Sham but was decreased in ACF+allopurinol vs ACF (p<0.05 in both cases) (Table 2). This occurred without a change in LV end systolic pressure (LVESP) and was associated with an elevated LV end diastolic pressure (LVEDP) in both ACF groups. LVEDD, LV end-diastolic volume (LVEDV), LV end-diastolic wall thickness (LVEDwt), LV end-systolic wall thickness (LVESwt), and LV end-diastolic wall stress, were all increased in both ACF and ACF + allopurinol groups vs. their respective Sham (Table 2). LVESD, an important marker of LV function and remodeling, was markedly increased in ACF vs Sham (p<0.05) and was significantly improved in ACF+allopurinol vs ACF (p<0.05) (Table 2).

LV EF, LV FS, and LV VCFr, key indexes of LV systolic contractility, were depressed at 8 weeks of ACF as compared to Sham (p<0.05 in all cases). Treatment with allopurinol normalized each of these parameters in the ACF+allopurinol group (Table 2, Figure 2). These data suggest allopurinol protects systolic function in chronic VO.
**LV Pressure-Volume Analysis**

LV high-fidelity pressure was matched to simultaneous echocardiographic dimensions to generate LV pressure-volume loops. Data collected during a 3-5 sec inferior vena cava occlusion yielded a set of LV pressure-volume loops at multiple LV preloads. The LV pressure-volume area (LV PVA) was increased 100% in ACF vs Sham and significantly decreased in ACF+allopurinol vs. ACF (p<0.05 in both cases). LV potential energy (LV PE), a measure of the unused energy in the cardiac cycle, was also increased 100% in ACF vs Sham and was decreased in ACF+allopurinol vs ACF (Figure 3). LV efficiency, or the ratio between LV PVA and LV PE, was increased in Sham+allopurinol vs Sham and ACF+allopurinol vs ACF (Figure 3). Analysis of the relatively load independent LV maximal elastance (LV Emax) demonstrated a decrease in LV contractility in the ACF group vs. Sham rats which was significantly improved in ACF+allopurinol rats (p<0.05) (Figure 2D). These data suggest that allopurinol treatment is associated with increased load independent LV contractility and efficiency in chronic VO.

**Allopurinol Improves Cardiomyocyte Efficiency in Chronic Volume Overload**

To determine the bioenergetic response of the myocardium in response to a chronic volume load, LV cardiomyocytes were isolated and studied using the XF24 analyzer. This XF24 measures the O₂ consumption rate (OCR) in live, adherent cells, which correlates to mitochondrial function. Adult rat cardiomyocytes isolated from 8 week sham and ACF rats ±allopurinol and were seeded into V28 plates at 7500 cells/well and their OCR was measured using the XF24 analyzer. Parameters of mitochondrial function
of the cardiomyocytes from each group were determined as described in Figure 4A. The maximal OCR of isolated cardiomyocytes in each group was measured following the injection of FCCP (1 µM) in the XF24. FCCP is a protonophore which stimulates maximal oxygen consumption of the cells by uncoupling the mitochondria and diminishing membrane potential. This leads to the rapid consumption of O$_2$ without the production of ATP. As seen in Figure 4B, the basal, maximal, and non-mitochondrial OCR in all groups were not significantly different. However, the maximal OCR trended to be lower in both sham and ACF treated with allopurinol as compared to their respective untreated group possibly reflecting metabolic changes in response to a decreased O$_2$ demand.

To assess myocardial contractile efficiency in the context of the isolated cardiomyocyte the ratio of the load independent LV contractility parameter maximal eleastance (EMax) to maximal OCR (EMax:Max$_{OCR}$) was determined. EMax:Max$_{OCR}$ ratio was decreased in chronic ACF compared to sham by 75% and was normalized with allopurinol treatment. This data suggests allopurinol protects the contractile efficiency of the myocardium in response to chronic VO.

Finally, in order to measure the non-mitochondrial OCR (OCR$_{non-mito}$) of the adult cardiomyocyte, antimycin A (10 µM) and rotenone (1 µM) were injected in the media simultaneously to inhibit the mitochondrial electron transport chain, resulting in a substantial decrease in OCR. The remaining non-mitochondrial OCR can be attributed to extramitochondrial sources of O$_2$ consumption, such as XO and the NAPDH oxidases. In Figure 4C, there was a negative directional change in the non-mitochondrial OCR with allourpinol treatment in both the sham and ACF, however this did not approach statistical
significance among all groups, likely due to insufficient resolution inherent in this measurement.

_Sarcomeric Protein Expression in Chronic 8 Week ACF_

To determine the expression of sarcomeric proteins that regulate cardiomyocyte Ca\(^{2+}\) sensitivity we studied LV tissue homogenates by western blot and LV tissue sections by immunohistochemistry. Western blot was performed on 8 week tissue from Sham and ACF ± allopurinol. Expression of the Ca\(^{2+}\) reuptake channel, SERCA2, was increased in ACF vs Sham (p<0.05) and was unchanged among other groups. PLN, a regulatory protein that binds to SERCA2 and decreases its affinity for Ca\(^{2+}\), was unchanged among all groups. SLN, a small molecular weight protein that also decreases SERCA2’s affinity for Ca\(^{2+}\), was studied by immunohistochemistry. SLN distribution was along the sarcoplasmic reticulum and staining was diffuse in-between the Z-lines. Staining was increased in ACF vs Sham, which was prevented with allopurinol (ACF+allopurinol). These data suggest increased SLN expression may inhibit SERCA2 sensitivity to Ca\(^{2+}\) and contribute to LV contractile dysfunction in chronic ACF.

**Discussion**

There were several major findings in this study. XO inhibition preserves LV contractile function in chronic ACF. The maintenance of LV contractility with allopurinol was associated with preserved LVESD in the face of an increasing LVEDD. The load independent measure of contractility LV Emax was decreased in VO rats and improved with allopurinol treatment. Decreased LV contractility in VO rats was
associated with increased SLN, a Ca\(^{2+}\) regulatory protein, which was also prevented with allopurinol. Allopurinol treatment in both Sham and ACF groups increased PV-loop derived LV efficiency. Finally, the ratio of LV Emax to maximal cardiomyocyte O2 consumption, \(\text{EMax:MaxOCR}\) was decreased in ACF and improved with allopurinol.

Inflammation and oxidative stress produce ROS through a number of enzymatic systems including XO. Recently, we have shown an acute increase in ROS and XO activity in the ACF rat concurrent with bioenergetic dysfunction.\(^{(39)}\) It is well known that mitochondria and sarcomeric proteins are sensitive targets of oxidative stress. This is important since VO produces an increase in PVA as shown in Figure 3, which is known to require higher myocardial oxygen consumption (MVO\(_2\)) and ATP consumption.\(^{(27)}\) This is particularly interesting in the context of XO since ADP, the precursor to its substrates xanthine and hypoxanthine, is elevated under increased bioenergetic demand and bioenergetic dysfunction.\(^{(25, 38)}\) We reasoned this combination of increased energy demand and ROS generation increases the susceptibility of the VO heart to failure. Indeed, we have recently established that XO inhibition in acute VO improves cardiac contractility and bioenergetic function. However, the effects of chronic XO inhibition in the setting of cardiac VO are unknown. In the current study, we found chronic VO causes decreased LV systolic function as early as 4 weeks which progressively worsens through 8 weeks. This is in agreement with previous studies in this model where LV contractility is depressed without changes in ventricular compliance.\(^{(8, 37)}\)

Since XO inhibition in acute ACF preserves LV function and bioenergetic function we reasoned that chronic XO inhibition would be cardioprotective and would be associated with improved LV metabolic properties. To test this hypothesis, allopurinol
was used to inhibit XO-derived ROS and we studied LV function and isolated cardiomyocyte respiration in the XF-24 Seahorse extracellular flux analyzer. Important sequential changes in LV chamber function and remodeling with ACF are shown in Table 1. LVEDD and LVESD are increased at 4 weeks and continue dilation through 8 weeks. The increased LVEDD reflects the augmented preload in ACF. Due to the Frank-Starling mechanism (FSM), a healthy heart responds with a compensatory increase in stroke volume and maintenance of LVESD. This can be observed in the acute stages of ACF where LVEDD is increased with no change in LVESD.(33) Allopurinol preserves the FSM in chronic ACF, suggesting XO-derived ROS are associated with the transition between acute compensation and the transition of failure in VO (Figure 1). This is supported by changes in LV contractile function in ACF that begin at 6 weeks and progress through 8 week (Table 1, 2). Allopurinol treatment prevents the decline in LV function evidenced by preserved LV EF, LV FS, LV VCFr, and the load independent LV Emax. The possible mechanisms by which ROS may alter contractility are many. Changes in Ca\(^{2+}\) cycling/sensitivity, ultrastructural changes in the cardiomyocyte, or alterations of the length-tension relationship, all may be important.(17) Indeed, isolated cardiomyocytes exposed to mechanical stretch(30) and LVs of patients with chronic mitral regurgitation(1) demonstrate loss of myofibrillar integrity and evidence of increased ROS both of which can affect Ca\(^{2+}\) sensitivity.(4) Interestingly, LVs exposed to 12 weeks of ACF demonstrate decreased Ca\(^{2+}\) responsiveness with similar increases in LVESD.(37) Taken together, these data suggest allopurinol’s preservation of FSM and contractility may be dependent on alterations in Ca\(^{2+}\) sensitivity.
Initiation of cardiomyocyte contraction depends on intracellular Ca\(^{2+}\) and is highly regulated. The majority of intracellular Ca\(^{2+}\) that causes contraction is released from sarcoplasmic reticulum and is taken back up by SERCA2 during diastole. Recently, the modulation of SERCA2 activity has become important field of study in heart failure.\(^{(22, 23)}\) Decreased SERCA2 activity is generally considered pathologic and has been implicated in dilated cardiomyopathy and congestive heart failure.\(^{(18, 24)}\) Aberrant Ca\(^{2+}\) handling from SERCA2 leads to decreased reuptake during diastole and subsequent decreased release in systole which negatively impacts contractility. In the current study, we demonstrate increased SERCA2 expression in ACF that is unaltered with allopurinol. Importantly, SERCA2 Ca\(^{2+}\) reuptake is negatively regulated by PLN and SLN by decreasing its affinity for Ca\(^{2+}\). PLN has been extensively studied and overexpression of PLN is associated with decreased contractile performance \textit{in vivo}.\(^{(19)}\) SLN is the more recently discovered regulatory protein for SERCA2 activity. SLN mRNA is upregulated in a mouse model of cardiac hypertrophy and heart specific SLN overexpression decreases contractility and causes ventricular hypertrophy.\(^{(2, 3, 29)}\) In the current study, PLN expression is not changed with ACF or allopurinol treatment. However, SLN staining is markedly increased in LV tissue from chronic ACF hearts which was prevented with allopurinol. This finding suggests increased SLN negatively impacts contractile function in chronic ACF by decreasing SERCA2 affinity for Ca\(^{2+}\).

Altered Ca\(^{2+}\) handling is also linked to cardiac contractile efficiency.\(^{(35)}\) Energy metabolism has a major role in heart failure and is dependent on effect production and use of ATP in the cardiomyocyte. Under normal conditions, ATP is produced by oxidation of fatty acids and carbohydrates in the mitochondria and is primarily consumed
by the contractile machinery and ionic pumps. The oxygen cost for the production of this ATP and its effective use determine myocardial efficiency. The increased worked load in ACF is manifested by an increase in the PVA which is directly correlated to oxygen consumption. In a recent study, animals subjected to 12 weeks of ACF demonstrated an increased O$_2$ cost per unit contractility suggesting decreased myocardial efficiency in VO. Interestingly, acute administration of allopurinol in patients with dilated cardiomyopathy decreased the MVO2 per unit of contractile function. In the current study, allopurinol treatment increased myocardial efficiency (SW/PVA) in both Sham+allopurinol and ACF+allopurinol as compared to their untreated counter parts (Figure 3). In the ACF+allopurinol group this change was driven by decreased LV PE and LV PVA compared to ACF. The decreased LV PE suggests less energy waste with allopurinol treatment. Isolated cardiomyocyte from Sham+allopurinol and ACF+allopurinol animals demonstrate a directional decrease in Max$_{OCR}$ when studied on the XF-24 Seahorse analyzer, although not significant. To assess myocardial contractile efficiency in the context of the isolated cardiomyocyte the ratio of the volume independent LV contractility parameter EMax to Max$_{OCR}$ (EMax:Max$_{OCR}$) was determined. EMax:Max$_{OCR}$ ratio was decreased in chronic ACF compared to sham by 75% and was normalized with allopurinol treatment. This data suggests allopurinol protects the contractile efficiency of the myocardium in response to chronic ACF which is in agreement with findings in dogs with rapid pacing and high cardiac work states and patients with dilated cardiomyopathy.

Whether allopurinol improves LV function in chronic VO solely through its effect on the expression of sarcomeric proteins cannot be conclusively determined in the current
study. This is due in large part to the fact that, we cannot rule out post-translational changes in metabolic enzymes and proteins or conversions to a fetal genetic profile both of which have been shown to alter contractile efficiency. (32) Indeed, sarcomeric proteins can themselves be oxidatively modified through carbonylation, tyrosine nitration, and thiol oxidation. (4) These modifications and impair cardiomyocyte sensitivity to Ca\(^{2+}\) and contraction. (4) Importantly, we have reported that both thiol oxidation and tyrosine nitration are elevated in acute ACF. (39) Further, a recent study demonstrated cardiomyocytes incubated with XO and its substrates alters structural proteins and was associated with changes in energetic metabolism. (11) In addition, in vitro studies demonstrate that XO depresses myofilament sensitivity to calcium and that it co-localizes with nitric oxide synthase-1 in the sarcoplasmic reticulum in the mouse cardiomyocyte, which can regulate excitation-contraction coupling as well as myofilament oxidative damage. (20, 31) Whether pathological changes in Ca\(^{2+}\) regulatory proteins occur at the level of protein expression or protein modification or a combination thereof is the focus of future work.

In conclusion, we demonstrate for the first time that XO mediates LV remodeling and functional abnormalities in chronic VO. VO is associated with deleterious changes in sarcomeric proteins and cardiomyocyte contractile efficiency which are prevented with XO inhibition. This study provides a possible therapeutic target in VO for which conventional heart failure therapies have failed to improve LV remodeling or systolic function.


29. Pashmforoush M, Lu JT, Chen H, Amand TS, Kondo R, Pradervand S, Evans SM, Clark B, Feramisco JR, Giles W, Ho SY, Benson DW, Silberbach M,


Figure 1. Allopurinol improves cardiac remodeling in response to chronic volume overload. Rats were subject to either sham or ACF surgery with or without allopurinol (100mg/kg/day) mixed in standard rat chow. Serial echocardiography was performed every 2 weeks. LVESD and LVEDD were plotted for each time point mean±SE for ACF (black) and ACF+allopurinol (red). The largest range for all time points for Sham data (mean±SE) is indicated by the cross-hatched box. LVESD=LV end systolic dimension, LVEDD=LV end diastolic dimension. *=p<0.05 vs ACF.
Figure 2. Allopurinol improves LV systolic function in response to chronic volume overload of 8 weeks of ACF. Rats were subject to either sham or ACF surgery with or without allopurinol (100mg/kg/day) mixed in standard rat chow. Simultaneous echocardiography and high-fidelity pressure catheterization was performed at 8 weeks. A 3-5 second transient inferior vena cava occlusion was performed and a family of pressure volume loops were generated and analyzed with computational software. LV EF=LV ejection fraction, LV FS=LV fractional shortening, LV VCfr=LV velocity of circumferential shortening, LV EMax=LV maximal elastance *=p<0.05 vs Sham, #=p<0.05 vs ACF.
Figure 3. Allopurinol increases contractile efficiency in chronic volume overload. Rats were subject to either sham or ACF surgery with or without allopurinol (100mg/kg/day) mixed in standard rat chow. Simultaneous high-fidelity pressure catheterization and echocardiography were performed at 8 weeks. A 3-5 second transient inferior vena cava occlusion was performed and a family of pressure volume loops were generated and analyzed with computational software. LV PE=LV potential energy, LV PVA=LV pressure volume area. *=p<0.05 vs Sham, #=p<0.05 vs ACF.
Figure 4. Maximum oxygen consumption and Emax in response to chronic volume overload. Cardiomyocytes were isolated from rats subject to either sham or ACF surgery with or without allopurinol. Myocytes (7500 cells/well) were plated in a Seahorse extracellular flux analyzer and basal, maximal, and non-mitochondrial oxygen consumption were determined (panel A-protocol panel B-group data). Simultaneous echocardiography and high-fidelity pressure catheterization was performed at 8 weeks. A 3-5 second transient inferior vena cava occlusion was performed and a family of pressure volume loops were generated and analyzed to determine maximum elastance. A surrogate for cardiomyocyte efficiency was computed as the ratio of LV EMax and Maximal O₂ consumption (panel C). LV Emax= LV maximal elastance. *=p<0.05 vs Sham, #=p<0.05 vs ACF.
Figure 5. Ca^{2+} regulation proteins in chronic volume overload. Western blot analysis was performed on 8 week LV tissue to determine sarcoendoplasmic reticulum calcium ATPase (SERCA2) and phospholamban (PLN) expression (A). Protein densitometry was normalized to β-tubulin densitometry (B and C). Immunohistochemistry analysis was performed on paraffin-embedded formalin-fixed LV tissue to determine expression of the small molecular weight protein Sarcolipin (Red) in Sham (D), ACF (E), Sham+allop. (F), and ACF+allop. (G). The nuclei are stained blue with DAPI. White bar represents 20 µm. *=p<0.05 vs Sham.
Table 1. LV Temporal Response to Chronic ACF

<table>
<thead>
<tr>
<th></th>
<th>2 Week</th>
<th></th>
<th>4 Week</th>
<th></th>
<th>6 Week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>ACF</td>
<td>Sham+Allop.</td>
<td>ACF+Allop.</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>396 ± 24</td>
<td>351 ± 17</td>
<td>406 ± 19</td>
<td>378 ± 8</td>
<td></td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>7.6 ± 0.2</td>
<td>9.45 ± 0.2</td>
<td>7.67 ± 0.2</td>
<td>8.92 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>4.62 ± 0.15</td>
<td>5.39 ± 0.27</td>
<td>4.59 ± 0.25</td>
<td>4.99 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>LV EF (%)</td>
<td>69 ± 3</td>
<td>72.2 ± 2.6</td>
<td>73.0 ± 2.0</td>
<td>74.1 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>LV FS (%)</td>
<td>39.2 ± 1.2</td>
<td>43.1 ± 2.0</td>
<td>40.2 ± 2.5</td>
<td>44 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>VCFr</td>
<td>7.6 ± 0.4</td>
<td>7.4 ± 0.5</td>
<td>7.5 ± 0.5</td>
<td>8.5 ± 0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>373 ± 12</td>
<td>351 ± 9</td>
<td>389 ± 6</td>
<td>384 ± 15</td>
<td></td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>8.19 ± 0.19</td>
<td>10.32 ± 0.2#</td>
<td>8.38 ± 0.35</td>
<td>9.75 ± 0.5§</td>
<td></td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>4.92 ± 0.24</td>
<td>6.15 ± 0.22#</td>
<td>4.83 ± 0.34</td>
<td>4.95 ± 0.42*</td>
<td></td>
</tr>
<tr>
<td>LV EF (%)</td>
<td>66.2 ± 2.3</td>
<td>66.5 ± 2.9</td>
<td>72.5 ± 2.7</td>
<td>75.8 ± 2.8*</td>
<td></td>
</tr>
<tr>
<td>LV FS (%)</td>
<td>40.1 ± 2.4</td>
<td>40.2 ± 2.3</td>
<td>42.6 ± 2.3</td>
<td>49.5 ± 2.21*</td>
<td></td>
</tr>
<tr>
<td>VCFr</td>
<td>7.5 ± 0.5</td>
<td>6.8 ± 0.6</td>
<td>8.7 ± 0.5</td>
<td>9.1 ± 0.7*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>346 ± 16</td>
<td>338 ± 8</td>
<td>395 ± 9</td>
<td>369 ± 5</td>
<td></td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>7.9 ± 0.15</td>
<td>11.14 ± 0.22#</td>
<td>8.14 ± 0.25</td>
<td>9.75 ± 0.5§</td>
<td></td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>4.59 ± 0.22</td>
<td>7.23 ± 0.33#</td>
<td>4.7 ± 0.3</td>
<td>5.05 ± 0.37*</td>
<td></td>
</tr>
<tr>
<td>LV EF (%)</td>
<td>69.4 ± 3.6</td>
<td>61.2 ± 3.1</td>
<td>71.3 ± 2.5</td>
<td>77.1 ± 2.4*</td>
<td></td>
</tr>
<tr>
<td>LV FS (%)</td>
<td>41.9 ± 2.4</td>
<td>35.1 ± 2.3#</td>
<td>42.5 ± 2.1</td>
<td>48.4 ± 2.3*</td>
<td></td>
</tr>
<tr>
<td>VCFr</td>
<td>8.0 ± 0.4</td>
<td>6.1 ± 0.5#</td>
<td>8.7 ± 0.4</td>
<td>9.9 ± 0.7*</td>
<td></td>
</tr>
</tbody>
</table>

n=8 7 8 7

HR=Heart Rate, LVEDD=LV end diastolic dimension, LVESD=LV end systolic dimension, LV EF=LV ejection fraction, LV FS=LV Fractional Shortening, VCFr=Velocity of circumferential shortening # p<0.05 vs. Sham, *=p<0.05 vs. ACF, §=p<0.05 vs. Sham+Allopurinol
### Table 2. LV Hemodynamic and Functional Parameters in Chronic 8 Week ACF

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>ACF</th>
<th>Sham+Allop.</th>
<th>ACF+Allop.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate</td>
<td>381 ± 11</td>
<td>349 ± 6#</td>
<td>376 ± 11</td>
<td>364 ± 6</td>
</tr>
<tr>
<td>MAP</td>
<td>98 ± 5.8</td>
<td>87.9 ± 4.6</td>
<td>106 ± 16#</td>
<td>79.3 ± 7§</td>
</tr>
<tr>
<td>LVESP</td>
<td>78.6 ± 5.5</td>
<td>72 ± 3.6</td>
<td>86 ± 14.8</td>
<td>68 ± 4.2</td>
</tr>
<tr>
<td>LVEDP</td>
<td>7.0 ± 1.4</td>
<td>12.7 ± 2#</td>
<td>4.6 ± 1.0</td>
<td>10.5 ± 0.7##§</td>
</tr>
<tr>
<td>LVES σ</td>
<td>108.2 ± 20.2</td>
<td>67.9 ± 8.0</td>
<td>72.2 ± 12.8</td>
<td>73.6 ± 11.6</td>
</tr>
<tr>
<td>LVED σ</td>
<td>11.6 ± 3.3</td>
<td>18.1 ± 1.7#</td>
<td>4.4 ± 1.0#</td>
<td>17.4 ± 2.0##§</td>
</tr>
<tr>
<td>LVESwt</td>
<td>2.38 ± 0.2</td>
<td>3.16 ± 0.17#</td>
<td>2.92 ± 0.16</td>
<td>3.05 ± 0.21#</td>
</tr>
<tr>
<td>LVEDwt</td>
<td>1.56 ± 0.13</td>
<td>1.97 ± 0.12#</td>
<td>1.92 ± 0.13#</td>
<td>1.73 ± 0.10</td>
</tr>
<tr>
<td>LVESD</td>
<td>4.36 ± 0.21</td>
<td>7.18 ± 0.26#</td>
<td>4.24 ± 0.31</td>
<td>6.09 ± 0.34##§</td>
</tr>
<tr>
<td>LVEDD</td>
<td>8.16 ± 0.27</td>
<td>11.3 ± 0.29#</td>
<td>7.69 ± 0.23</td>
<td>10.91 ± 0.26#</td>
</tr>
<tr>
<td>LV EF</td>
<td>75.9 ± 1.8</td>
<td>63.3 ± 1.6#</td>
<td>74.2 ± 3.0</td>
<td>72.5 ± 2.6*</td>
</tr>
<tr>
<td>LV FS%</td>
<td>46.6 ± 1.6</td>
<td>36.6 ± 1.2#</td>
<td>45.3 ± 2.9</td>
<td>44.3 ± 2.4*</td>
</tr>
<tr>
<td>VCFr</td>
<td>9.8 ± 0.5</td>
<td>6.2 ± 0.4#</td>
<td>10.4 ± 1.0</td>
<td>9.3 ± 0.5*</td>
</tr>
</tbody>
</table>

LVESP=LV end systolic pressure, LVEDP=LV end diastolic pressure, LVES σ=LV End systolic stress, LVED σ=LV end diastolic stress, wt=wall thickness, LVEDD=LV end diastolic dimension, LVESD=LV end systolic dimension, LV EF=LV ejection fraction, LV FS%= LV Fractional Shortening, VCFr= Velocity of circumferential shortening

# = p<0.05 vs. Sham, *=p<0.05 vs. ACF, §=p<0.05 vs Sham+Allopurinol

### Table 3. Morphometric Data on 8 Weeks of ACF

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>ACF</th>
<th>Sham+Allop.</th>
<th>ACF+Allop.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>443±11</td>
<td>422±10</td>
<td>363±15#</td>
<td>373±10*</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.43±0.16</td>
<td>2.41±0.13#</td>
<td>1.20±0.04</td>
<td>2.33±0.14§</td>
</tr>
<tr>
<td>LV weight (g)</td>
<td>0.97±0.09</td>
<td>1.38±0.06#</td>
<td>0.82±0.03</td>
<td>1.45±0.07§</td>
</tr>
<tr>
<td>RV weight (g)</td>
<td>0.24±0.04</td>
<td>0.46±0.03#</td>
<td>0.18±0.01</td>
<td>0.41±0.03§</td>
</tr>
<tr>
<td>Lung weight (g)</td>
<td>1.7±0.1</td>
<td>2.4±0.2#</td>
<td>1.7±0.1</td>
<td>2.5±0.3§</td>
</tr>
<tr>
<td>Heart/Body Weight (x100)</td>
<td>0.32 ± 0.04</td>
<td>0.57 ± 0.03#</td>
<td>0.33 ± 0.014</td>
<td>0.62 ± 0.04§</td>
</tr>
</tbody>
</table>

# = p<0.05 vs. Sham, *=p<0.05 vs. ACF, §=p<0.05 vs Sham+Allopurinol
DISCUSSION

Introduction

When exposed to a volume stress, a set of changes are set forth within the myocardium involving its shape, size, and tissue composition. These changes typically follow an eccentric remodeling pattern with increases in LV chamber diameter that outpace changes in LV wt. While it is thought these changes are an effort to normalize the stresses placed on the ventricle, they never fully compensate for the increased work load and result in a futile feed forward cycle of remodeling that ends with a dilated failure phenotype. Attempted treatment strategies for VO have largely been borrowed from concepts that have met success in pressure overload. However, due to fundamental differences between the disease pathologies these treatments have failed.

To date there is no standard medical therapy that slows or stops the progression of VO. VO is clearly associated with increased myocardial energy demand and therapies directed towards protection or improvements in bioenergetics are gaining interest. The goal of these therapies is centered around maintaining the amount of cardiac work produced per unit of oxygen consumed. This relationship depends not only on normal production of ATP from the mitochondria but also on factors that regulate cardiac contractility. These factors include; cardiac geometry, cardiomyocyte sensitivity to Ca\(^{2+}\), and myofibrillar integrity. Importantly, oxidative stress is integrally connected to bioenergetics due to the high sensitivity of the mitochondria to ROS and independent mitochondrial production of ROS. As energy demand and hence ATP consumption is
increased due to VO the purine degradation pathway becomes central. As the final enzyme in the purine metabolic pathway, XO degrades its substrates, hypoxanthine and xanthine, into uric acid. In doing so, XO generates ROS when oxygen is used as an electron acceptor during the hydroxylation of xanthine or hypoxanthine. Classically an enzyme targeted in gout for its formation of uric acid, interest in XO as a therapeutic target due to its contribution to ROS has emerged in multiple fields of research including; cancer, neurodegeneration, immunology, and cardiovascular disease.\(^{135}\)

The case for XO involvement in VO is supported by three major concepts (Figure 1). First, as myocardial energy demand is increased, ATP is consumed and the purine degradation pathway is activated. This pathway provides increased substrates that are shuttled through XO. This is conceptually similar to the robust interest in XO in the process of ischemia reperfusion injury. In this setting, ATP breakdown products are accumulated during ischemia and are quickly metabolized by XO upon reperfusion, thereby generating superoxide and hydrogen peroxide. Secondly, the progression of VO is largely thought to be due to stretching forces placed on the myocardium due to the increased volume load. Recent evidence suggests XO is activated by mechanical stretch.\(^{150}\) Indeed, cardiomyocyte stretch was shown to increase ROS production via an unknown pathway.\(^{151}\) Taken together, this supports a role for XO in the myocardium’s response to stretch in VO. Lastly, both animal models and patients with VO demonstrate evidence of oxidative stress suggesting a role for enzymes involved in pro-oxidant pathways.\(^{61, 71}\) This finding is of particular importance in the case of XO due a mechanism of XO activation. XO activity can be increased in response to ROS via thiol oxidation. This sets up a possible feed forward cycle of XO ROS generation and
increased XO activation. As a progressive disease, concepts involving VO pathogenesis must consider processes that are feed-forward in nature.

The data presented in this dissertation examines the role of XO in both acute and chronic VO and its impact on cardiac function. XO inhibition with allopurinol was utilized in both settings. A combined in vitro and in vivo approach was taken to address important concepts in each case. Well defined end-points were measured with advanced technological approaches to assess cardiac function and the cardiomyocyte’s bioenergetic state in response to VO. The first set of experiments focused on the early events in VO and uncovered a potential role for XO in the acute setting of 24 hrs ACF in vivo. These data describe the cardiac response to VO and the beneficial effects of allopurinol treatment. To examine a potential mechanism of XO activation in acute ACF we studied the response of isolated cardiomyocytes exposed to mechanical stretch in vitro. Additional studies were conducted to determine the temporal response of allopurinol treatment in chronic VO using serial echocardiography through 8 weeks of ACF. Further, we utilized in vitro extracellular flux technology in combination with in vivo cardiac functional analysis to examine the role of cardiac efficiency and its interaction with XO in disease progression.

The implications and relevance of the data presented in this dissertation will be discussed with a focus on XO as a central factor in VO pathogenesis. The validity of XO as a therapeutic target in VO will be examined along with possible future directions of these projects and their application to other pathologies.
Figure 1. XO as a Central Factor in VO. XO is central the pathology of VO by being activated by three major processes that occur in VO. First, myocardial energy demand is increased in VO and provides increased substrates for XO. Second, increased diastolic wall stress exposed the myocardium to increased stretch which activates XO. Third, increased ROS from sources such as the mitochondria can activate XO by thiol oxidation.
Interactions Between Mitochondria, XO, and LV Function in Acute Cardiac VO

Initially, we characterized the response of the myocardium to acute VO utilizing the ACF rat animal model. Tissue analysis from these animals determined mitochondrial function and XO activity and distribution. This of data shows systolic and diastolic cardiac dysfunction is present within 24 hrs of ACF. This was associated with increased XO activity and bioenergetic dysfunction of isolated mitochondria. Allopurinol treatment normalized both systolic and diastolic function concurrent with improved mitochondrial function. Taken together, these data demonstrate a novel interaction between XO, mitochondria, and LV function in the setting of acute VO.

XO Activation and Distribution in LV Tissue in Acute VO

XO activity was increased in LV tissue homogenate from 24 hrs ACF. The method used to determine XO activity provides the necessary substrates and cofactors that are needed for XO metabolism and results in production of uric acid which is measured by HPLC and specificity is verified by allopurinol inhibition. Cardiomyocyte XO activity was approximately 10% of the XO activity in whole LV tissue homogenates after normalization to sample protein content. ACF LV tissue homogenates demonstrated a modest 15% increase in XO activity compared to Sham LV homogenates. Interestingly, XO activity from isolated ACF cardiomyocytes was increased 300% compared to sham cardiomyocytes. This suggests that ACF causes greater XO activation in cardiomyocytes even though other cellular locations of XO such as the endothelium...
and interstitial cells may contain higher basal levels of the enzyme. Changes in XO activity occurred without an increase in total XOR expression or cleavage as determined by western blot, suggesting a post-translational activation event. XO can be activated by either proteolytic cleavage or thiol oxidation of XDH.\textsuperscript{135} Both modifications in XDH structure change the site where electrons are transferred during the hydroxylation of either xanthine or hypoxanthine and thereby convert the enzyme to its oxidase form or XO. Data presented in stretched cardiomyocytes implicates thiol oxidation as the potential mechanistic explanation for this finding and is discussed in more detail later.

It is important to note that the measurement of XO activity is carried out under saturating substrate levels. The physiological interpretation of this data must also consider alterations in intracellular substrate concentrations. It is possible that VO produces an environment in which XO activity is increased in the setting of enhanced substrate availability thus amplifying XO’s role. This contention is supported by an increased PVA in VO which directly correlates to an increased rate of \( \text{O}_2 \) consumption.\textsuperscript{191} Further, free ADP concentration is increased in the failing heart and is associated with increased \( \text{O}_2 \) consumption.\textsuperscript{192} ADP is degraded in the purine metabolic pathway ultimately generating hypoxanthine. These data support a role for XO in VO by either a direct increase in XO activation, increased substrate availability, or importantly a combination thereof.

Immunohistochemistry from sham and ACF rats demonstrated extensive XO/XDH distribution in endothelial cells and interstitial cells and along Z-lines in cardiomyocytes which is consistent with our previous report in the human heart.\textsuperscript{71} Subcellular localization of XO has not been fully characterized due in part to differences
in detection methods thus yielding inconsistent results. However, identification of XOR in endothelial cells has been previously reported in multiple studies. Further, XOR has also been previously indentified in interstitial cells such as leukocytes and mast cells. The extensive XOR staining present in endothelial cells and interstitial cells may explain the large difference in XO activity found in LV tissue compared to that found in isolated cardiomyocytes.

Preferential staining of XOR along the cardiomyocyte’s Z-line is of particular interest due to the function of the Z-line and its connection to large structural sarcomeric proteins. The Z-line defines the lateral boundaries of the sarcomere and is responsible for force transmission in-between cardiomyocytes. Along with providing an anchor point for structural proteins, recent evidence suggests proteins that form the Z-line can interact with nuclear signaling. For example, myopalladin is highly expressed at actin filament anchorage points and its overexpression results in disruption of both the Z-line and sarcomere. Interestingly, in cardiac biopsies taken from normal patients and those with chronic VO, XO was uniformly distributed along the Z-line in normal tissue but was associated with areas of myofibrillar disarray in VO tissue. This suggests XO’s close proximity to important structural components of the cardiomyocyte may predispose them to oxidative forces resulting from XO metabolism. This concept will be discussed in further detail involving experiments of cardiomyocyte mechanical stretch.
Role of XO Inhibition on LV Function in Acute VO

Previous studies involving acute VO of 24 hr ACF have not described abnormalities in LV systolic function. However, these studies only utilized echocardiography to assess LV contractility. LV PV-loop analysis allows for a more robust examination of LV contractility that is places traditional echocardiography measurements in the context of ventricular pressures. Data presented in this dissertation, demonstrates systolic function is depressed in acute VO evidenced by decreased LV ESPVR. ESPVR characterizes the maximal pressure that can be developed by the ventricle at any given LV volume. As the contractility of the myocardium increases, the slope of the ESPVR line increases. Treatment with allopurinol preserved the LV ESPVR while traditional measures of LV contractility such as LV EF did not change. These findings suggest that the LV never fully compensates for the increased load it faces in ACF and that allopurinol treatment is cardioprotective at the onset of disease.

The acute change in venous return from ACF results in LV VO. This is reflected by an increased LV dimension and volume at the end of diastole (LVEDD and LVEDV, respectively). This acute volume change is characterized by diastolic dysfunction with increased LVEDP and LVED wall stress (σ). LVED σ characterizes the forces placed on the heart at end-diastole in context of the geometrical shape of the LV. Importantly, it is thought that LVED σ is the driving force behind the progressive deleterious response the LV exhibits under VO. Both LVEDP and LVED σ were normalized in ACF animals treated with allopurinol. Given that LVEDP is a component of the equation that derives LVED σ, it is important to note the both the end diastolic dimension and wall thickness
did not change with allopurinol treatment. This suggests that alterations in LVEDP are solely responsible for the improvement in LVED $\sigma$ with allopurinol.

LVEDP depends primarily on two factors; venous return or preload, and the compliance of the LV at end diastole. In this setting, given that mean arterial pressure in both ACF groups were similar, it is unlikely that allopurinol affects LV preload. Therefore, LV end diastolic compliance likely drives the beneficial diastolic effects of allopurinol treatment. The relationship between compliance and pressure of the LV is analogous to the stiffness of an inflatable object, where as the compliance decreases or stiffness increases the pressure required to inflate the object to a certain volume increases. In the case of an inanimate object, compliance is driven solely by the viscoelastic properties of the material of which it is comprised. However, in the case of the LV, compliance depends on the viscoelastic tissue properties of the extracellular matrix (ECM) and cardiomyocytes, as well as, the dynamic intracellular events surrounding diastolic relaxation.\textsuperscript{203, 204} Studies of ECM in acute VO demonstrate collagen dissolution that is unaltered by allopurinol treatment (unpublished observations). Diastolic relaxation is an active event that requires ATP to release the actin-myosin crossbridges formed during cardiomyocyte contraction. Further, increased cardiac ADP levels are linked to diastolic dysfunction by outcompeting ATP at the actin-myosin crossbridge site. This impairs the rate limiting step of crossbridge cycling and relaxation by delaying ADP dissociation from the crossbridge site.\textsuperscript{205} Indeed, artificially altered ADP levels directly correlate with increased LVEDP in the rat heart.\textsuperscript{206} This implicates bioenergetic processes involved in ATP/ADP regulation as contributors to diastolic dysfunction in acute VO and a possible pathway by which allopurinol may intervene (Figure 2).
Figure 2. Bioenergetics and Diastolic Function. Bioenergetic dysfunction is associated with an increased ADP/ATP ratio. High ADP levels can inhibit actin-myosin crossbridge cycling and decrease LV diastolic function. This results in decreased LV compliance and is evidenced by increased LV end diastolic wall stress (LVED σ) and LV end diastolic pressure (LVEDP).
Bioenergetic Dysfunction and XO in Acute VO

It is well known that the mitochondria are both targets and sources of oxidative stress. VO creates a unique cardiac environment in which an increased stroke volume must be achieved in order to maintain normal systemic perfusion. This places an increased demand on the mitochondria for ATP production, in a setting of activated XO. Studies in isolated mitochondria demonstrated a decrease in State 3 mitochondrial function in response to VO which is prevented by allopurinol treatment. State 3 mitochondrial function represents the maximal ability of the mitochondria to consume oxygen at saturating substrate levels. When exposed to ROS, components of the mitochondria are highly sensitive to damage including; mt DNA, proteins, and lipids. This can result in inefficient ATP production, alterations in the mitochondrial membrane potential, mitochondrial swelling, or apoptosis. Indeed, mitochondria from VO patients display swelling and altered architecture when examined by transmission electron microscopy. It is tempting to speculate that allopurinol preserves mitochondrial function by limiting XO-derived ROS damage.

While the mitochondria may become inefficient ATP producers in the setting of VO, energetic demands are unrelenting and ATP levels remain normal until the very last stages of HF. Under these circumstances more substrates are shuttled through the mitochondria and the ADP/ATP ratio increases. Increased ADP, as discussed previously, can independently act as a signaling molecule disrupting cardiac function and provide additional substrates to purine degradation pathways such as that involving XO. Together, these concepts setup a catastrophic cycle (Figure 3) of mitochondrial functional decline, further ROS generation, and cardiac dysfunction.
Figure 3. Link between VO, XO, and the Mitochondria. VO increases energy demand subsequently increasing substrates for XO. XO causes oxidative stress and damages the mitochondria. The mitochondria then become less efficient which generates more XO substrates and generates ROS which further activates XO. This interplay is purposed to contribute to the progressive remodeling and dysfunction in VO.
Protein Nitration in Acute VO

Certain amino acids are sensitive to oxidation by ROS and RNS. These modifications can alter the properties of these proteins changing their catalytic activity, conformation, intracellular location, and susceptibility to proteolysis. One example of these modifications is combined ROS/RNS action that adds a nitro group (NO$_2$) on aromatic residues such as tyrosine. There is extensive studies detailing increased nitrotyrosine modifications in heart disease and our lab recently reported similar findings in both ACF rats and VO patients. Nitrotyrosine staining was increased in LV tissue sections from 24 hr ACF compared to Sham. This increase was prevented by allopurinol treatment. Interestingly, eNOS and iNOS were not increased in response to ACF suggesting XO-derived ROS independently increase nitrative modifications without changes in NOS expression. This is of particular interest as XO has been shown to co-localize with nitric oxide synthase-1 in the sarcoplasmic reticulum and can decrease excitation-contraction coupling by nitration of Ca$^{2+}$ handling proteins. It should also be noted that nitrotyrosine staining was closely associated with the Z-line sharing a similar pattern as XO. These data suggest a causative role for XO in nitrative modifications possibly contributing to cardiomyocyte dysfunction in VO.

Mechanical Stretch Induces XO Activity by a Mitochondrial ROS Dependent Pathway

Studies were conducted to explore a potential mechanism for XO activation in response to VO. An in vitro approach was taken utilizing isolated adult cardiomyocytes and exposing them to short-term mechanical stretch. The rational for this experiment was
based upon findings related to stretch and ROS. Mechanical stretch has been associated with ROS generation in smooth muscles cells, aortic endothelial cells, pulmonary endothelial cells, skeletal muscle, and cardiomyocytes.\textsuperscript{61, 150, 151, 211-213} In the case of cardiomyocyte stretch, a causative factor for ROS production has not been previously determined. Therefore, we stretched isolated cardiomyocytes for 3 hrs of 5% sinusoidal strain and assayed the cells for XO activity. Indeed, XO activity was increased in cardiomyocytes subjected to cyclical stretch. To determine the effect of increased XO activity, we performed transmission electron microscopy of stretched cardiomyocytes and demonstrated mitochondrial swelling and myofibrillar degeneration localized to the Z-line which as discussed above displays the highest intensity of XO staining when studied by immunohistochemistry (Figure 4). Therefore, it is tempting to speculate that XO-derived ROS play a causative role in the degeneration of the myofibril in response to stretch. We have previously reported increased matrix metalloproteinase (MMP) activation at 24 hours of ACF which is associated with increased ROS formation.\textsuperscript{61} Interestingly, transgenic mice with heart specific overexpression of MMP-2 demonstrate Z-line breakdown and disruption of the mitochondrial architecture.\textsuperscript{214} While not explored in this dissertation, it is possible that MMP activation is mediated by ROS and causes the myofibrillar degeneration that has now been identified in the rat, dog, and human with isolated VO.\textsuperscript{61, 71, 80, 95}
Figure 4. XO Distribution is Localized to Areas of Damage in Response to Stretch. Immunohistochemical demonstrates XO is localized to the Z-line the cardiomyocyte. Stretched cardiomyocytes display increased XO activity which is associated with Z-line disarray (black arrows) and mitochondrial disruption (white arrows). This suggests XO’s close proximity to structural proteins in the Z-line may be important in myocardial stretch of VO.
To determine if XO-derived ROS plays a causative role in the myofibrillar degeneration of stretched cardiomyocytes, we pretreated cells with allopurinol. Indeed, allopurinol prevents myofibrillar degeneration and protected the mitochondrial architecture. Increased ROS is associated with mitochondrial swelling and addition of general antioxidants is protective.\textsuperscript{215, 216} Further, genetic-based animal models of cardiac hypertrophy have also demonstrated myofibrillar degeneration at the Z-line concurrent with mitochondrial disarray suggesting a possible connection between the two that is upstream of XO.\textsuperscript{217, 218} Since XO activation can occur through ROS dependent-thiol oxidation, we hypothesized that the mitochondria may provide the early event responsible for activation of XO. This hypothesis was tested using pretreatment with the mitochondrial ROS inhibitor MitoQ in stretched cardiomyocytes. MitoQ accumulates specifically in the mitochondria and in its quinol form acts as an antioxidant. MitoQ pretreatment prevented the stretch induced increase in XO activity and also preserved the integrity of both the myofibril and the mitochondria. These data suggest that mitochondrial ROS are responsible for XO activation in response to mechanical stretch likely through a thiol oxidation dependent mechanism and not increased XOR proteolysis. This is in agreement with previously discussed data regarding XO activation \textit{in vivo}. LV tissue from 24 hr ACF animals displayed increased XO activity without an increase in total XOR protein or cleavage products suggesting a post-translational activation event. Taken together, these data suggest a combination therapy of allopurinol and MitoQ may have a synergistic effect \textit{in vivo}. 
Inhibition of XO Improves LV Function and Remodeling in Chronic VO

The temporal response of allopurinol treatment in chronic VO was characterized utilizing the 8 wk ACF rat animal model of VO. Serial echocardiography was performed bi-weekly through 8 wks of ACF. This data demonstrates progressive adverse LV remodeling and deterioration of cardiac function in response to ACF both of which are prevented with allopurinol treatment. Further, *in vitro* extracellular flux technology in combination with *in vivo* cardiac functional analysis was utilized to examine the role of cardiac efficiency and its interaction with XO in disease progression. Chronic ACF was associated with decreased cardiac contractile efficiency which was improved with allopurinol. LV tissue analysis suggests Ca$^{2+}$ handling proteins are aberrantly altered in chronic VO and normalized with allopurinol.

*Effect of Allopurinol on the LV Temporal Response to Chronic VO*

To establish the LV response to chronic VO in the context of allopurinol treatment, serial echocardiography was performed at 2, 4, 6, and 8 weeks of ACF. ACF demonstrates the three phases of cardiac failure. The acute phase, as discussed previously, is characterized by an increase in LVED σ and a modest increase in LVEDD. This occurs without changes in LV ejection fraction or LVESD. During this phase LV, wt remains constant at both end-systole and end-diastole. The chronic compensated phase is characterized by increases in LVED σ, LVEDD, and now LVESD. The eccentric remodeling pattern becomes more apparent as increases in chamber diameter outpace changes in wt. LV contractility as characterized by traditional echocardiography methods remains normal. Data presented previously suggests this phase starts by 2
weeks of ACF and lasts through 4 weeks. In ACF animals LVEDD and LVESD both are increased by 4 weeks concurrent with contractility parameters LV EF, LV VCFr, and LV FS. This suggests that the heart is dilating in response to the increased volume, but is able to maintain LV function. The Frank-Starling relationship allows a compensatory increase in stroke volume in response to increased preload.\(^27\) This mechanism along with increased adrenergic drive is thought to be responsible for LV compensation during this phase.\(^28, 200\) However, as discussed previously, it should be noted that more sensitive measurements of LV function (ESPVR) demonstrate LV contractile abnormalities, as earlier as, 24 hrs of ACF. This contractile dysfunction may be masked due to ventricular loading conditions inherent in both ACF and patients with MR.\(^219\)

The chronic decompensated phase of cardiac failure is characterized by further increases in LVED \(\sigma\), LVEDD, and LVESD with overt contractile dysfunction marked by decreased LV EF, LV VCFr, and LV FS. The transition between the chronic compensated and chronic decompensated phase appears to begin between 4 and 6 wks of ACF, as contractile function is preserved at 4 wks and depressed by 6 wks. This is evidenced by further eccentric remodeling by 6 wks coupled with decreased LV FS and LV VCRr, which progresses through 8 wks. This pattern of remodeling follows closely to previous reports in this animal model.\(^200, 220\) While the distinctions between each phase of failure may appear somewhat arbitrary and are a moving target, clinical evidence supports differing outcomes for patients in each phase.\(^1, 221\)

For example, VO patients with MR demonstrate decreased survival following reparative surgery once LV EF falls below 60%.\(^219, 221, 222\) Therefore it is important to determine what biological processes dictate the transition between each phase.
We demonstrated allopurinol effectively protects LV contractility and LV end-systolic remodeling through 8 weeks of ACF. The major indices of LV systolic function (LV EF, LV FS, LV VCFr) in allopurinol treated ACF rats remain similar to Sham levels at all studied time points. This occurred with similar LV diastolic dilation in both ACF groups suggesting similar LV preloads. The fact that ACF and ACF+allopurinol displayed similar diastolic remodeling suggests that improvements in LV contractility were due to protective forces on the myocardium and not changes in venous return. Further, patency of the fistula was verified at time of animal sacrifice. Importantly, LVESD was significantly protected through 8 weeks in ACF+allopurinol. However, by 8 weeks LVESD, while still improved compared untreated ACF, was significantly higher than Sham. Clinically, LVESD serves as a key indicator for timing of mitral valve repair. In the case of asymptomatic severe MR, surgical intervention is recommended once LVESD ≥ 40mm.\(^{223}\) While it is apparent allopurinol exerts a cardioprotective effect in chronic VO these benefits may not be indefinite. However, these findings do indicate that allopurinol mitigates the transition from chronic compensated HF to chronic decompensated HF in ACF.

*Allopurinol Improves Contractile Efficiency in Chronic VO*

The foundation for an interaction between VO, bioenergetics, and XO was laid in our acute studies of ACF and is continued through the chronic studies. It was demonstrated that 8 wks of ACF is associated with increased LV PVA compared to Sham. LV PVA directly correlates to increased LV \(O_2\) consumption.\(^{191}\) With analysis of PV loop data, it is possible to estimate the potential energy (LV PE) stored in the
ventricle that is not utilized to produce ejection. The LV PE is represented by the area of a triangle bound by the ESPVR on the top-left, the end diastolic pressure volume relationship on the bottom, and the PV loop on the right. As LV contractility is increased, the slope of the ESPVR increases. This effectively decreases wasted cardiac PE and is reflected by area changes in the PE triangle as the x-axis intercept of the top-left bounding line encroaches towards the PV loop (Figure 5). Therefore an isolated increase in LV contractility, as defined by the ESPVR, is reflected by a decrease in LV PE.

8 wk ACF animals demonstrate a 100% increase in PVA and PE compared to Sham which was significantly attenuated with allopurinol treatment. Since PVA=Stroke Work+PE and stroke work is relatively unchanged with allopurinol treatment, changes in PE are likely the driving force responsible for changes in PVA. These data suggest chronic ACF is associated with increased O2 consumption which is prevented by allopurinol possibly by decreasing wasted cardiac PE.

Since it is possible to calculate surrogate markers for both the energy utilized (PVA) and energy wasted (PE) during the cardiac cycle, it is also possible to determine cardiac efficiency. The equation LVefficiency=SW/PVA determines how effectively the myocardium utilizes energy to produce a given heart beat. We demonstrated that allopurinol treatment is associated with improved LV efficiency in both 8 wk Sham and ACF rats compared to their untreated counterparts. In both cases, this was due to a decrease in LV PE. These data suggest that allopurinol treatment improves LV
Figure 5. Cardiac Contractile Efficiency in Chronic VO. PV loop analysis demonstrates increase LV potential energy, a surrogate marker for wasted energy in chronic ACF. Allopurinol administration normalized LV PE. LV PE is the area bound by the ESPVR (red line), EDPVR (blue line), and the PV loop.
contractile efficiency in both normal and chronic VO rats. Indeed, acute administration of allopurinol to both HF dogs and HF patients decreases the $O_2$ consumed per unit of cardiac work suggesting improved cardiac efficiency.\textsuperscript{148, 157} It is also important to note that LV efficiency in 8wk untreated Sham and ACF animals did not differ. Given the marked increase in LV PE in ACF animals, this finding was due to a compensatory increase in SW in the ACF group, suggesting the ACF is still partially compensated at 8wks. Importantly, longer term studies of chronic ACF demonstrate depressed cardiac efficiency by 12 wks.\textsuperscript{220} Nevertheless, chronic ACF is associated with increased waste of cardiac energy which is prevented by allopurinol. These data provide insight into a possible mechanism by which allopurinol preserves cardiac function in chronic VO.

To gain further insight into the bioenergetic state of the chronic ACF, an \textit{in vitro} approach utilizing the XF-24 extracellular flux analyzer was used. Isolated cardiomyocytes from 8wk sham and ACF rats with and without allopurinol were studied for basal $O_2$ consumption rate (OCR), maximal OCR ($\text{Max}_{\text{OCR}}$), and non-mitochondria OCR. All parameters exhibited a directional, albeit statistically insignificant, decrease in OCR in both Sham and ACF rats with allopurinol treatment compared to their untreated counterparts. Of particular interest is the $\text{Max}_{\text{OCR}}$ which represents the FCCP-stimulated maximal OCR of the cardiomyocytes. Allopurinol decreased $\text{Max}_{\text{OCR}}$ 40\% in the treated ACF group compared to untreated ACF. To assess myocardial contractile efficiency in the context of the isolated cardiomyocyte the ratio of the volume independent LV contractility parameter $E_{\text{max}}$ to $\text{Max}_{\text{OCR}}$ ($E_{\text{max}}:\text{Max}_{\text{OCR}}$) was determined. $E_{\text{max}}:\text{Max}_{\text{OCR}}$ was significantly decreased by 75\% in chronic ACF compared to sham and was normalized with allopurinol treatment. There are two possible interpretations of this data
which rely on the determination of what MaxOCR physiologically represents. On one hand, MaxOCR could be viewed as a measurement of bioenergetic health of the cardiomyocyte where high MaxOCR would be desired. On the other hand, MaxOCR could be viewed as a reflection of in vivo demands for ATP and therefore O2 consumption. In the case of the latter changes in MaxOCR would depend on intracellular/intramitochondrial adaptations to current O2 demands. Given that allopurinol treatment directionally decreases MaxOCR and is associated with improved LV contractility, remodeling, and efficiency as determined by PV loop analysis, it is tempting to speculate that increased Emax:MaxOCR reflects improved contractile efficiency at the cardiomyocyte level. This interpretation would be in agreement with findings in HF dogs and HF patients where allopurinol improves myocardial efficiency but further studies are needed and are discussed in more detail below.148,157

Sarcomeric Protein Expression in Chronic VO

Cardiomyocyte Ca2+ regulation affects both contractile function and contractile efficiency. To initiate cardiomyocyte contraction, intracellular Ca2+ concentration is rapidly increased by Ca2+ release from the SR. SERCA2 is responsible for the reuptake of intracellular Ca2+ back to the SR causing cardiomyocyte relaxation. Aberrant Ca2+ handling from SERCA2 leads to decreased reuptake during diastole and subsequent decreased release in systole, which negatively impacts contractility. To determine if Ca2+ handling is involved in chronic VO, protein expression of SERCA2 and its regulatory proteins PLN and SLN were studied in LV tissue from 8 week ACF rats. SERCA2 and PLN expression did not vary between Sham and ACF with or without allopurinol
treatment. SLN, a newly discovered inhibitor of SERCA2 activity, was increased in ACF and normalized with allopurinol treatment. SLN is a small molecular weight (6 kD) protein that decreases SERCA2’s affinity for Ca\(^{2+}\). Increased SLN impairs contractility and contributes to ventricular hypertrophy. Interestingly, chronic ACF of 12 wks demonstrates decreased contractile responsiveness to infused Ca\(^{2+}\) and is associated with depressed contractile efficiency. These data suggest an interaction between XO and Ca\(^{2+}\) handling in chronic VO and may provide new therapeutic targets directed at modulating SERCA2 activity.

Whether allopurinol improves LV function in chronic VO solely through its effect on the expression of sarcomeric proteins cannot be conclusively determined in the current work. We cannot rule out post-translational changes in metabolic enzymes and proteins or conversions to a fetal genetic profile both of which have been shown to alter contractile efficiency. Further, sarcomeric proteins can be oxidatively modified through carbonylation, tyrosine nitration, and thiol oxidation which impairs cardiomyocyte sensitivity to Ca\(^{2+}\) and contraction. Whether pathological changes in Ca\(^{2+}\) regulatory proteins occur at the level of protein expression or protein modification or a combination thereof is the focus of future work.

Future Studies

*Direct Bioenergetic Measurements in Chronic VO with Allopurinol*

The studies presented in this dissertation heavily implicate pathological energetic involvement in cardiac VO. While these studies provide novel insight into possible therapeutic approaches involving XO inhibition in VO, a direct measurement of cardiac energetics *in vivo* would be preferable. Such studies have been conducted in HF patients
treated with acute allopurinol administration. However, with exception to the work presented this dissertation, it is unknown whether chronic allopurinol administration affects cardiac efficiency. Due to small vessel size and limited experimental equipment, this is a technically complicated task to achieve in rodents. To produce these data, the O\textsubscript{2} concentration of blood going into the heart muscle and the concentration of the heart’s venous return must be determined in the context of how much contractile work the heart produced for that amount of O\textsubscript{2} consumed. Utilizing a donor rat to perfuse the heart of the studied rat, it is possible to make these measurements in rodents. Indeed, this experimental design has demonstrated a decrease in cardiac efficiency in the 12 wk ACF. Future studies utilizing this technique can provide direct evidence that XO inhibition in chronic ACF improves cardiac efficiency.

\textit{Determine if MitoQ Therapy is Beneficial in VO}

MitoQ prevents XO activation in response to mechanical stretch. In addition, stretched-induced myofibrillar integrity and mitochondrial swelling are prevented with MitoQ. This suggest mitochondrial ROS are at least the initial event responsible for stretch-induced XO activation. Therefore, studies that investigate whether MitoQ prevents LV dysfunction in chronic VO are warranted. Further, while XO inhibition preserves LV contractility in 8 wk ACF rats, the trajectory of the LV end systolic dimension by 8 wks of allopurinol treatment suggests improvements will eventually be exhausted. Therefore, combination therapies utilizing both MitoQ and allopurinol may result in a synergetic effect that maintains or prolongs cardioprotection. Indeed, multiple studies suggest a role for MitoQ therapy in cardiovascular disease.
**Determine if Ca\(^{2+}\) Handling Proteins are Post-Translationally Modified in VO**

Data presented in this dissertation suggests Ca\(^{2+}\) regulation interacts with XO in chronic VO. This is based on expression levels of proteins involved in Ca\(^{2+}\) cycling. However, in the setting of increased oxidative stress it is possible that post-translational modifications may also play a role in Ca\(^{2+}\) regulation. Extensive work has been conducted regarding expression of SERCA2, PLN, and recently SLN in HF.\(^{14, 16, 21, 229}\) However, information about how sarcomeric protein modifications are involved in HF is incomplete. In this dissertation, nitrotyrosine staining is increased in acute ACF in a similar fashion to patients with chronic MR.\(^71\) Nitrotyrosine modification of SERCA2 is present in the aging heart and is associated with decreased Ca\(^{2+}\) uptake.\(^{230}\) These studies could be conducted by immunoprecipitation of sarcomeric proteins and then probing for modifications such as carbonylation, tyrosine nitration, and thiol oxidation. If modifications are identified they could be further characterized with mass spectrophotometry to identify altered residues which may have functional significance. For example, age dependent tryrosine nitration in SERCA2 occurs primarily on Tyr294-Tyr295 which is close sites responsible for Ca\(^{2+}\) translocation. Studies of these modifications could provide novel therapeutic targets in any cardiac disease involving Ca\(^{2+}\) handling abnormalities.

**Examine the Role of Mitochondria-Sarcoplasmic Reticulum Crosstalk in VO**

Bioenergetics and the mitochondria are implicated in this dissertation in both acute and chronic VO with possible associations with sarcomeric proteins. The spatial organization of the mitochondria is of interest due to its proximity to the sarcoplasmic
reticulum. This organization allows for a possibility of Ca\(^{2+}\) mediated crosstalk between the mitochondria and the sarcoplasmic reticulum. Ca\(^{2+}\) reuptake during diastole is regulated by the sarcoplasmic protein SERCA2 which requires large amounts of ATP to be provided by the mitochondria.\(^{229}\) In addition, mitochondrial Ca\(^{2+}\) signaling plays an integral role in apoptosis, cell death, and regulation of the permeability transition pore.\(^{231}\) Many studies have linked heart failure, aging, and other cardiovascular insults to apoptosis.\(^{232}\) Therefore the regulation of Ca\(^{2+}\) in connection with effective ATP production could be interconnected with cardiovascular pathologies especially in the setting of increased energy demand inherent to VO. Future studies should explore this possible interplay which may regulate cardiomyocyte function and death in HF.

**Determine the Point of No Return for XO Inhibition in VO**

This dissertation demonstrated that allopurinol therapy started at the beginning of VO in the rat preserved cardiac function and prevented systolic remodeling. To expand the application of this therapy, studies that define the point at which allopurinol treatment confers no benefit should be conducted. Relevant questions that should be addressed are: Is XO inhibition beneficial if started later in disease progression? and What is the role of XO inhibition following removal of VO by either repair of the ACF or the mitral valve in patients? These questions are of great importance due a recent clinical trial involving allopurinol’s metabolite, oxypurinol. In the OPT-CHF trial, oxypurinol failed to reverse HF in patients with advanced disease.\(^{165}\) Therefore studies that begin allopurinol treatment during the chronic compensated state and before overt failure ensues would provide valuable information that has a translational benefit. Indeed, early allopurinol treatment but not late allopurinol treatment was beneficial in a model of myocardial
In addition to these studies, it should be determined whether allopurinol improves cardiac function after removal of VO. Indeed, MR patients operated on under current guidelines demonstrate cardiovascular abnormalities and dysfunction after mitral valve repair. Therefore, it is possible that allopurinol may provide a therapeutic cardioprotective modality for VO patients prior to surgical repair. These studies could be conducted in the ACF model utilizing allopurinol prior to repair of the fistula vs untreated animals following repair.

Conclusions

This dissertation investigated pathogenic mechanisms mediated by XO in both acute and chronic VO in a combined in vivo and in vitro approach. The work presented in this dissertation contributes important information to the understanding of the progression and treatment of VO in patients. The description of in vivo changes in LV function and remodeling and the beneficial effects of allopurinol help determine critical time points for therapeutic intervention. Using advanced LV functional analysis we have demonstrated that the ACF is never fully compensated and systolic dysfunction is present by 24 hrs. Acute ACF was associated with increased energy demand and bioenergetic dysfunction. These data, combined with in vitro studies, suggests XO is activated by a myocardial stretch-dependent pathway which is initiated by ROS from the mitochondria. Together these findings establish a possible feed-forward vicious cycle present in acute ACF that may drive the progressive cardiac remodeling/dysfunction in VO. This contention is supported as chronic XO inhibition slowed the progression of LV remodeling and prevented LV dysfunction through 8 wks. Therefore, these studies support the hypothesis that XO plays a causative role in the myocardial
damage/dysfunction in both acute and chronic VO. Moreover, these studies provide a possible therapeutic target in VO for which conventional heart failure therapies have failed to improve LV remodeling or systolic function.
GENERAL LIST OF REFERENCES


11. Periasamy M, Huke S. Serca pump level is a critical determinant of ca(2+)homeostasis and cardiac contractility. *J Mol Cell Cardiol.* 2001;33:1053-1063


Yamaguchi O, Peterson A, Backx PH, Kurihara S, Hori M, MacLennan DH. Cardiac-specific overexpression of sarcolipin inhibits sarco(endo)plasmic reticulum Ca2+ ATPase (SERCA2a) activity and impairs cardiac function in mice. Proc Natl Acad Sci U S A. 2004;101:9199-9204


73. Samad Z, Kaul P, Shaw LK, Glower DD, Velazquez EJ, Douglas PS, Jollis JG. Impact of early surgery on survival of patients with severe mitral regurgitation. *Heart.* 2011;97:221-224


86. Sawyer DB, Siwik DA, Xiao L, Pimentel DR, Singh K, Colucci WS. Role of oxidative stress in myocardial hypertrophy and failure. *J Mol Cell Cardiol.* 2002;34:379-388
87. Tziomalos K, Hare JM. Role of xanthine oxidoreductase in cardiac nitroso-redox imbalance. *Front Biosci.* 2009;14:237-262


98. Li JM, Gall NP, Grieve DJ, Chen M, Shah AM. Activation of nadph oxidase during progression of cardiac hypertrophy to failure. *Hypertension*. 2002;40:477-484


110. Sorescu D, Griendling KK. Reactive oxygen species, mitochondria, and nad(p)h oxidases in the development and progression of heart failure. *Congest Heart Fail.* 2002;8:132-140

111. Sazanov LA. Respiratory complex i: Mechanistic and structural insights provided by the crystal structure of the hydrophilic domain. *Biochemistry.* 2007;46:2275-2288


171. Strauer BE. Cardiac energetics in clinical heart disease. Basic Res Cardiol. 1987;82 Suppl 2:389-402


200. Ryan TD, Rothstein EC, Aban I, Tallaj JA, Husain A, Lucchesi PA, Dell'Italia LJ. Left ventricular eccentric remodeling and matrix loss are


204. Lalande S, Johnson BD. Diastolic dysfunction: A link between hypertension and heart failure. *Drugs Today (Barc).* 2008;44:503-513


228. Supinski GS, Murphy MP, Callahan LA. Mitoq administration prevents endotoxin-induced cardiac dysfunction. *Am J Physiol Regul Integr Comp Physiol*. 2009;297:R1095-1102


APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORMS
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: September 22, 2009

TO: Dell'Italia, Louis J.
    BMR2 438 2180
    934-3969

FROM: [Signature]
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Xanthine Oxidase and TNF-Alpha in ACF Repair
        Sponsor: Internal
        Animal Project Number: 090908585

On September 22, 2009, the University of Alabama at Birmingham Institutional Animal Care and
Use Committee (IACUC) reviewed the animal use proposed in the above referenced application.
It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>B</td>
<td>312</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from September 2009. Approval from the IACUC
must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate
granting agency.

Refer to Animal Protocol Number (APN) 090908585 when ordering animals or in any
correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at
934-7692.
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF RENEWAL

DATE: April 26, 2011

TO: LOUIS J DELL’ITALIA, M.D.
BMR2-432 2180
FAX: (205) 996-2586

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Xanthine Oxidase and Bioenergetic Function in Volume Overload
Sponsor: NIH
Animal Project Number: 110409070

As of April 26, 2011, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>B</td>
<td>672</td>
</tr>
</tbody>
</table>

Animal use must be renewed by April 4, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 110409070 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7092.