ROLES AND MECHANISMS OF OXIDANT STRESS IN CARDIOVASCULAR DISEASE

DISSERTATION

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

By

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ABSTRACT

My primary thesis focus deals with understanding the pathology behind cardiovascular disease across disease states; retroviral (HIV/AIDS), diabetes related cardiovascular complications, and finally development of atrial arrhythmias (Section I, II & III), in an attempt to provide novel mechanistic insight into these complications so that specialized therapy can be developed to treat these complications. Section I: HIV related cardiovascular disease: The first four chapters deal with cardiovascular disease in AIDS, a field that has gained substantial importance over the last 5 years. With the prolongation of life expectancy therapy and increased quality of life due to HAART, a variety of HIV-related complications that are apparently not directly related to immunodeficiency or opportunistic infections have become apparent. HIV-related cardiovascular complications represent increasingly important contributors to the overall morbidity and mortality of HIV/AIDS patients. Chapter 2 reviews the emergent field of HIV related vascular abnormalities, the most prominent of which is an advanced/premature development of atherosclerosis and vascular lesion formation, primarily related to alterations in the vascular endothelium. The endothelium is now recognized as a uniquely vulnerable target in HIV infection,
because of its role as a critical regulator of access to major organs, and a potential reservoir of virus, as well as in of itself. The HIV virus itself, viral products and paradoxically components of antiretroviral therapies can have deleterious effects on the endothelium, maybe even acting synergistically. The mechanism behind the toxicities poorly defined, though many possible avenues for study have been suggested. Since the relationship between human HIV infection and cardiovascular disease can be complicated by various factors, demonstration of relevant animal models for the investigation of retrovirus-related cardiac dysfunction and pathologies could provide opportunities for further mechanistic insight and therapeutic intervention. In Chapter 3, we employed a well-established murine model of retroviral infection (LPBM5 virus) and defined the time-dependencies of retroviral progression and cardiac dysfunction, in an attempt to extend its relevance as a disease model. An additional goal was to test the hypotheses that altered NOS isoform expression and/or endothelial protein nitration are associated with time dependent vascular dysfunction during AIDS related vasculopathy in murine tissues and corroborated to our findings in human tissues. At 1 and 5 weeks we observed statistically significant decreased in KCL contractility and time dependent contractile deficits in response to the alpha adrenergic agonist PE, Emax reduced by approximately 40% (213±15 mg control vs. 133±16 mg at 10weeks), and EC50 values (102±7.3ng control vs.190±37ng at 5 weeks vs. 130±22ng at 10weeks, p<0.05). We also tested vascular relaxant responses and found a decreased endothelium dependent relaxation to ACH (EC50 control 120±27nM vs. 343±94nM at 10 weeks), while
the response to an exogenous NO donor SNP remained unchanged, suggesting a specific endothelial dysfunction. Histochemical investigations into the same tissues showed increased protein 3NT, ICAM, NOS-2, NOS-3 and XO. These findings were corroborated in concurrent experiments in a cohort of well-catalogued human cardiac micro-vascular tissues. In conclusion, we have demonstrated, for the first time, a specific functional vasculopathy with smooth muscle and endothelial involvement in a murine model of AIDS. Further, this observed vasculopathy was shown to be associated with and correlated with increased oxidative stress and specific endothelial activation.

As discussed in review chapter 2, hyperlipidemia and advanced atherosclerosis have recently been identified as side effects of HIV protease inhibitor (HIV-PI’s) therapies. However, the mechanisms involved are unclear. Endothelial cell dysfunction is now recognized as an initiating event in vascular lesion formation and atherogenesis and a contributor to dyslipidemia. In chapter 4 we tested the hypothesis that HIV-PI’s impose direct detrimental effects on vascular endothelium. Ritonavir (5µM) caused significant increase in ACH EC₅₀ (0.65±0.06 µM vs. 0.23±0.07 µM in control, p<0.05) but had no effect on Eₘₐₓ in mouse thoracic aorta segments. Saquinavir (5 µM) caused about 60% reduction in ACH Emax whereas it did not affect the EC50. After 18 hr incubation, Saquinavir, Indinavir and Ritonavir at 5 µM each caused significant increase of reactive oxygen species (2-4 fold increases, p<0.05). This increase was prevented by N-acetylcysteine (20 mM). These drugs also promoted necrosis and apoptosis in vitro, (annexin V only and annexin V/6-carboxy-fluorescein
diacetate double-label respectively). These results provide the first evidence for direct toxicities of HIV-PI’s to vascular endothelium. HIV-PI’s tested showed endothelial effects to varying degrees, suggesting that different HIV-PI’s may have differing mechanisms of endothelial toxicity. We demonstrated in this preliminary set of experiments that Ritonavir and Saquinavir showed more cytotoxicity than Indinavir. In Chapter 5 we investigated a role for direct endothelial toxicity induced by Saquinavir (SAQ), the first HIV-PI drug marketed in the US and still an important component of HAART therapies. Studies using human endothelial cells in culture at clinically relevant concentrations (5 and 10µM, 2-48 hrs) demonstrated concentration dependent increases in cell death, mainly via apoptosis rather than necrosis (determined via Annexin-V positive membrane labeling). Live cell imaging also demonstrated increased intracellular oxidant production (as measured by DCF fluorescence), which could be abrogated by incubation with the antioxidant N-acetylcysteine (NAC). NAC also prevented SAQ induced apoptotic cell death. These data demonstrate that SAQ has direct toxicological effects on human endothelial cells, and that the toxicity apparently involves apoptotic pathway activation via reactive oxygen and/or nitrogen species. Section II deals with cardiovascular complications of diabetes in a mouse model of streptozotocin induced hyperglycemia, a type I diabetes model. Clinically Type I diabetes mellitus is complicated by severe progressive cardiovascular diseases, including hypertension, congestive heart failure, coronary artery disease and arrhythmias. Time-dependencies of clinically relevant parameters of cardiac performance in a mouse model would be useful,
but have not been previously investigated. Hyperglycemia was induced in male CF-1 mice weighing 16-18g with a single dose of streptozotocin and animals were studied longitudinally at 0, 1 week and 5 weeks post-STZ, for assessment of both diastolic and systolic performance by non-invasive echocardiography, as well as electrocardiographically for conduction abnormalities. Significant and rapid hyperglycemia was observed at 1 week post-STZ and persisted throughout the 5 week study (avg 1w 254±33, 5w 308±41. mg/dl). Statistically significant impairment in LV fractional shortening was observed at both 1 week and 5 weeks (down ~20%). We also observed an early reduction in the E wave (Control 102.99±3.59, 1 week 88.43±5.17) and E/A ratios (control 3.53±0.25, 1 week 3.04±0.24) at 1 week, but at 5 weeks the diastolic parameters were back to control. ECGs from control and diabetic mice showed a significant decrease in the P-wave amplitude, P- duration however was unaltered. We noted a consistent time dependent increase in atrio-ventricular conduction time as shown by the increased QRS complex duration (Control 20.0±0.4ms, 1 week diabetic 22.39±0.53, 5 week STZ 25.16±0.84ms, p<0.005) and an increase in the QT interval, which persisted after heart rate correction, (QTcF, Control 126.4±2.3, 5w STZ 134.53±1.57). This study suggests that the STZ mouse model is appropriate for the mechanistic study of Type I diabetic cardiomyopathy, and provides time-dependent, clinically relevant assessments of cardiac performance (systolic, diastolic and electrocardiographic) as a foundation for further mechanistic studies which are shown in chapter 7. Since we carefully cataloged the data obtained in our functional studies, we had a unique opportunity to carry out histochemical
analysis that could then be correlated to diastolyic, systolic, conduction data on each individual animal and thus attempt to investigate the mechanisms involved in deteriorating cardiac. We observed protein 3NT levels increase dramatically (48%), but no concurrent increase in NOS2 or COX2 were observed. Specific induction of XO (44% increase), and increased C-RP (25%), was observed, as well as a significant reduction in number of PECAM stained microvessels (26%). Though there was no increase in Cx40 or Cx43 content, we also saw striking increases (60-70 fold) in mid-myocyte staining for cardiac Cx43, suggesting alteration of tightly regulated gap junctions in diabetes. Thus we found evidence supporting a role for increased reactive nitrogen species, through the induction of a specific oxidant enzyme XO, but not NOS, thus supporting the use of antioxidant therapies in diabetic cardiomyopathy.

In the final section, consisting of chapter 8, appendix A1 and appendix A2 we looked at mechanisms of cardiac dysregulation and development of arrhythmias in canine models. Atrial Fibrillation (AF) is the most common sustained arrhythmia in the United States currently affecting about 2 million Americans. While AF occasionally occurs in the absence of other cardiovascular disease (termed lone AF), it is more commonly seen in the presence of definite risk factors. Mitral Regurgitation (MR, present in 1-2% of general adult population) in particular is a common primary risk factor for development of AF. Data from animal models suggest that chronic AF developing as a result of MR is different from lone AF arising in the absence of detectable structural disease. In Appendix A1 we tested the hypothesis that these two models had differing underlying
mechanisms of structural remodeling, resulting in development of substrate for AF. Rapid pacing induced AF, results in acute changes in cellular hypertrophy, increased specific immune cell infiltration, and possibly oxidant production. Fibrosis along with cellular hypertrophy and immune cell infiltration appear to be involved in the long term remodeling associated with MR. In Chapter 8, we looked in more detail at the MR Dog model. We studied the development of AF susceptibility induced by mitral regurgitation in the canine model over time, to isolate the mechanisms of dysfunction. While the average degree of MR in our dogs was mild to moderate, the inducibility of AF in these dogs by right atrial pacing showed a marked increase in the MR vs. control dogs at both time-points (3/6 MR dogs at 1 month and 3/5 at 6 months). These studies showed the MR dog model to closely resemble clinical MR, with similar changes in atrial dilatation, P wave durations and K current changes. Immunohistochemistry showed regional increases in fibrosis. We also found data suggesting that increased oxidant stress and a chronic inflammation and infiltration are present.

We show that merely increased fibrosis is not sufficient to explain the mechanical and electrical abnormalities seen in MR, but rather, oxidative stress and inflammatory mechanisms might also play important roles by modulating various components. Finally in Appendix 2 we tested a novel therapeutic agent, ALT-711 that specifically targets fibrosis stability for its therapeutic value in chronic atrial dilatation (MR) induced AF. Advanced glycation end products (AGEs), accumulate and form crosslinks on long-lived vascular and myocardial structural proteins in normal aging e.g. collagen. Cross-linking results in
increased stability of fibrosis. Structural factors decreasing compliance (fibrosis) have been implicated in atrial fibrillation (AF). We used the dog model of mitral regurgitation (MR) induced AF. Electrophysiological experiments failed to show a significant effect of ALT-711 on MR induced changes in Ito and Iksus. MR induced site-specific increases in myocyte hypertrophy, interstitial fibrosis, myotubular content, and oxidant stress, but not increases in AGE formation. While ALT-711 succeeded in reducing fibrosis formation and also resulted in lower AGE staining, there was no effect on hypertrophy and oxidant stress markers. In conclusion, ALT-711 does not seem to be of major therapeutic value in this setting. Furthermore, decreases in fibrosis failed to reduce vulnerability to arrhythmia, suggesting that increased fibrosis is not enough to explain the development of arrhythmias in chronic atrial dilatation models.

Taken together, these studies show that increased oxidative stress is a common factor in cardiovascular dysfunction across disease states, and therapeutic agents targeting oxidative damage might have unique importance to cardiovascular health.
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PUBLICATIONS

Research Publications


FIELDS OF STUDY

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<td>α</td>
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<tr>
<td>AF</td>
<td>Atrial Fibrillation</td>
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<td>β</td>
<td>beta</td>
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<td>°C</td>
<td>degrees Celsius</td>
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<td>Cx</td>
<td>Connexin</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>HIV-PI</td>
<td>HIV protease Inhibitors</td>
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<td>INDI</td>
<td>Indinavir</td>
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<td>Abbreviation</td>
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<tr>
<td>MR</td>
<td>Mitral Regurgitation</td>
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<td>NAC</td>
<td>N-Acetly Cysteine</td>
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<td>3NT</td>
<td>3-nitrotyrosine</td>
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<td>RITO</td>
<td>Ritonavir</td>
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<tr>
<td>SAQ</td>
<td>Saquinavir</td>
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<td>XO</td>
<td>Xanthine Oxidase</td>
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CHAPTER 1
INTRODUCTION AND THESIS OBJECTIVES
Roles and Mechanisms Of Oxidative Stress In Cardiovascular Disease
Since the chapters presented herein are either already submitted for publication, or are formulated for submission, each chapter contains its own introduction and a brief rationale for the research presented. As such each chapter is considered to be a reasonably independent entity. Therefore this introduction chapter is intended to provide only a brief introduction to the subject matter that this thesis will deal with, followed by a brief overview of each chapter, in the context of existing knowledge in the research area.

The central theme of the thesis is the involvement of oxidative stress in cardiovascular pathology across disease states. However, as a graduate student in Dr. John Bauer’s lab, I have had the unique opportunity to be involved in several projects involving various aspects of cardiovascular disease, which is the leading cause of death in the United States. Traditional cardiovascular therapy, often very expensive, has often failed to ameliorate the cardiovascular complications due to some unique pathological contributors. Therefore, novel therapies to treat expensive and unique pathologies of heart disease are warranted.

My primary thesis focus deals with understanding the pathology behind cardiovascular disease across disease states; retroviral (HIV/AIDS), Diabetes related cardiovascular complications, and finally development of atrial arrhythmias (Section I, II & III), in an attempt to provide novel mechanistic insight into these complications so that specialized therapy can be developed to treat these complications.
Animal Models of cardiovascular disease are of unique importance, as they provide an opportunity to study cardiovascular disease in the absence of confounding factors that are prevalent and exceedingly difficult to control in the human population (diet, exercise, genetics, smoking, etc). Hence we have used a variety of mouse and canine models of disease. However, animal findings need to be corroborated to human data, and hence wherever possible we have tried to do so.

Section I: HIV related cardiovascular disease
The first four chapters deal with cardiovascular disease in AIDS, a field that has gained substantial importance over the last 5 years(1). In the US, the last decade has shown an important shift in the HIV related disease presentation, morbidity and mortality. This shift transpired as a result of the dramatic success of Highly Active Anti Retroviral Therapy (HAART) regimens that were introduced in 1993. However, with this same prolongation of life expectancy and increased quality of life, a variety of HIV-related complications that are apparently not directly related to immunodeficiency or opportunistic infections have become apparent. For example, HIV-related cardiovascular complications represent increasingly important contributors to the overall morbidity and mortality of HIV/AIDS patients. The major goals of chapter 2 are to address the following questions: What are the major vascular complications in HIV/AIDS? How important are they? How do these changes relate to disease progression? What are the potential mechanisms involved? What are the therapeutic issues?
The most prominent form of HIV related vascular abnormalities appear to be an advanced/premature development of atherosclerosis and vascular lesion formation and overall, the vascular changes observed in HIV patients seem to be primarily related to alterations in the vascular endothelium.(2-6)

We present a brief discussion of some potentially important mechanisms involved in this setting: Direct infection of endothelial cells by HIV virus, Toxicity of HIV related proteins, Systemic (non-specific) response to viral infection, Related drug toxicities (direct vs. indirect).

While the contribution of HAART drugs to this condition is implicated(7-13), reports of endothelial damage in the AIDS population precede the introduction of HAART(14), suggesting a complex pathological pathway of endothelial damage in the HIV patient population, with possible contributions of virus, resulting immune response and HAART drugs all playing a role. Dysregulation of endothelium, and subsequent release of endothelial factors may also play a role in this condition.

In conclusion, the endothelium is now recognized as a uniquely vulnerable target in HIV infection, because of its role as a critical regulator of access to major organs, and a potential reservoir of virus, as well as in of itself.

The HIV virus itself, viral products and paradoxically components of antiretroviral therapies can have deleterious effects on the endothelium, maybe even acting synergistically. The mechanism behind the toxicities remain poorly defined, though many possible avenues for study have been suggested.
Furthermore, even in this uniquely vulnerable population, the effect of traditional risk factors such as smoking, age, diet and exercise are still important and must not be overlooked. Whatever the mechanism of toxicity, it appears, that much like the diabetic population, the HIV+ population, is at high risk for development of cardiovascular disease, especially atherosclerosis, and hence should be prospectively managed as a pre-cardiac patient population.

Since the relationship between human HIV infection and cardiovascular disease can be complicated by the various factors mentioned above, demonstration of relevant animal models for the investigation of retrovirus-related cardiac dysfunction and pathologies could provide opportunities for further mechanistic insight and therapeutic intervention. In Chapter 3, we employed a well-established murine model of retroviral infection (LPBM5 virus) and defined the time-dependencies of retroviral progression and cardiac dysfunction, in an attempt to extend its relevance as a disease model. The LPBM5 model of retroviral infection is commonly called the "murine AIDS" model, due to its high similarity to many of the immune-related complications seen during human HIV infection, including aberrant cytokine release, changes in T-cell populations, and increased susceptibility to opportunistic infections.

An additional goal of Chapter 3 was to test the hypotheses that altered NOS isoform expression and/or endothelial protein nitration are associated with time dependent vascular dysfunction during AIDS related vasculopathy in murine tissues and corroborated our findings in human tissues. At 1 and 5 weeks we observed statistically significant decreased KCL contractility and time dependant
contractile deficits in response to the alpha adreneric agonist PE, Emax reduced by approximately 40% (213±15 mg control vs. 133±16 mg at 10weeks), and EC50 values (102±7.3ng control vs.190±37ng at 5 weeks vs. 130±22ng at 10weeks, p<0.05). We also tested vascular relaxant responses and found a decreased endothelium dependant relaxation to ACH (EC50 control 120±27nM vs. 343±94nM at 10 weeks), while the response to an exogenous NO donor SNP remained unchanged, suggesting a specific endothelial dysfunction. Histochemical investigations into the same tissues showed increased protein 3NT, ICAM, NOS-2, NOS-3 and XO. These findings were corroborated in concurrent experiments in a cohort of well catalogued human cardiac micro-vascular tissues. In conclusion, we have demonstrated, for the first time, a specific functional vasculopathy with smooth muscle and endothelial involvement in a murine model of AIDS. Further, this observed vasculopathy was shown to be associated with and correlated with increased oxidative stress and specific endothelial activation. And finally, this finding was echoed in a relevant population of human HIV/AIDS patients. Research into the sources and intracellular targets of oxidants in this disease could provide important mechanistic insights and may reveal new therapeutic opportunities for this increasingly important cardiovascular disease state, and may even afford an advantage of protecting vasculature (and perhaps more specifically the vascular endothelium) as well as stabilizing immune dysfunction.

As has been discussed in review chapter 2, hyperlipidemia and advanced atherosclerosis have recently been identified as side effects of HIV protease
inhibitor (HIV-PI’s) therapies. However, the mechanisms involved are unclear. Endothelial cell dysfunction is now recognized as an initiating event in vascular lesion formation and atherogenesis and a contributor to dyslipidemia. In chapter 4 we tested the hypothesis that HIV-PI’s impose direct detrimental effects on vascular endothelium. Ritonavir (5µM) caused significant increase in ACH EC\textsubscript{50} (0.65±0.06 µM vs. 0.23±0.07 µM in control, p<0.05) but had no effect on E\textsubscript{max} in rat thoracic aorta segments. Saquinavir (5 µM) caused about 60% reduction in ACH E\textsubscript{max} whereas it did not affect the EC50. Isolated murine endothelial cells were treated with HIV-PI’s in vitro and intracellular oxidant production was evaluated by microscopy and digital image analysis (DCF fluorescence). Cell death was also examined with dual labeling of carboxyl-fluoroscein and rhodamine-conjugated annexin V. After 18 hr incubation, Saquinavir, Indinavir and Ritonavir at 5 µM each caused significant increase of reactive oxygen species (2-4 fold increases, p<0.05). This increase was prevented by N-acetylcysteine (20 mM). These drugs also promoted necrosis and apoptosis in vitro, (annexin V only and annexin V/6-carboxy-fluorescein diacetate double-label respectively). These results provide the first evidence for direct toxicities of HIV-PI’s to vascular endothelium. These actions may contribute to PI related atherosclerosis. Furthermore we demonstrated that thought all the HIV-PI’s tested showed endothelial effects, they did so to varing degrees, thus suggesting that different HIV-PI’s may have differering mechanisms of endothelial toxicity. We demonstrated in this preliminary set of experiments that Ritonavir and Saquinavir
showed more cytotoxicity than Indinavir. Further, we wanted to corroborate our findings of cytotoxicity on murine cells in humans.

Hence in Chapter 5 we investigated a role for direct endothelial toxicity induced by Saquinavir (SAQ), the first HIV-PI drug marketed in the US and still an important component of HAART therapies. In initial studies using isolated vascular tissues we observed selective impairment of endothelium dependent vasodilation with no effect on contractile responses. Subsequent studies using human endothelial cells in culture at clinically relevant concentrations (5 and 10µM, 2-48 hrs) demonstrated concentration dependent increases in cell death, mainly via apoptosis rather than necrosis (determined via Annexin-V positive membrane labeling). Live cell imaging also demonstrated increased intracellular oxidant production (as measured by DCF fluorescence), which could be abrogated by incubation with the antioxidant N-acetylcysteine (NAC). NAC also prevented SAQ induced apoptotic cell death. These data demonstrate that SAQ has direct toxicological effects on human endothelial cells, and that the toxicity apparently involves apoptotic pathway activation via reactive oxygen and/or nitrogen species.

Section II: Diabetes related cardiovascular disease

In continuation of my study of cardiovascular pathogenesis mechanisms across disease states, Section II deals with cardiovascular complications of diabetes in a mouse model of streptozotocin induced hyperglycemia, a type I diabetes model.
Type I diabetes mellitus is complicated by severe progressive cardiovascular diseases, including hypertension, congestive heart failure and coronary artery disease (15-17). Diabetes is also a major risk factor for the development of arrhythmias (18). Mechanistic studies are often difficult to conduct in this patient population and can be confounded by co-existing variables. Time-dependencies of clinically relevant parameters of cardiac performance in a mouse model would be uniquely useful (for transgenic studies, etc), but have not been previously investigated. In chapter 6 we tested the hypothesis that the STZ diabetic mouse model, time mimics events observed in Type I non-atherogenic cardiomyopathy in a functionally relevant and linear manner. Hyperglycemia was induced in male CF-1 mice weighing 16-18g with a single dose of streptozotocin (STZ, 150 mg/kg i.p. prepared daily in citrate buffer pH 4.5 for maximal stability). Animals were studied longitudinally at 0 and 5 weeks post-STZ (n=18 each time point), for assessment of both diastolic and systolic performance by non-invasive echocardiography, as well as electrocardiographically for conduction abnormalities. Significant and rapid hyperglycemia was observed at 1 week post-STZ and persisted throughout the 5 week study (avg 1w 254±33, 5w 308±41. mg/dl). Statistically significant impairment in LV fractional shortening was observed at both 1 week and 5 weeks (down ~20%). We also observed and early reduction in the E wave (Control 102.99±3.59, 1 week 88.43±5.17) and E/A ratios (control 3.53±0.25, 1 week 3.04±0.24) at 1 week, but at 5 weeks the diastolic parameters were back to control. ECGs from control and diabetic mice showed a significant decrease in the P-wave amplitude, P- duration however was
unaltered. We also observed a consistent time dependant increase atrio-
ventricular conduction time as shown by the increased QRS complex duration 
(Control 20.0±0.4ms, 1 week diabetic 22.39±0.53, 5 week STZ 25.16±0.84ms, 
p<0.005). Further, we observed an increase in the QT interval, which persisted 
after heart rate correction, (QTcF, Control 126.4±2.3, 5w STZ 134.53±1.57).

This study suggests that the STZ mouse model is appropriate for the 
mechanistic study of Type I diabetic cardiomyopathy, and provides time-
dependent, clinically relevant assessments of cardiac performance (systolic, 
diastolic and electrocardiographic) as a foundation for further mechanistic 

Since we carefully cataloged the data obtained in our functional studies, we had 
a unique opportunity to carry out histochemical analysis that could then be 
correlated to diastolyic, systolic and conduction data on each individual animal 
and thus attempt to investigate the mechanisms involved in deteriorating cardiac 
function which are detailed in Chapter 7. After sacrifice, the apical portion of the 
heart was equatorially bisected just distal to the mitral valve and immersed in 
10% formalin. Immunohistochemistry for protein 3-nitrotyrosine(3NT), Nitric 
Oxide Synthase-2 (NOS2), Cyclooxygenase-2 (COX2), Xanthine Oxidase (XO), 
Glucose transporter (GLUT4), C-reactive protein (CRP), Platelet Endothelial Cell 
Adhesion Molecule (PECAM), Connexin40 (Cx40) and Connexin43 (Cx43) was 
then carried out. We saw protein 3NT levels increase dramatically (48%), but no 
concurrent increase in NOS2 or COX2 were observed. Specific induction of XO 
(44% increase), and increased C-RP (25%), was observed, as well as a
significant reduction in number of PECAM stained microvessels (26%). Though there was no increase in Cx40 or Cx43 content, we also saw striking increases (60-70 fold) in mid-myocyte staining for cardiac Cx43, suggesting alteration of tightly regulated gap junctions in diabetes. Thus we found evidence supporting a role for increased reactive nitrogen species, through the induction of a specific oxidant enzyme XO, but not NOS 2, thus supporting the use of antioxidant therapies in diabetic cardiomyopathy. Furthermore we found evidence of increased myocardial CRP, which might be of interest as a potential pathophysiological agent as well as a marker of inflammation and cardiovascular risk. We observed a significant reduction in myocardial microvessel density, suggesting an explanation for the increased ischemic risk in diabetes, even in the absence of overt macrovascular coronary artery disease. And finally we show that cardiac ventricular Cx43 distribution is altered during diabetic cardiomyopathy, and suggest that these alterations may mediate some of the electrophysiological abnormalities associated with this condition.

**Section III: Mechanistic investigations in Canine Models of atrial fibrillation**

In the final section, consisting of chapter 8, appendix A1 and appendix A2 we looked at mechanisms of cardiac dysregulation and development of arrhythmias in canine models.

Atrial Fibrillation (AF) is the most common sustained arrhythmia in the United States currently affecting about 2million Americans(19). While AF occasionally occurs in the absence of other cardiovascular disease (termed lone AF), it is more commonly seen in the presence of definite risk factors. Mitral Regurgitation
(MR, present in 1-2% of general adult population) in particular is a common primary risk factor for development of AF(20). About 50% of patients with mitral valve disease will develop AF. Data from animal models suggest that chronic AF developing as a result of MR is different from lone AF arising in the absence of detectable structural disease. We had unique access to animal (dog) models of both rapid pacing induced AF and surgically induced MR resulting in AF. In Appendix A1 we tested the hypothesis that these two models had differing underlying mechanisms of structural remodeling, resulting in development of substrate for AF. In conclusion, it appears that discrete mechanisms might be in play in the two different models of AF. Rapid pacing induced AF, results in acute changes in cellular hypertrophy, increased specific immune cell infiltration, and possibly oxidant production. On the other hand, fibrosis along with cellular hypertrophy and immune cell infiltration appear to be involved in the long term remodeling associated with MR. In Chapter 8, we looked in more detail at the MR Dog model. Mitral valve disease, in particular MR has a reported incidence of ~20-50% in patients with AF, and is one of the most common underlying co-morbidities associated with AF. Surgical correction of the valve rarely eliminates the arrhythmia, with approximately 75% of the patients continuing to have AF after surgery. We studied the development of AF susceptibility induced by mitral regurgitation in the canine model over time, to isolate the mechanisms of dysfunction. While the average degree of MR in our dogs was mild to moderate, the inducibility of AF in these dogs by right atrial pacing showed a marked increase in the MR vs control dogs at both time-points (3/6 MR dogs at 1 month
and 3/5 at 6 months). These studies showed the MR dog model to closely resemble clinical MR, with similar changes in atrial dilatation, P wave durations and K current changes. Immunohistochemistry however only showed regional increases in fibrosis, but not to the magnitude that would be required to produce the global conduction changes required to affect P wave duration (a whole atrial phenomena). We however also found data suggesting that the atrial as a whole is subjected to increased oxidant stress and a chronic globalized inflammation and infiltration. Furthermore we also saw changes in Cx content and distribution; Cx40 showed changes in both content and distribution, Cx 43 on the other hand showed no changes in distribution. Interestingly, this was the opposite of our findings in diabetic mouse ventricle, suggesting that Cxs might be differentially regulated in the atria.

We show that merely increased fibrosis is not sufficient to explain the mechanical and electrical abnormalities seen in MR, but rather, oxidative stress and inflammatory mechanisms might also play important roles by modulating various components, and heterologous expression of connexin protein might be involved in development of arrhythmia substrate. Finally in Appendix 2 we tested a novel therapeutic agent, ALT-711 that specifically targets fibrosis stability for its therapeutic value in chronic atrial dilatation (MR) induced AF. Advanced glycation end products (AGEs), are products of non-enzymatic glycation and oxidation of proteins and lipids. They have been shown to accumulate and form crosslinks on long-lived vascular and myocardial structural proteins in normal aging e.g. collagen. Cross-linking results in increased stability of fibrosis by decreasing
collagen breakdown. Structural factors decreasing compliance (fibrosis) have been implicated in atrial fibrillation (AF). We used the dog model of mitral regurgitation (MR) induced AF. MR was surgically induced in 12 dogs and after 6 months of MR, n=6 dogs received one dose of ALT-711. Tissue samples from left atrial appendage and left atrial free wall were also isolated and immediately formalin fixed for immunohistochemistry. Electrophysiological experiments failed to show a significant effect of ALT-711 on MR induced changes in Ito and Iksus. MR also induced site-specific increases in myocyte hypertrophy, interstitial fibrosis, myotubular content, and oxidant stress. However, there does not seem to be a corresponding increase in AGE formation. While ALT-711 succeeded in reducing fibrosis formation and also resulted in lower AGE staining, there was no effect on hypertrophy and oxidant stress markers. In conclusion, ALT-711 does not seem to be of major therapeutic value in this setting. Furthermore, decreases in fibrosis failed to reduce vulnerability to arrhythmia, suggesting that increased fibrosis is not enough to explain the development of arrhythmias in chronic atrial dilatation models.

Taken together, these studies show that increased oxidative stress is a common factor in cardiovascular dysfunction across disease states, and therapeutic agents targeting oxidative damage might have unique importance to cardiovascular health.
REFERENCE:


4. Lewis W: Atherosclerosis in AIDS: potential pathogenetic roles of antiretroviral therapy and HIV. *J Mol Cell Cardiol* 32:2115-2129, 2000


19. Levy S: Atrial fibrillation, the arrhythmia of the elderly, causes and associated conditions. *Anadolu Kardiyol Derg* 2:55-60, 2002

CHAPTER 2
VASCULAR ABNORMALITIES IN HIV PATHOGENESIS

This chapter has been prepared for submission to *Cardiovascular Toxicity* and is presented in the style appropriate for the journal.
ABSTRACT
In the US the last decade has shown an important shift in the HIV related disease presentation, morbidity and mortality. This shift transpired as a result of the dramatic success of Highly Active Anti Retroviral Therapy (HAART) regimens that were introduced in 1993. However, with this same prolongation of life expectancy and increased quality of life, a variety of HIV-related complications that are apparently not directly related to immunodeficiency or opportunistic infections have become apparent. For example, HIV-related cardiovascular complications represent increasingly important contributors to the overall morbidity and mortality of HIV/AIDS patients. The major goals of this review chapter are to address the following questions: What are the major vascular complications in HIV/AIDS? How important are they? How do these changes relate to disease progression? What are the potential mechanisms involved? What are the therapeutic issues? The most prominent form of HIV related vascular abnormalities appear to be an advanced/prefmature development of atherosclerosis and vascular lesion formation and overall, the vascular changes observed in HIV patients seem to be primarily related to alterations in the vascular endothelium. We present a brief discussion of some potentially important mechanisms involved in this setting: Direct infection of endothelial cells by HIV virus, Toxicity of HIV related proteins, Systemic (non-specific) response to viral infection, Related drug toxicities (direct vs. indirect). While the contribution of HAART drugs to this condition is implicated, reports of endothelial damage in the AIDS population precede the introduction of HAART, suggesting a complex pathological pathway of endothelial damage in the HIV patient population, with possible contributions of virus, resulting immune response and HAART drugs all playing a role. Dysregulation of endothelium, and subsequent release of endothelial factors may also play a role in this condition. In conclusion, the endothelium is now recognized as a uniquely vulnerable target in HIV infection, because of its role as a critical regulator of access to major organs, and a potential reservoir of virus, as well as in of itself. The HIV virus itself, viral products and paradoxically components of antiretroviral therapies can have deleterious effects on the endothelium, maybe even
acting synergistically. The mechanism behind the toxicities poorly defined, though
many possible avenues for study have been suggested. And furthermore, even in
this uniquely vulnerable population, the effect of traditional risk factors such as
smoking, age, diet and exercise are still important and must not be overlooked.
Whatever the mechanism of toxicity, it appears, that much like the diabetic
population, the HIV+ population, is high risk for development of cardiovascular
disease, especially atherosclerosis, and hence should be prospectively managed as
a pre-cardiac patient population.
INTRODUCTION

With the worldwide incidence of HIV infection approaching an estimated 5,000,000 cases annually, the human immunodeficiency virus (HIV) and resultant development of acquired immunodeficiency syndrome (AIDS) may represent the most pressing medical challenge of the last 20 years (1). Although important improvements in survival have occurred in the western world and developed countries (mainly due to improved antiretroviral drug therapies), unless HIV vaccination strategies prove effective the global crisis of HIV/AIDS is not likely to subside.

In the US the last decade has shown an important shift in the HIV related disease presentation, morbidity and mortality. This shift transpired as a result of the dramatic success of Highly Active Anti Retroviral Therapy (HAART) regimens that were introduced in 1993. HAART, a cocktail of several anti-retroviral drugs that interrupt various stages of the replication cycle of HIV, has significantly improved overall survival and increased time of AIDS-free living for patients infected with HIV (2). However, with this same prolongation of life expectancy and increased quality of life, a variety of HIV-related complications that are apparently not directly related to immunodeficiency or opportunistic infections have become apparent. For example, HIV-related cardiovascular complications represent increasingly important contributors to the overall morbidity and mortality of HIV/AIDS patients(3). Shown in Table 1 is a timeline of the events that shaped the current understanding of HIV-related vascular complications. Outlined in Table 2 is a collection of clinical and retrospective studies describing the presence of vascular abnormalities in AIDS. The
major goals of this review chapter are to address the following questions: What are the major vascular complications in HIV/AIDS? How important are they? How do these changes relate to disease progression? What are the potential mechanisms involved? What are the therapeutic issues?

**VASCULAR COMPLICATIONS OF HIV/AIDS**

Shown in Table 2 is a listing of several studies documenting vascular abnormalities in HIV infected individuals. Many of the initial observations of vascular involvement were made in autopsy related case reports, whereas more recent studies have prospectively evaluated the incidence of vascular structural or functional abnormalities. Collectively these studies have illustrated that vascular complications may be an important contributor to retroviral disease progression and mortality.

The most prominent form of HIV related vascular abnormalities appears to be an advanced development of atherosclerosis and vascular lesion formation. For example, autopsy studies have shown an 8% prevalence of accelerated atherosclerosis in HIV+ patients, even in the absence of other risk factors. Consistent with this enhanced vascular lesion development, acute coronary events in this patient population are known to occur almost a decade earlier than non-infected populations (4; 5). Unlike ‘traditional’ Acute Coronary Syndrome (ACS), HIV+ patients are more likely to have single vessel disease, suggesting a different etiology. There is also some evidence that these patients may be uniquely susceptible to restenosis after treatment (5), all of which suggest that vascular
complications during HIV infection are important phenomena in disease progression and overall mortality.

In addition to prevalence of atherosclerotic plaques in major vessels, other related vascular disorders such as vasculitis and morphological changes in the endothelium in HIV have also been documented. These include disturbed intima, increased leukocyte adherence, cellular morphology changes along with increased expression of endothelial activation markers(6-9). All of these features are consistent with a more rapid progression of the atherosclerosis process (e.g. the ‘response to injury hypothesis)(10; 11). Another interesting fact is that cerebrovascular involvement is also observed in this population with a stroke incidence of about 15% according to some reports (12-14). Thus, the vascular abnormalities that occur in HIV/AIDS patients may represent a syndrome of inter-related conditions. Given the chronic and progressive nature of these conditions it is likely that vascular complications will be of increased importance as the worldwide incidence of HIV infection expands.

ENDOTHELIAL INVOLVEMENT IN HIV

Overall the vascular changes observed in HIV patients seem to be primarily related to alterations in the vascular endothelium. The endothelium is now recognized as not just the monolayer of cells forming a protective barrier between circulating blood and tissue, but as a multifunctional organ. It functions as a biological sieve, selectively allowing bi-direction passage of molecules and gases. Further it is a potent endocrine organ, sensing haemodynamic changes and maintaining vascular haemostasis by secreting various bioactive factors, including NO, endothelin –1 and
angiotensin –2. It expresses adhesion molecules, receptors for various local and circulating factors, growth factors and cytokines. A healthy endothelium provides a balance between vasoconstriction and vasodilation, platelet activation and inhibition, as well as pro and anti coagulation. Endothelial dysfunction upsets this balance, therefore predisposing the vessel to leukocyte adherence, platelet activation, thrombosis, impaired coagulation, vascular inflammation, smooth vessel proliferation and atherosclerosis(15; 16). It is interesting to note that many studies have demonstrated that endothelial dysfunction precedes structural changes in vasculature. An endothelium that is structurally intact but dysfunctional can be the first stage of vasculopathy and atherogenesis. The endothelium also plays a central role in the maintenance of the blood-brain barrier, and hence leaky inflamed endothelium may be involved in the occurrence of AIDS related dementia, which is reported in up to 20% of infected adults. Several recent reports have suggested that endothelial dysfunction may occur in HIV patients even in patients with well controlled disease, in the absence of overt immunodeficiency or reduction in CD4+ cells, suggesting that endothelial perturbations may occur early in disease pathogenesis(17).

MECHANISMS OF ENDOTHELIAL DYSFUNCTION IN HIV/AIDS

Although there is now substantial evidence of endothelial abnormalities in this patient population, the mechanisms by which these alterations occur are not well defined. Better understanding of HIV related vascular disease is necessary for the development of treatment options and perhaps will provide new insight regarding
immuno-vascular interactions. Below is a brief discussion of some potentially important mechanisms involved in this setting.

1. Direct infection of endothelial cells by HIV virus:

2. Toxicity of HIV related proteins:

3. Systemic (non-specific) response to viral infection:

4. Related drug toxicities (direct vs. indirect):

**Direct Effects Of HIV On Endothelium**

The question of whether HIV can productively infect endothelial cells is still debated. To enter a cell HIV-1 requires binding to both CD4 and one of the seven transmembrane G-protein coupled chemokine receptors which act as co-receptors. Although endothelial cells do not express CD4, HIV virus may still be able to enter the cells through a CD4 independent mechanism through CXCR4 receptors that are constitutively expressed in this cell type as shown in in-vivo experiments(18-21).

Another complication is that it appears that location plays an important role in the susceptibility of endothelial cells to HIV. Reports suggest that liver sinusoidal endothelial cells are can sustain a productive HIV infection as perhaps can endothelium from adipose tissue, though there is still controversy over endothelial cells in other sites (22-25). Direct in vitro infection of brain endothelial cells by HIV has been shown through binding of gp120 to a CD4-independent receptor or other CD4 independent mechanism (26; 27). Some pathological studies have also demonstrated evidence for HIV-1 related nucleic acids in brain endothelial cells of AIDS and HIV+ patients.(28) Similarly in vitro studies have shown that HIV-1 can
penetrate coronary artery endothelial cells by transcytosis, (22) however, vascular endothelial cells appear not to support proliferation of HIV. Instead it appears that after a couple of days the virus enters a dormant state, though possibly serving as a reservoir of virus for re-infection (29). There is some suggestion thought that the presence of proinflammatory cytokines can serve to stimulate and prolong virus replication, as shown by Conaldi et al in HUVECS, incubated with IL1-β and TNFα (30). Another study showed that in the presence of T cells and monocytes there can be a rescue effect leading to a productive HIV infection, an effect that is enhanced by interferon (IFN) γ and RANTES. (31)

So if the consensus is that productive HIV infection does not occur in vascular endothelial cells, what is the cause of the observed endothelial impairments observed in this patient population? A variety of mechanisms have been proposed, including endothelial injury caused by HIV products, HIV antiretroviral therapies, increased secondary infections, concurrent drug use (both legal and not), or secondary injuries caused by lipid dysregulations (either therapy or HIV related), immune dysregulation, increased oxidant stress, etc, or as seems likely a combination of some or all of the above, as will be discussed subsequently.

**TOXICITY OF HIV RELATED PROTEINS**

**HIV Proteins Tat and gp-120**

HIV associated protein Tat, trans –activator of viral transcription, is produced early in infection, and is essential for viral gene expression and replication (32). Tat can also be secreted in a soluble form, which circulates in the blood stream of infected
individuals and, once taken up by uninfected cells, alters cellular physiology by
influencing gene transcription (32-34). Gp-120 is a viral envelope glycoprotein that
binds to CD4 surface marker and is directly responsible for viral entry into T helper
cells. Both can be detected in the plasma of HIV patients.

During active infection of T cells by HIV (35; 36), Tat can act as a mediator effecting
the physiological function of T cells, B cells, monocytes, chondrocytes, neurons, etc.
In addition Tat is a highly potent activator of pro-inflammatory and angiogenic
pathways in the vascular system. Tat been implicated as a prime mediator of the
angiogenesis associated with kaposi’s sarcoma, as well as the microangiopathy
seen in AIDS (29; 33; 34; 37-40). Tat residues 40-60, essential for its transactivating
properties and stability, also have similarity to the heparin binding domain of VEGF
and other heparin binding growth factor (32; 34; 41; 42). Tat has been shown to
mediate the release of bFGF from Kaposi Sarcoma cells, inducing angiogenesis
(32; 42). Further, tat can directly activate VEGF receptor VEGFR-2 (KDR/Flk), thus
promote KDR mediated neovascularization and through VEGFR-1 (Flt-1) stimulate
monocyte chemotaxis(43; 44).

Tat incubation has been shown to increase expression of adhesion molecules
including VCAM, ICAM, and ELAM, in endothelial cells as well as on monocytes,
resulting in increased adhesion of monocytes to endothelium, leading to disruption of
monolayers mediated perhaps by MMP-9 which is shown to be up regulated in
monocytes by Tat. [dhawan s (9; 45-49). Increased expression of e-selectin, which
can lead to increased leukocyte adherence, has also been shown in the presence of
tat (9). Further, increased adhesion molecule expression and the increased
adhesion of immune cells to endothelium appear to be enhanced in the presence of cytokines, which themselves can be induced by exposure to tat. Treatment of macrophages with Tat protein results in increased secretion of IL-1β, IL-6, IL-8 and TNF α, all of which themselves can activate the endothelium (46; 50). Furthermore tat can also modulate angiogenesis and endothelial cell morphogenesis and proliferation, most significantly in Karposi’s Sarcoma (33; 34; 37; 39; 51) Tat in the presence of inflammatory cytokines causes proliferation and migration of vascular endothelial cells and degradation of basement membranes probably mediated through the Flk-1/KDR (VEGFR2) receptor (46). Other studies have shown that tat can mediate some of its effects through activation of NFκB and subsequent changes in gene expression flores, (49) including e-selectin up regulation in endothelial cells, IL-6 in astrocytes, IL-1beta and MMP-9 in monocytes.

Some reports also suggest that HIV tat can trigger apoptosis of cells, either through induction of TNFalpha as in PC12 neuronal cells (52) or through activation of caspases as reported by Groopman et al, in endothelial cells (53).

The other HIV protein that has been extensively studied in relation to endothelial damage, Gp120 is a viral envelope glycoprotein responsible for the binding of the virus to CD4 surface marker and viral entry into T helper cells. It also has direct cytotoxic effects as well as mediating endothelial function and expression of inflammatory cytokines and adhesion molecules.

Studies with rat brain endothelium and gp120 transgenic mice(54) have demonstrated that gp120 appears to alter endothelial cell permeability, leading to leakiness of the blood brain barrier, perhaps through a substance P dependent
mechanism, as well as through induction of proinflammatory cytokines including ICAM-1 and VCAM1 on the endothelium, which can lead to enhanced leukocyte recruitment into inflammatory sites and into tissue (26; 27; 55). Furthermore, gp120 can also have direct effects on monocytes, leading to the expression of proinflammatory cytokines, namely prostaglandin E2 and IL-1. (56) These cytokines in turn can have potent effects on endothelial function. Gp120 also influences the adhesive properties of monocytes, inducing expression of CD31, CD38 and CD49d, as well as enhancing the activation of NF-κB resulting in a further increase in pro-inflammatory cytokine production (56-58). The effect on monocytes has particular relevance for the vascular system as monocyte–endothelial adhesion is considered an early initiating event in atherogenesis (16).

In addition to brain endothelium, in vivo studies with HUVECs show that incubation with gp120 can lead to endothelial apoptosis, perhaps mediated by chemokine receptors CCR5 and CXCR4, and involving induction of caspases (20; 59-61) leading to damage to the endothelial layer.

In summary Tat and gp120 can lead to alteration of endothelial function in a variety of ways, including induction of endothelial cell apoptosis, activation of cytokines by monocytes, increasing recruitment of immune cells, enhancement of monocyte-endothelial cell adhesivity and increased endothelial cell permeability.
SYSTEMIC (NON-SPECIFIC) RESPONSE TO VIRAL INFECTION

Role of Cytokines and Oxidative Stress

As has been discussed above, HIV and HIV proteins can influence the secretion of a variety of vasoactive molecules, including Endothelin-1 (ET-1), IL-1β, IL—6, TNFα, etc. from monocytes/macrophages as well as endothelial cells. Indeed HIV infected patients have been shown to have elevated pro-inflammatory cytokine levels, (TNF α, IL-6) in their plasma (62; 63), which can act to induce cell proliferation, inflammatory cell migration, local imbalance between pro-coagulant and anti-coagulant factors, as well causing direct activation of endothelial cells.

In addition, many reports suggest that HIV+ patients have increased levels of Reactive Oxygen Species (ROS), and Reactive Nitrogen Species (RNS) (64-66). Reactive nitrogen species (RNS) are a family of biologically relevant oxidants derived from the interaction of nitrogen-based intermediates (most notably NO) with reactive oxygen species (e.g. superoxide anion, hydroxyl radical, hydrogen peroxide). Nitric oxide (NO) is a key mediator of both immune function and cardiovascular homeostasis, and may be an important pathophysiologic link between immune and cardiovascular system control. Increased oxidant stress may play a role in the pathogenesis of disease, increasing viral replication, impairing Tcell responsiveness and increasing apoptosis. The increase in ROS and RNS are also associated with a progressive depletion of antioxidants, especially glutathione, (64; 65; 67). Furthermore, there appears to be a strong association between reduced thiol levels in plasma and decreased survival, suggesting clinical relevance of the impaired antioxidant defense in these patients(68), and many studies have looked at
N-Acetyl Cysteine (NAC) supplementation to replenish GSH levels and there is some evidence that NAC treatment can raise plasma GSH levels and increase T and NK cell function (69; 70).

Whether the increased oxidant stress in HIV+ patients is due to virus or due to antiretroviral treatment is a little controversial, in one study (71) oxidant stress as measured by plasma F2 isoprostane was increased in patients with lower HIV RNA and associated with antiretroviral therapy (esp. efavirenz), suggesting a drug effect. While other studies have shown that antiretroviral treatment is associated with decreased oxidant stress and improved antioxidant status, though still at abnormal oxidant levels (72). As will be discussed in a later chapter, we have noted that incubation of endothelial cells with protease inhibitors can result in increased oxidants in vivo, which might also be a contributing factor. Whatever the mechanism, it appears that this patient population shows elevated oxidant levels and depleted antioxidants in both patients on antiretroviral therapy and naïve patients (67; 68).

In addition to diverting NO from its physiological activities into RNS formation, ROS and RNS can have profound cellular effects and toxicities, including the avid capacity to cause nitration of tyrosine residues, both protein bound and free, resulting in the stable formation of 3-nitrotyrosine residues. Protein-3NT formation has been demonstrated to be a potent structural and functional post-translational protein modification, has deleterious effects on cardiac contractility and vascular function, and has been observed in a variety of cardiovascular disease states.
Indeed, one study suggests that antioxidant supplementation in HIV resulting in a boosted host antioxidant system can stop the increase in endothelial dysfunction measured by markers such as plasma von Willebrand factor (73).

We have recently shown that a mouse model of retroviral infection displays time-dependent increases in aortic RNS formation during retroviral progression and that the extent of protein-3NT formation was inversely correlated to aortic performance (chapter 2). Our human studies recapitulated this phenomenon as cardiac prevalence of microvascular protein-3NT was drastically increased in HIV+/CVD+ patients as compared to HIV+/CVD- and HIV-/CVD+ as will be discussed in a later chapter.

**Lipidostrophy and Glucose Metabolism**

Even in the pre–HAART era, lipid anomalies had been noticed in HIV+ patients primarily hypercholestremia and hypertriglyceredemia (74; 75). However, recently there have been many studies showing that patients on HAART show a specific lipidostrophy syndrome consisting of peripheral fat loss, central fat accumulation, hypertriglyceridemia, hypercholestremia, elevated free fatty acids and insulin resistance, at a prevalence rate of 30-80% from study to study(76-82) as will be discussed in the next section. However, reports of endothelial damage in the AIDS population precede the introduction of HAART (7; 8). Pre HAART data suggests that HIV positive patients show abnormal lipid metabolism, (elevated triglycerides) which can have negative effects on endothelial health. Further, various biological makers of endothelial dysfunction such as VCAM-1, ICAM-1 and von Willebrand
factor have been described to be elevated in the HAART drug naïve patient population (83). Indeed there are reports (84) suggesting that introduction of antiretroviral therapy reduced markers of endothelial activation in patients with HIV, at least in the short term while many other clinical studies of long term HAART treated patients suggest increased cardiovascular risk, suggesting a complex pathological pathway of endothelial damage in the HIV patient population, with possible contributions of virus, resulting immune response and HAART drugs all playing a role.

While the actual prevalence and mechanism of this dysregulation is still debated, the fact that a large number of HIV patients suffer from lipid dysregulation is uncontroversial. Lipid dysregulation can have a deleterious effect on endothelial function. Increased levels of plasma lipids especially LDL particles can cause endothelial dysfunction, impair vasodilatory capacity, maintain inflammatory infiltration of the plaque, and facilitate aggregation and coagulation, supporting the increased risk of vascular damage in these patients. Moreover, there have also been reports of changes in glucose metabolism as well as insulin resistance (85-90) in HIV+ patients on antiretroviral therapy, as will be discussed later, which in themselves can lead to deleterious effects on endothelial health.

RELATED DRUG TOXICITIES (DIRECT VS. INDIRECT)

In this patient population, there is an urgent need for therapeutic agents that could interrupt the rapid progression to AIDS in HIV patients. Hence, a number of anti-retrovirals were developed and “fast-tracked” for use in humans. HAART therapy is
the most significant advance in HIV therapy, often resulting in undetectable plasma viral loads. However, even HAART cannot eradicate HIV from latent reservoirs forcing the therapy to be continued indefinitely, which has resulted in a number of unexpected toxicities going hand in hand with greater survival of patients. Drugs that are currently in use to treat HIV infection and its related complications have been documented to have undesirable cardiovascular effects in both human and animal studies. There appear to be two mechanisms by which these therapeutic agents cause endothelial dysfunction, either by direct cellular damage, or by indirect effects through dysregulation of lipid metabolism.

**Nucleoside Reverse Transcriptase Inhibitors (NRTI’s)**

Zidovudine (AZT) was one of the first drugs introduced to combat HIV infection and now is currently used in HAART along with newer drugs of the same class like stavudine. While evidence of cardiac dysfunction in mouse models of retroviral infection treated with AZT alone and in combination with other HAART drugs (Lamividune & Indinavir), has been shown, recent reports suggest that AZT treatment can also have endothelial effects leading to dysfunction, probably through a mitochondrial, superoxide dependent mechanism, as shown by lewis et al in an in-vivo mouse aorta experiment (91). Further, though most studies suggest that HIV-PI’s are the major contributing factors to HAART- related lipidystrophy, a few studies show that HIV-PI naïve but NRTI treated patients, show a progressive lipidystrophy indistinguishable from that normally associated with HAART, suggesting that NRTI’s have an independent contribution to this condition (78; 92-94). Furthermore, some
studies at the cellular level have shown depleted mitochondrial DNA and mitochondrial proliferation in adipocytes in patients treated with NRTI’s. An especially greater significance was also found in stavudine treated as compared to Zidivudine treated patients, and severity of fat wasting was found to be related to the duration of NRTI treatment. (93; 95) Though there are no studies connecting NRTI use and endothelial dysfunction in humans, it follows that if NRTI’s do contribute to lipid abnormalities, they could then indirectly result in decreased endothelial function.

HAART and protease inhibitors

The implementation of HIV-protease inhibitor drugs (HIV-PI’s) into combination therapy regimens has been central to the development of improved care and outcomes for HIV/AIDS patients. These agents have proven highly effective in providing a sustained suppression of viral load, and have dramatically reduced the incidence and severity of opportunistic infection in HIV/AIDS patients. However, these agents have also been implicated in the development of a variety of drug toxicities, including hepatic injury, metabolic complications, insulin resistance and lipodystrophies.

In fact, a specific syndrome of fat redistribution (peripheral wasting with central accumulation) associated hyperlipidemia involving cholesterol, triglycerides and insulin resistance has been noticed in this population, generally associated with antiretroviral treatment, most often HAART involving HIV-PI’s though some characteristics have also been noted on patients just treated with NRTI’s (78; 96). These characteristics have been linked in the general population to cardiovascular
disease and have sparked the current spate of cardiovascular health studies in HIV patients.

Indeed, the use of HIV-PI’s have been linked to cardiovascular toxicities, including atherogenesis, vasculopathies, and cardiac structural and/or functional changes and some suggestion of myocardial infarctions, though the last remains controversial. In a recent large clinical study (DAD study) of HIV patients on HAART regiments that either included HIV-PI’s or not, it was found that the HIV-PI treated group had a 10 fold higher increased in CAD related events (9.8/1000 to 0.8/1000), as well as increased incidence of myocardial infarction (5.1/1000 to 0.4/1000)(97). HIV-PI use has also recently been shown to be associated with in vivo endothelial dysfunction in humans as measured by carotid ultrasonography showing increased intimal media thickness(98), decreased flow mediated dilation (17) as well increased plasma levels of endothelial damage markers (99), though mostly in conjunction with lipid dysregulation.

While the mechanisms remain unclear, increased levels of plasma lipids especially LDL particles can cause endothelial dysfunction, impair vasodilatory capacity, maintain inflammatory infiltration of the plaque, and facilitate aggregation and coagulation, supporting the increased risk of cardiovascular damage in these patients (16; 100). Some reports suggest that the increased plasma triglyceride and cholesterol levels were caused by increased fatty acid and cholesterol biosynthesis in adipose and liver, through accumulation of activated forms of sterol regulatory binding protein (SREBP)-1 and -2 in the nucleus of liver and adipose, resulting in elevated expression of lipid metabolism genes (101). Other studies showed that in
presence of HIV-PI nelfinavir there is an increase in lipolysis in adipocytes, possibly due to decreased expression of the lipolysis regulator perilipin suggesting an effect of HIV-PI’s on lipid catabolism (102). Investigators have also focused on the effects of HIV-PI’s on adipocyte differentiation through regulation of transcription factors critical for adipogenesis especially CCAAT/enhancer binding protein α (CEBPα) and peroxisome proliferator-activated receptor γ (PPARγ) which have been shown to be reduced by HIV-PI treatment, in particular Nelfinavir (102; 103). These in-vitro studies are also supported by a study by a clinical study (104) which showed that HIV-1-infected patients with antiretroviral-induced lipoatrophy showed greatly reduced SREBP1c expression, smaller adipocytes, reduced mRNA concentrations of the adipogenic differentiation factors CCAAT-enhancer binding protein (C/EBP) beta and alpha, peroxisome proliferator-activated receptor (PPAR) gamma.

An hypothesis has also been proposed that because HIV-protease shares amino acid sequence homology with low density lipoprotein-receptor related protein (LRP) and cytoplasmic retinoic-acid binding protein type I, HIV-protease inhibitors interact with or mimic these proteins, resulting in reduced differentiation and increased apoptosis of peripheral adipocytes (105) though this is based purely on sequence homology and has not been proved in vivo.

Other reports have also linked HIV-PI use to decreased oxidative glucose metabolism, insulin sensitivity, and increased insulin resistance (79; 85; 89; 90). While the mechanisms behind this phenomena have not yet been elucidated, it should be noted that increased plasma free fatty acids (due to lipolysis) are known to be critical mediators of insulin resistance in obesity and type-2 diabetes(106).
On the other hand, studies with healthy volunteers taking HIV-Pis point to a direct
effect of HIV-Pis on glucose metabolism (90; 107; 108). One potential mechanism
was suggested by an invitro study that showed reduced Glut-4 transport activity but
not translocation using rat adipocytes and Xenopus oocytes. (89)
Also, as discussed earlier, in patients with HAART associated lipidodystrophy there are
reduction of PPARγ, which might also affect insulin sensitivity. In fact there have
been a couple of successful pilot studies looking at the effects on PPARγ agonists
(rosiglitazone) in these patients, with treated patients showing increased glucose
sensitivity and even reduced central fat accumulation (109; 110).
In addition to indirect toxicities through metabolic changes, HIV-Pis might also have
direct effects on endothelium, including endothelial dysfunction and cell death(111),
as will be discussed in further chapters in this thesis (Chapter 2, Baliga RS in press).
In conclusion, it appears that both HIV-Pis and NRTI’s may have deleterious effects
on cardiovascular health, and indeed it would not be surprising to except
synergistical interactions between the various HAART therapy components, as
suggested by a few recent studies (92; 112) which show that dyslipidemia and
insulin resistance are worse in patients receiving a combination of HIV-PI and NRTI
than either drug alone.
Given the importance of the HAART therapy and the widespread and chronic use of
these agents in an expanding patient population, further understanding of this
potential drug toxicity is imperative.
Protease inhibitors, most notably ritonavir, have the ability to inhibit several
metabolizing enzymes of cytochrome P450 pathway 3A4 (CYP3A4). Several lipid
lowering drugs, statins, follow the same pathway, which might lead to increased levels of the statin drugs exacerbating their toxicity (rhabdomyolysis and myopathy). Therefore, this unique population of patients must be carefully evaluated before employing lipid-lowering therapy.

CONFOUNDING FACTORS

As with all clinical studies, there are many factors that might influence the progression of disease/toxicities but are more related to patient characteristics than actual disease/toxicity agents. These factors are often present as limitations of clinical data and a brief summary of relevant factors in studying cardiovascular disease in AIDS are presented below.

Lifestyle Related Toxicities

In spite of the effects of virus and drug induced toxicities, traditional lifestyle risk factors like smoking, diet etc still play an important role in HIV-related cardiovascular disease. Studies on smoking in HIV+ patients have shown that a high percentage (70%) of HIV+ patients smoke and further 80% of current HIV+ smokers have no immediate plans to quit (113; 114). The increased cardiovascular risks that are associated with smoking even in the general population are well known and might have even greater significance in these patients (115).

There is also some suggestion that the lipid dystrophy and glucose intolerance produced by PI treatment can be worsened by increased dietary fat intake, at least in rodents (101; 116) suggesting that like in the non infected population, diet and exercise may play a role in the development of cardiovascular disease in HIV/AIDS.
as well. Another factor is the age related increase in abdominal fat, and cardiovascular risk that occurs even in healthy patients and with many HIV patients surviving into middle age, which might also be buried in this data. Another potentially relevant factor is that with better control of the disease many HIV+ patients begin regaining weight (117). However, a disproportionately higher gain in fat mass as compared to lean tissue has been observed after malnutrition and weight loss(118; 119), though there is substantial individual variation. Increased fat to lean mass ratio has been shown to be a risk factor in the general population, and stands to reason would also be harmful in HIV+ patients.

Potential Importance Of Genetics?

An interesting statistic in all the clinical studies is that depending on the study parameters, 30-70% of HIV+ HAART treated patients show signs of lipid dystrophy, and a significant population stays lipidystrophy free, even with prolonged treatment. While rigorous, large clinical trials are needed to study the population showing signs of endothelial damage, if this statistic holds, the lifestyle characteristics and genotypes of the endothelial damage resistant population might suggest some unique protective avenues.

There has been a lot of interest in determining the genotypes that might afford increased tolerance, however, to date there has been little success. An initial study suggested that the ApoE4 genotype might be important (120), however later studies have disputed this finding (121). A recent report suggests that the ApoC might be more important, carriers of -455C variant had 30% lower levels of HDL-cholesterol than non-carriers, and the measurements of lipids before and after the use of PI
demonstrated synergistic effects of the treatment and apo C-III variants on triglyceride levels. (122)

Other studies have looked at TNF-alpha promoter region gene polymorphisms in HIV-positive patients with lipodystrophy, and suggest that the -238 (but not the -308) promoter region TNF-alpha gene polymorphism is a determinant in the development of HIV-related lipodystrophy (123).

Yet another study identified a single-nucleotide polymorphism (3'322C/G) in the gene encoding a key cholesterol/triglyceride regulator, sterol-regulatory element-binding protein 1c (SREBP-1c), as associated with hyperlipoproteinamia, specifically the heterozygous/homozygous SREBP-1c-3'322C genotype as compared to the normal homozygous SREBP-1c-3'322G. However this mutation did not translate to a difference in the functional protein (124), and another later study failed to find the same association (125).

Still another avenue under study is the genotype of the HIV virus itself. A study by Anderson et al observed an association of mutations at reverse transcriptase codons M41 and T215 of the HIV virus with hypertriglyceridemia, and implied that the HIV genotype itself may be a significant etiologic factor in antiretroviral-associated lipodystrophy (126). However, these mutations were also associated with antiretroviral resistance, suggesting that this subset of patients had been extensively pretreated, which in itself is a risk factor for lipid dystrophy.

A common drawback of all the genotype studies is that they have all been to date small studies of 50-100 patients and the results need to be confirmed in larger numbers of patients as well as in an ethnically diverse population.
While the most studies in the HIV/AIDS field have focused on adults, of the 42 million HIV cases in the world a substantial 3.2 million are children (1). Daily, 2,000 children worldwide are infected with AIDS, and as the profile of AIDS infection shifts to a largely heterosexual transmission, this number is expected to increase, unless there is a drastic increase in antiretroviral care to prevent mother to child transmissions. Unfortunately, like the adult population, the pediatric population too seems to be vulnerable to the full spectrum of lipodystrophy noted in adults, including fat redistribution, hyperlipidemia and insulin resistance. (82; 88; 127; 128). This syndrome may be of particular significance in the pediatric population, given that they are still growing (developmental toxicities?) and further might potentially be on retroviral therapy all their life.

CONCLUSIONS

While the contribution of HAART drugs to this condition is implicated (17), reports of endothelial damage in the AIDS population precede the introduction of HAART (7; 8). Pre HAART data suggests that HIV positive patients show abnormal lipid metabolism, (elevated triglycerides) which can have negative effects on endothelial health. Further, various biological makers of endothelial dysfunction such as VCAM-1, ICAM-1 and von Willebrand factor have been described to be elevated in the HAART drug naïve patient population (129). Indeed there are reports (84) suggesting that introduction of antiretroviral therapy reduced markers of endothelial activation in patients with HIV, at least in the short term, while many other clinical studies of long term HAART treated patients suggest increased cardiovascular risk,
suggesting a complex pathological pathway of endothelial damage in the HIV patient population, with possible contributions of virus, resulting immune response and HAART drugs all playing a role. Dysregulation of endothelium, and subsequent release of endothelial factors may also play a role in this condition.

In conclusion, the endothelium is now recognized as a uniquely vulnerable target in HIV infection because of its role as a critical regulator of access to major organs, and a potential reservoir of virus, as well as in of itself. The HIV virus itself, viral products and paradoxically components of antiretroviral therapies can have deleterious effects on the endothelium, maybe even acting synergistically. The mechanism behind the toxicities remain poorly defined, though many possible avenues for study have been suggested.

And furthermore, even in this uniquely vulnerable population, the effect of traditional risk factors such as smoking, age, diet and exercise are still important and must not be overlooked.

Whatever the mechanism of toxicity, it appears, that much like the diabetic population, the HIV+ population, is at high risk for development of cardiovascular disease, especially atherosclerosis, and hence should be prospectively managed as a pre-cardiac patient population.
<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1981</td>
<td>1st reported case of &quot;unknown&quot; case of PCP in LA, that was later associated with AIDS</td>
<td>(130)</td>
</tr>
<tr>
<td>1983-1984</td>
<td>Isolation of AIDS-associated virus</td>
<td>(131; 132),</td>
</tr>
<tr>
<td>1984</td>
<td>HIV (HTLV-III) identified as the causative agent in AIDS</td>
<td>(133)</td>
</tr>
<tr>
<td>1986</td>
<td>Brain endothelial cells infected by AIDS virus</td>
<td>(24)</td>
</tr>
<tr>
<td>1990</td>
<td>Endothelial cells: target for the HIV1 virus?</td>
<td>(134)</td>
</tr>
<tr>
<td>1992</td>
<td>Endothelial cell dysfunction in HIV infection</td>
<td>(7)</td>
</tr>
<tr>
<td>1992</td>
<td>Lipids, lipoproteins, triglyceride clearance and cytokines are altered in human immunodeficiency virus infection and the acquired immunodeficiency syndrome</td>
<td>(74; 135)</td>
</tr>
<tr>
<td>1993</td>
<td>Coronary artery lesions and human immunodeficiency virus infection</td>
<td>(8)</td>
</tr>
<tr>
<td>1996</td>
<td>Aortic endothelium in HIV-1 infection: chronic injury, activation, and increased leukocyte adherence</td>
<td>(6)</td>
</tr>
<tr>
<td>1997</td>
<td>Soluble adhesion molecules and endothelial cell damage in HIV infected patients</td>
<td>(129)</td>
</tr>
<tr>
<td>1999</td>
<td>Diagnosis, prediction, and natural course of HIV-1 protease-inhibitor-associated lipodystrophy, hyperlipidaemia, and diabetes mellitus: a cohort study</td>
<td>(78; 79)</td>
</tr>
<tr>
<td>2000</td>
<td>HIV-1 penetrates coronary artery endothelial cells by transcytosis</td>
<td>(22)</td>
</tr>
<tr>
<td>2000</td>
<td>Endothelial Dysfunction noted in HIV+ patients by Brachial Echocardiography</td>
<td>(17)</td>
</tr>
</tbody>
</table>

(PCP: Pneumocystis carinii Pneumonia, LA: Los Angeles, HTLV: Human T-cell Lymphotrophic Virus)

TABLE 2.1: Time-line of events that outline AIDS related vascular dysfunction
<table>
<thead>
<tr>
<th>Ref.</th>
<th>Incidence of Cardiovascular complications in an HIV+ patient population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
</tr>
<tr>
<td>(3)</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>(136; 137)</td>
<td>Myocarditis</td>
</tr>
<tr>
<td>(138; 139)</td>
<td>Pericarditis/Pericardial Effusions</td>
</tr>
<tr>
<td>(140; 141)</td>
<td>Infective Endocarditis</td>
</tr>
<tr>
<td>(137; 142)</td>
<td>Non bacterial thrombotic endocarditis</td>
</tr>
<tr>
<td>(13; 14)</td>
<td>Cerebrovascular disease</td>
</tr>
<tr>
<td>(8; 143; 144)</td>
<td>Accelerated Atherosclerosis</td>
</tr>
</tbody>
</table>

TABLE 2.2: Incidence of AIDS related Cardiovascular diseases
<table>
<thead>
<tr>
<th>Year</th>
<th>Patient #</th>
<th>Cardiovascular Marker Studied</th>
<th>HIV, HIV-PI Durat.</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>125 HIV+, 30 HIV-</td>
<td>VWF, tPA</td>
<td>Not Reported (NR)</td>
<td>Hypercoaguable state and endothelial damage markers in sera</td>
</tr>
<tr>
<td>2000</td>
<td>55 HIV+ve/HIV-PI +ve 47 HIV+ve, no HIV-PI 104 HIV-ve</td>
<td>IMT(intima-media thickness), epiaortic vessels</td>
<td>HIV-PI mean duration 22.5 months HIV infection mean 5-6yrs</td>
<td>Higher that expected prevalence of carotid vascular lesions in HIV-PI treated</td>
</tr>
<tr>
<td>2001</td>
<td>894 HIV+/Indinavir only, 886 HIV+/only NRTI 900 HIV+ on both</td>
<td>Adverse Cardiovascular event (MI, angina, death, stroke, and peripheral vascular disease)</td>
<td>HIV mean Duration NR HIV-PI treatment duration 1922.5 person-years</td>
<td>Retrospective person time analysis of phase III data No significant increase in short term risk with therapy containing HIV-PI Indinavir, compared to NRTI</td>
</tr>
<tr>
<td>2001</td>
<td>136 HIV +ve/HIV-PI 32 HIV +ve/HIV-PI –ve 68 HIV-ve</td>
<td>Atherogenic plaque defined as IMT ≥ 1200mm in peripheral arteries</td>
<td>HIV-PI mean duration 26.3±8.9 months</td>
<td>Significantly more plaques in HIV+ve that HIV-ve, no association with HIV-Pis, only conventional factors</td>
</tr>
<tr>
<td>2002</td>
<td>423 HIV+</td>
<td>IMT, Lipid Dystrophy</td>
<td>48.2% on HAART with HIV-PI Mean HIV-PI duration 18.78</td>
<td>Univariate linear regression, IMT significantly (p&lt;0.05) correlates to HAART, multivariate regression only correlation to traditional factors</td>
</tr>
<tr>
<td>2002</td>
<td>28 HIV+/HIV-PI+, 15 HIV+/HIV-PI-ve, 16 HIV-ve</td>
<td>Carotid IMT</td>
<td>HIV-PI duration 28.7months</td>
<td>HIV-PI treated group had a significantly increased IMT, triglyceride, HDL and apo B than controls.</td>
</tr>
<tr>
<td>2003</td>
<td>92 HIV+, 30 HIV-ve</td>
<td>VWF, t-PA, IL-6, TNF-α</td>
<td>NR</td>
<td>Viral Load in AIDS patients is significantly correlated to elevated circulating vWF, and t-PA</td>
</tr>
<tr>
<td>2003</td>
<td>840 HIV+</td>
<td>Coronary event rate (angina pectoris, unstable angina, acute(MI))</td>
<td>5yrs of HIV-PI</td>
<td>17 patients had coronary events, 3 time higher event rate (575 per100,000) and lower mean age (45.6yr) than database registry for France.</td>
</tr>
<tr>
<td>2004</td>
<td>68 HIV+/ ACS+, Control 68 HIV-ve/ACS+ve</td>
<td>Acute Coronary Syndrome (ACS)</td>
<td>HIV mean duration 8.5±5.3, 52.94% HIV+ve on HAART</td>
<td>HIV+ significantly more likely younger (50±8 v/s61±11), male, smokers, low HDL, with single vessel disease and greater restenosis risk, less likely diabetic.</td>
</tr>
</tbody>
</table>

(VWF: Von Willedebrant’s Factor, tPA: tissue plasminogen activator, IMT: intimal media thickness)

TABLE 2.3: Summary of clinical data of HIV/HIV-PI and vascular heath association
REFERENCE:


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100. Laroia ST, Ganti AK, Laroia AT, Tendulkar KK: Endothelium and the lipid metabolism: the current understanding. *Int J Cardiol* 88:1-9, 2003


150. de Larranaga GF, Petroni A, Deluchi G, Alonso BS, Benetucci JA: Viral load and disease progression as responsible for endothelial activation and/or injury in human immunodeficiency virus-1-infected patients. *Blood Coagul Fibrinolysis* 14:15-18, 2003

CHAPTER 3

AIDS RELATED VASCULOPATHY: EVIDENCE FOR OXIDATIVE AND INFLAMMATORY PATHWAYS IN MURINE AND HUMAN AIDS

(This chapter has been prepared for submission to Cardiovascular Toxicity and is presented in the style appropriate for the journal.)
ABSTRACT

According to The Centers for Disease Control and Prevention (CDC) estimates, there are between 850,000 to 950,000 HIV infected patients in the United States. Highly Active Anti Retroviral Therapy (HAART) has significantly improved overall survival and increased time of AIDS-free living for patients infected with HIV but this same prolongation of life expectancy has lead to increased evidence of complications that are apparently not directly related to immunodeficiency or opportunistic infection. For example autopsy studies suggest that HIV+ patients have increased risk of vascular lesions leading to arteriosclerosis, even in the absence of other risk factors. It is now recognized that the vascular endothelium plays a major role in maintenance of cardiovascular health and early impairment in this cell monolayer has been associated with, and in some cases may initiate, many forms of disease. Here we therefore tested the hypothesis that vascular dysfunction occurs in this model and evaluated potential mechanisms. To further investigate the relevance of our results we also studied vascular tissues procured from HIV/AIDS cases. an additional component of this study was to test the hypotheses that altered NOS isoform expression and/or endothelial protein nitration are associated with time dependent vascular dysfunction during AIDS related vasculopathy in murine tissues and corroborated our findings in human tissues. At 1 and 5 weeks we observed statistically significant decreased in KCL contractility and time dependant contractile deficits in response to the alpha adreneric agonist PE, Emax reduced by approximately 40% (213±15 mg control vs. 133±16 mg at 10weeks), and EC50 values (102±7.3ng control vs.190±37ng at 5 weeks vs. 130±22ng at 10weeks,
p<0.05). We also tested vascular relaxant responses and found a decreased endothelium dependant relaxation to ACH (EC50 control 120±27nM vs. 343±94nM at 10 weeks), while the response to an exogenous NO donor SNP remained unchanged, suggesting a specific endothelial dysfunction. Histochemical investigations into the same tissues showed increased protein 3NT, ICAM, NOS-2, NOS-3 and XO. These findings were corroborated in concurrent experiments in a cohort of well catalogued human cardiac micro-vascular tissues. In conclusion, we have demonstrated, for the first time, a specific functional vasculopathy with smooth muscle and endothelial involvement in a murine model of AIDS. Further, this observed vasculopathy was shown to be associated with and correlated with increased oxidative stress and specific endothelial activation. And finally, this finding was echoed in a relevant population of human HIV/AIDS patients. Research into the sources and intracellular targets of oxidants in this disease could provide important mechanistic insights and may reveal new therapeutic opportunities for this increasingly important cardiovascular disease state, and may even afford an advantage of protecting vasculature (and perhaps more specifically the vascular endothelium) as well as stabilizing immune dysfunction.
INTRODUCTION

According to The Centers for Disease Control and Prevention (CDC) estimates, there are between 850,000 to 950,000 HIV infected patients in the United States, with approximately 40,000 new infections occurring in the US every year(1). In addition to new infections, enhanced survival in already infected individuals has led to substantial numbers of patients living with HIV/AIDS. Furthermore, the frequency of new infections worldwide has steadily increased, with an estimated total number of 5,000,000 new cases worldwide.

Highly Active Anti Retroviral Therapy (HAART) has significantly improved overall survival and increased time of AIDS-free living for patients infected with HIV (2; 3) but this same prolongation of life expectancy has lead to increased evidence of complications that are apparently not directly related to immunodeficiency or opportunistic infections(3; 4) For example, HIV-related cardiovascular complications represent increasingly important contributors to the overall morbidity and mortality of HIV/AIDS patients(5-7) with a prevalence as high as 80%, and an estimated 5-20% progressing to severe dilated cardiomyopathy and cardiac failure (3; 7). Other studies have illustrated that vascular complications may be an important contributor to retroviral disease progression and mortality. For example autopsy studies suggest that HIV+ patients have increased risk of vascular lesions leading to arteriosclerosis, even in the absence of other risk factors and Acute Coronary Syndrome (ACS) in this patient population is known to occur almost a decade earlier than non-infected populations (8; 9). There is also some evidence that these patients may be uniquely susceptible to restenosis after treatment (9), all of which suggest that vascular
complications during HIV infection are important phenomena in disease progression and overall mortality.

Although there is substantial evidence of vascular abnormalities in HIV/AIDS patients the mechanisms responsible are not well defined. Direct HIV infection of vascular cells (most notably vascular endothelium) remains controversial(10-12) and toxicities of HIV associated proteins (gp120, Tat protein), increased cytokine exposures, and more recently toxicities of HIV drug regimens have all been suspected as participants. A key feature of many previous reports is the study of such events in vitro and/or in cell culture systems and demonstration of relevant animal models for the investigation of retrovirus-related cardiac vasculopathy, which could provide opportunities for further mechanistic insight and therapeutic intervention. In this study, we employed a well-established murine model of retroviral infection (LPBM5 virus), which we and others have demonstrated to recapitulate all features of cardiac dysfunction observed in HIV/AIDS patients(13). In this report we investigated the time-dependencies of retroviral progression and vascular dysfunction, in an attempt to extend its relevance as a disease model. The LPBM5 model of retroviral infection is commonly called the "murine AIDS" model, due to its high similarity to many of the immune-related complications seen during human HIV infection, including aberrant cytokine release, changes in T-cell populations, and increased susceptibility to opportunistic infections(13). We therefore tested the hypothesis that vascular dysfunction occurs in this model and evaluated potential
mechanisms. To further investigate the relevance of our results we also studied vascular tissues procured from HIV/AIDS cases.

It is now recognized that the vascular endothelium plays a major role in maintenance of cardiovascular health and early impairment in this cell monolayer has been associated with, and in some cases may initiate, many forms of disease(14; 15). Low basal levels of nitric oxide (NO) derived from the constitutive isoform of nitric oxide synthase (NOS) 3 in endothelial cells have been shown to be a critical modulator of local vascular tone and thrombus formation under normal physiological conditions(15). In contrast, elevated levels of NO production, via an inflammation-related induction of NOS2 are associated with several forms of vascular dysfunction and infectious disease(16-20). Once formed, NO rapidly reacts with available superoxide anion to form peroxynitrite, a reactive nitrogen species known to oxidize proteins, promote DNA damage and cause cellular apoptosis and necrosis. We and others have demonstrated that reactive nitrogen species formation may be an important participant in the initiation and/or progression of a wide array of cardiac and vascular pathological settings and that protein nitration contributes to perturbations in endothelial cells and cardiac myocytes in these settings(13; 20-24). For these reasons an additional component of this study was to test the hypotheses that altered NOS isoform expression and/or endothelial protein nitration are associated with time dependent vascular dysfunction during AIDS related vasculopathy in murine tissues and corroborate our findings in human tissues.
METHODS

Murine AIDS Model and Study Design:

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

Pathogen-free female C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) were housed in a sterile cage rack system with HEPA filtered air circulation (approximately 50 air changes/hour, Allentown Caging Inc., Allentown, PA).

Following 1-2 weeks of acclimatization LP-BM5 retrovirus was dosed via a single intraperitoneal injection (100 µl dose containing 200 reverse transcriptase units). Control animals received an identical injection of vehicle. At the time of injection all mice were six weeks old and weighed 16-18g. To account for time variations, animal age and body weight, two groups of control animals were studied at 0 and 10 weeks post injection (n=6) per group. No statistically significant differences were observed in these two groups and hence they were pooled for further comparisons to retrovirus-infected mice. Spleen weight was measured as an index of retroviral progression at times of sacrifice, as documented by others.
**Reverse transcriptase-polymerase chain reaction (RT-PCR) for LPBM5:**

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

**Isolated Vascular Function Studies**

At 5 and 10 weeks, animals were sacrificed with an i. p. dose of 100mg/kg Nembutal Sodium (Abbott Laboratories). Thoracic aortas were rapidly isolated for functional
evaluations using methods similar to those previously described (Watt, Faseb).

Briefly, thoracic aorta segments (2-3mm) were mounted on isometric force
transducers (Grass Instruments, Quincy, Mass.) and incubated in 10ml organ baths
containing Krebs buffer bubbled with 95% O₂ at 37°C. After 90 minutes equilibrium
(resting tone 1gm), maximal contractile force was evaluated for each segment using
a high potassium concentration (modified Krebs’ Buffer containing 90mM KCL).
Cumulative contractile responses were then evaluated for cumulative bath additions
of phenylephrine.

After pre-contraction with phenylephrine to 80% of maximum, relaxant responses to
Acetylcholine (an NO mediated endothelium dependent relaxant) and sodium
nitroprusside (an NO mediated endothelium independent relaxant) were also
determined. Finally response of segments to L-Nitro Arginine incubation (L-NA, 200
µM) was also recorded.

Contractile and relaxant response data were fit to the 4-parameter logistic equation
using GraphPad Prism Software (GraphPad Software Inc., San Deigo, Calif.)

Following functional studies the aortic segments were immediately weighed, and
then formalin fixed so that functional parameters could be related to in situ staining
results described below.

**Histology and Immunohistochemistry**

Following functional analyses, aortas were rapidly processed for
immunohistochemical studies using standard protocols. Aortic protein 3-nitrotyrosine
(anti-3NT antibody, Upstate Biotechnology, Lake Placid, NY, 1:200 dilution),
Intracellular Adhesion molecule (anti-ICAM), Nitric Oxide Synthase 3 (anti-NOS3, Transduction Labs, Lexington, KY) anti Xanthine Oxidase (anti-XO, RDI labs) and Nitric oxide Synthase 2 (anti-NOS2, Transduction Labs, Lexington, KY, 1:400 dilution) were assessed in aortic cross-sections. General morphology and extent of fibrosis deposition were assessed using Masson’s Trichrome stain (cytoplasm, red; collagen, blue; nuclei, black) with a kit based approach (Sigma Chemical). Staining controls included antibodies preadsorbed with purified 3NT or murine NOS2; addition of antigen eliminated positive staining in each case, demonstrating antibody specificity. Diaminobenzidine (0.06% w/v) was used to provide visualization of immunoreactivity, with methyl green counterstaining.

Throughout these studies individual vascular tissue segments from each animal were carefully accounted for, allowing isolated segment functional responses to be directly related to immunohistochemical analyses from the same segment. This provided us an opportunity to relate various vascular endpoints statistically using correlation analysis (see below).

**Digital Image Analysis**

Digital images were acquired using a Polaroid DMC camera and Olympus microscope (model BX40) and transferred to Image Pro Plus software (Media Cybernetics, Silver Spring, MD) for both area and intensity analyses. In protein 3NT, NOS2, NOS3 and ICAM studies, aortic images were captured at 800x, and relative intensity was determined using image threshold analysis, as we have previously described. At this magnification individual endothelial cells can be identified and
individually analyzed for endothelial versus smooth muscle immunostaining. Similarly in human cardiac samples, images were shot around microvessels at 800x, in order to distinguish endothelial cells.

**HIV/AIDS Cardiac Specimens**

Left ventricular sections (LV anterior wall) were obtained as paraffin-embedded autopsy specimens from AIDS patients and non-HIV infected controls from the National Cancer Institute AIDS Cancer and Specimen Resource. All autopsy samples studied were collected within 4 hours of death. Autopsy samples were collected between 1983 and 1998. Patient histories were reviewed to sub classify samples into 4 groups: non-HIV infected patients with no evidence of cardiovascular disease (HIV-/CVD-, n=6), HIV infected patients with no documented evidence of cardiovascular disease (HIV+/CVD-, n=18), and HIV infected patients with documented evidence of cardiovascular disease (HIV+/CVD+, n=27). Age at autopsy was not different between patient groups (CTRL: 40±2.3 yrs, HIV-DCM: 36.5±3.2yrs, p=NS), and was not a significant factor for cardiovascular disease. Average ejection fraction in HIV+ patients with dilated cardiomyopathy was 27.5±7.5%, mean CD4+ counts were 123.5±46.5 cells/mm$^3$. The microvasculature in the cardiac specimens was then assessed for evidence of NOS2 protein-3NT, XO and ICAM,, using immunohistochemical methods identical to those described above.
**Statistical Analysis**

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

As described in our previous publication, at 5 and 10 weeks, an immunosuppressive profile closely resembling AIDS was observed during LPBM5 infection, with significantly decreased total circulating blood monocytes (13). The mice displayed hallmark features of AIDS related cardiomyopathy with left ventricular fractional shortening decreasing significantly from 49.4±0.3% in controls to 43.8±0.5% by 10 weeks of retroviral infection, cardiac output decreasing from 10.5±0.1 to 6.9±0.3 ml/min, and stroke volume reducing from 21.9±0.3 to 15.1±0.4 µL in the same time period.

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

Splenomegaly is consistently observed in murine AIDS and used as a marker of retroviral progression and immune dysfunction. Figure 3.1 (top panel), illustrates increases in organ size. Total spleen weights in control vs. retrovirus infected animals at sacrifice are shown in the figure. Spleen weight was significantly increased, indicating retroviral progression and immune dysfunction. Additional staining with Masson’s trichrome did not reveal evidence of splenic fibrosis (data not shown).

LPBM5 infectivity is facilitated by the gag, pol, and env genes. The p12 region in the gag gene is unique to the LPBM5 virus and important to infectivity. Shown in Figure are representative RTV expression bands in splenic tissue from RTV infected mice at five and ten weeks. The 229bp RT-PCR product, specific for the p12gag region of LPBM5, was confirmed by restriction enzyme analysis with Sma I (data not shown).
Viral load is depicted in Figure. Virus was present in splenic tissue (homogenates) in RTV-treated animals at 5 and 10 weeks post-treatment.

**Isolated vascular function: Contractile responses**

Shown in Figure 3.2 are the time dependant effects of LPBM5 virus infection on isolated vascular function. Statistically significant decreases in maximal vascular contractile responses to total depolarization (90mM KCL) were noted at 1 and 5 weeks post retroviral injection as shown in figure 2.2. At 10 weeks, though tending to decrease no statistical significance was observed.

Cumulative contractile responses to α-receptor agonist PE were also reduced in a time dependent manner as shown in Figure 3.2. Curve fitting of the cumulative concentration exposures provided statistically significant changes in PE Emax and EC50 values (p<0.05). Emax reduced by approximately 40% (213±15 mg control vs. 133±16 mg at 10weeks), and EC50 values were also altered (102±7.3ng control vs.190±37ng at 5 weeks vs. 130±22ng at 10weeks, p<0.05).

**Isolated vascular function: Relaxant responses**

Significant endothelium specific alterations in relaxant responses were also observed in a time dependant manner as shown in figure. Emax was significantly decreased at 1 and 5 weeks post retrovirus injection and EC50 at 10 weeks showed a statistically significant increase (120±27nM vs. 343±94nM at 10 weeks).
In contrast to diminished acetylcholine responses no significant change in vasorelaxant response to the endothelium independent and ‘spontaneous’ nitric oxide liberating agent nitroprusside (Figure 3.3).

**Isolated vascular function: Inhibition of NOS**

Shown in figure 3.4 are time dependent changes in response to 1mM L-Nitro-arginine, a non-isoform specific NOS inhibitor. At 1 week there was a significant contraction in response to L-NA incubation, p <0.05.

**Histochemical studies: Oxidative and Inflammatory pathways**

Extensive protein 3NT was observed in the aortic segments isolated from the retrovirus treated animals, especially at the later time point. Furthermore, this immunostaining appeared to be concentrated in the endothelial layer (representative images in Figure 2.5). Digital image analysis showed statistically significant increases in the endothelial layer and smooth muscle layer with time, about a 10-fold increase from control. Correlation analysis relating regional nitration intensity to vascular function was also carried out (data not shown). Smooth muscle protein 3NT was observed to be inversely correlated to the maximal PE response. (r= -0.69, p<0.01), as well as to maximal KCL response (r= -0.2516, p<0.05). There was however no significant correlation between the extent of endothelial 3NT staining and ACH maximal relaxation response (NS).

Endothelial activation marker ICAM was also observed to be increased in the endothelial layer and smooth muscle layers, but only at 10weeks (see Figure 3.5).
The endothelial ICAM prevalence was also inversely correlated to ACH maximal relaxation response (ACH Emax vs. endothelial ICAM prevalence, r = -0.3272, p<0.05).

Significant time dependant increases were also seen in endothelial and smooth muscle XO content, at 5 and 10 weeks, p<0.05).

**Histochemical studies: NOS isoforms**

Figure 3.6 shows digital image analysis data of NOS2 immunostaining in the endothelial and smooth muscle layers of the aortic segments. As seen in the figure, we observed a significant time dependant increase in NOS2 staining in both the endothelial and the smooth muscle layers. This increase in smooth muscle NOS2 prevalence was correlated to the observed contractile response to PE (r = -0.72, p<0.05) and also to KCL response (r = -0.66, p<0.05), while the increased endothelial NOS2 was not significantly correlated to the Ach response.

(supplementary figure I)

Immunohistochemistry for NOS3 showed staining almost exclusively in the endothelium, which was upregulated at 5 and 10 weeks, suggesting an activated endothelial state in the retroviral aortas. Correlation analysis also showed correlation between increased NOS3 immunostaining and ACH maximal contraction (r= -0.2702, p<0.05).
**Corroborative evidence in isolated human vascular tissues**

Image analysis of the immunostaining showed increased endothelial 3NT staining in the HIV+/CVD+ group, suggesting that the increased oxidative stress shown in the murine model is also seen in the human disease (figure 3.7). We also observed similarly increased endothelial NOS2 staining in the HIV+/CVD+ group as compared to the HIV-/CVD- group, again as predicted by the murine model. We also observed an increased ICAM staining in the human cardiac microvascular endothelium suggesting a unique endothelial involvement in this condition. Finally we also observed an increase in XO staining in the cardiac endothelium from HIV+/CVD+ patients, further supporting increased oxidative stress in this condition.
DISCUSSION

Over the last decade, the importance of the vascular endothelium for maintenance of cardiovascular health has become evident and early injury and/or dysfunction has been associated with many cardiovascular disease states (14; 15; 17; 23; 25). Vascular disorders such as vasculitis and morphological changes in the endothelium in HIV are documented and include disturbed intima, increased leukocyte adherence and cellular morphology changes along with increased expression of endothelial activation markers (26-31). In recent years, the endothelial dysfunction has increasingly been reported in the HIV/AIDS population (28; 32-36). While the contribution of HAART drugs to this condition is implicated (32) reports of endothelial damage in the AIDS population precede the introduction of HAART (30; 37). Various biological makers of endothelial dysfunction such as VCAM-1, ICAM-1 and von Willebrand factor have been described to be elevated in the HAART drug naïve patient population (33; 38). Indeed there are reports (39) suggesting that introduction of antiretroviral therapy reduced markers of endothelial activation in patients with HIV, at least in the short term while many other clinical studies of long term HAART treated patients suggest increased cardiovascular risk, suggesting a complex pathological pathway of endothelial damage in the HIV patient population, with possible contributions of virus, resulting immune response and HAART drugs all playing a role (28; 30; 32-36; 40). Further potential pathological contributions of opportunistic infections, other therapeutic drugs, as well as illegal drugs are also often present in a clinical HIV +ve population, which have also to be taken into consideration (41; 42). Thus it appears that this is a condition in which animal models
have a unique applicability in figuring out individual contributions of various potential pathways of endothelial damage and also possible synergies.

As described in our previous paper, at 5 and 10 weeks, our animal model (LPBM5) displayed an immunosuppressive profile closely resembling AIDS, with significantly decreased total circulating blood monocytes. Severe immunodeficiency occurs post 10 wk(13). Here we observed progression similar to others, and consistent with our previous studies. We followed disease progression in this model by monitoring spleen weights and splenic viral load in the mice at 0, 5 and 10 weeks and saw increased viral load as well as splenomegaly demonstrating disease progression. However, we observed no correlation between the spleen weights and observed decreased vascular function, though the vascular deficiency did show significant correlation to markers of oxidative stress and endothelial activation, as described later, suggesting that the observed vasculopathy may be more a function of immune response to virus rather than direct effects of virus per se. Again, the similar observations in the Murine LPBM5 and Human AIDS tissues further corroborate the immune response rather than direct action of virus theory.

At 1 and 5 weeks we observed statistically significant decrease in KCL contractility suggesting a general lack of tone in the smooth muscle. Further we also observed time dependant contractile deficits in response to the alpha adreneric agonist PE, suggesting a generalized vasculopathy in these mice. In our functional studies we also tested vascular relaxant responses and found a decreased endothelium dependant relaxation to ACH while the response to an exogenous NO donor SNP
remained unchanged, suggesting a specific endothelial dysfunction. We also investigated the effect of L-NA a non-isoform specific NOS inhibitor, and found an interesting contractile effect only at 1 week, suggesting an increased NO tone at this specifically this time-point. This contrasted with our vessel data, which suggests decreased NO availability at all post infection timepoints.

Given our initial findings at the intact vessel level, we then conducted immunohistochemical analysis to provide further mechanistic insight. Increased reactive oxygen species are often seen in various settings of endothelial dysfunction. Further a previous report from a small clinical study suggested that antioxidant supplementation can reduce endothelial damage markers in human HIV patient plasma (43). Also though a recent clinical study of HIV patients showed that HAART treated HIV+ have higher plasma levels of oxidative stress than untreated HIV+ve patients suggesting that HAART treatment is more important a source of oxidative stress in this patient population than HIV. The actual changes in oxidative stress between HIV+ and HIV- patients is still to be determined(44; 45). We investigated the prevalence of a stable biomarker of oxidative stress, protein 3 nitrotyrosine (3NT). Digital image analysis demonstrated statistically significant increases in the endothelial layer and smooth muscle layer with time, about a 10fold increase from control. Further correlation analysis showed significant negative correlation between increased smooth muscle protein 3NT and decreased
contractile response to PE $(r= -0.69, p<0.01)$, as well as decreased KCL response $(r= -0.2516, p<0.05)$.

Since we observed a decrease in endothelial NO related responses, we carried out immunohistochemical studies of NOS isoforms NOS2 and NOS3. NOS3 was found to be almost exclusively in the endothelium, and was upregulated in the endothelium at 5 and 10 weeks, suggesting an activated endothelial state in the retroviral aortas. Correlation analysis also demonstrated an inverse correlation between NOS3 immunostaining and ACH maximal contraction $(r= -0.2702, p<0.05)$, suggesting perhaps increased content of NOS3 but perhaps decreased activity. Another explanation for this contra-intuitive finding could be an activated endothelium leading to an increase in oxidant production in these aortas, which would result in more NO being diverted into reactive nitrogen species pathways, with the ultimate result being, reduced NO available for physiological functions. The finding of increased protein 3NT in the aortas supports this hypothesis. The finding of increased protein 3NT in the aortas supports this hypothesis. To further investigate this possible mechanism, we looked at XO prevalence in these tissues and found an increase in XO content at 5 and 10 weeks. This increase in XO could also explain the L-NA data. At 1 week with NOS3 up, and XO tending up but not significant, there could be increased NO availability, thus the contraction in response to L-NA, and at later timepoints, with XO up, NO produced could be diverted into RNS pathways, thus not available to influence tone. Furthermore, evidence of an activated endothelial state is provided by another endothelial activation marker ICAM, which went up late in the
disease progression but showed a strong correlation with the decreased endothelial function seen in the mouse model (p=0.0388). Collectively these findings suggest that early post infection in this animal preparation a local vascular inflammatory/oxidative process occurs, and this may play an important role in the observed vascular dysfunction.

To test the relevance of these murine findings we then investigated the same analytes in our bank of human HIV+ cardiac samples. Through collaboration with Dr. Leona Ayers we had unique access to a large well-documented library of HIV patient cardiac tissue, left anterior ventricular wall, obtained within 4 hours of death. Patient histories were reviewed to sub classify samples into 3 groups: non-HIV infected patients with no evidence of cardiovascular disease (HIV-/CVD-, n=6), HIV infected patients with no documented evidence of cardiovascular disease (HIV+/CVD-, n=18), and HIV infected patients with documented evidence of cardiovascular disease (HIV+/CVD+, n=27). We then conducted histochemical analysis and observed that the human HIV+ microvascular endothelium showed similar increases in oxidative stress and endothelial activation markers. Protein 3NT, XO, ICAM and NOS2 were increased only in the HIV+ patients that had evidence of cardiovascular involvement. Thus we saw corroboration of our murine model data in Human HIV+ tissue.

In conclusion, we have demonstrated, for the first time, a specific functional vasculopathy with smooth muscle and endothelial involvement in a murine model of AIDS. Further, this observed vasculopathy was shown to be associated with and correlated with increased oxidative stress and specific endothelial activation. Finally,
this finding was echoed in a relevant population of human HIV/AIDS patients. 
Research into the sources and intracellular targets of oxidants in this disease could provide important mechanistic insights and may reveal new therapeutic opportunities for this increasingly important cardiovascular disease state, and may even afford an advantage of protecting vasculature (and perhaps more specifically the vascular endothelium) as well as stabilizing immune dysfunction.
REFERENCE:


23. Mihm MJ, Jing L, Bauer JA: Nitrotyrosine causes selective vascular endothelial
induced nitrification and inactivation of myofibrillar creatine kinase in experimental heart
HIV-1 infection: chronic injury, activation, and increased leukocyte adherence. Am J
Pathol 149:1887-1898, 1996
28. Bonnet D, Aggoun Y, Szezepanski I, Bellal N, Blanche S: Arterial stiffness and
endothelial dysfunction in HIV-infected children. AIDS 18:1037-1041, 2004
29. Lewis W: Atherosclerosis in AIDS: potential pathogenetic roles of antiretroviral
therapy and HIV. J Mol Cell Cardiol 32:2115-2129, 2000
Quilichini R, Aubert L, Tamalet C, Juhan-Vague I, Gastaut JA: Endothelial cell
31. Drouet L, Scrobohaci ML, Janier M, Baudin B: Endothelial cells: target for the
32. Stein JH, Klein MA, Bellehumeur JL, McBride PE, Wiebe DA, Otvos JD, Sosman
JM: Use of human immunodeficiency virus-1 protease inhibitors is associated with
atherogenic lipoprotein changes and endothelial dysfunction. Circulation 104:257-
262, 2001
33. Chironi G, Escaut L, Gariepy J, Cogny A, Teicher E, Monsuez JJ, Levenson J,
Simon A, Vittecoq D: Brief report: carotid intima-media thickness in heavily
40. de Larranaga GF, Petroni A, Deluchi G, Alonso BS, Benetucci JA: Viral load and disease progression as responsible for endothelial activation and/or injury in human immunodeficiency virus-1-infected patients. *Blood Coagul Fibrinolysis* 14:15-18, 2003
42. CDC: Drug associated HIV transmission continues in the United States. 2002.
44. Aukrust P, Muller F, Svardal AM, Ueland T, Berge RK, Froland SS: Disturbed glutathione metabolism and decreased antioxidant levels in human

FIGURE LEGENDS

Figure 3.1: Splenomegaly and expression of LP-BM5 during murine retroviral pathogenesis. Top Panel: Spleen weight increases with time of infection. Bottom panel: Viral load in RTV mice (●), Control mice show no virus (○). Band intensity was assessed using imaging software (UVP-Labworks analysis), normalized to α-actin expression in each tissue.

Figure 3.2: Isolated vascular contractile responses. A statistically significant reduction in maximal response to total depolarization (90mM KCL) was observed at 1 and 5 weeks post retrovirus infection (71% of max control response at 1 week and 74% at 5 weeks).

Contractile responses to an α agonist phenylephrine were also significantly reduced, at all time points post infection. At 5 weeks there was a significant right shift in the dose response curve, p<0.05.

Figure 3.3: Isolated vascular relaxation responses. A statistically significant reduction in Emax was seen at 1 and 5 weeks in response to Acetylcholine (Ach), an endothelium dependant vascular relaxant (this response has been shown to be mediated by nitric oxide production from the endothelial NOS3 isoform). At 10 weeks the maximal relaxation response was normal, but there was a right shift of the dose response curve. Sodium nitroprusside response is unaltered in the murine AIDS model.

Figure 3.4: Time dependent changes in response to 1mM L-Nitro-arginine, a non-isoform specific NOS inhibitor. At 1 week there was a significant contraction in response to L-NA incubation, p <0.05.
Figure 3.5: Representative photomicrographs of vascular immunostaining for 3-NT are shown. Panel A: Time and site dependent increases in protein nitration, maximal staining for 3-NT observed in the endothelial layer. Panel B: Significant upregulation in ICAM a marker of endothelial activation, at 10 weeks, both the endothelial layer and smooth muscle. Panel C: Statistically significant upregulation of the NOS3 isoform only in endothelium. Panel D: XO, upregulated, in both smooth muscle and endothelium, and reached statistical significance at 5 and 10 weeks.

Figure 3.6: Statistically significant time and site-specific upregulations in NOS isoforms. Top Panel: Averaged OD values for NOS2 elevated in both smooth vessel and endothelium. Bottom panel, NOS3 elevated in endothelium.

Figure 3.7: Histological examination of microvasculature from human autopsy samples. Panel A: Protein 3NT in HIV+/CVD+ group, Panel B: ICAM, Panel C: NOS2 expression, Panel D: XO. Statistically significant elevations only in the HIV+/CVD+ patient group.

Supplementary Figure 1: Correlations between Vascular dysfunction and smooth muscle staining for each individual ring, p<0.05 for significant correlations.
Figure 3.1: Splenomegaly and expression of LP-BM5 during murine retroviral pathogenesis.
Figure 3.2: Isolated vascular contractile responses
Figure 3.3: Isolated vascular relaxation responses
Figure 3.4: Time dependent changes in response to 1mM L-Nitro-arginine
Figure 3.5: Vascular immunostaining for oxidative and inflammatory markers
Figure 3.6: Statistically significant time and site-specific upregulations in NOS isoforms
Figure 3.7: Histological examination of microvasculature from human autopsy samples
Supplementary Figure 1: Correlations between Vascular dysfunction and smooth muscle staining for each individual ring
CHAPTER 4

DIRECT EFFECTS OF HIV-PI'S ON ENDOTHELIAL CELLS. CLASS VS. INDIVIDUAL EFFECTS OF SAQUINAVIR, INDIANAIR AND RITONAVIR.
ABSTRACT

Hyperlipidemia and advanced atherosclerosis have recently been identified as side effects of HIV protease inhibitor (PI's) therapies; the mechanisms involved are unclear. Endothelial cell dysfunction is now recognized as an initiating event in vascular lesion formation and atherogenesis and a contributor to dyslipidemia. Here we tested the hypothesis that PI's impose direct detrimental effects on vascular endothelium. Ritonavir (5µM) caused significant increase in ACH EC\textsubscript{50} (0.65±0.06 µM vs. 0.23±0.07 µM in control, p<0.05) but had no effect on E\textsubscript{max} in rat thoracic aorta segments. Saquinavir (5 µM) caused about 60% reduction in ACH E\textsubscript{max} whereas it did not affect the EC50. Isolated endothelial cells were treated with PI's in vitro and intracellular oxidant production was evaluated by microscopy and digital image analysis (DCF fluorescence). Cell death was also examined with dual labeling of carboxyl-fluoroscein and rhodamine-conjugated annexin V. After 18 hr incubation, saquinavir, indinavir and ritonavir at 5 µM each caused significant increase of reactive oxygen species (2-4 fold increases, p<0.05). This increase was prevented by N-acetylcysteine (20 mM). These drugs also promoted necrosis and apoptosis in vitro, (annexin V only and annexin V/6-carboxy-fluorescein diacetate double-label respectively). These results provide the first evidence for direct toxicities of PI's to vascular endothelium. These actions may contribute to PI related atherosclerosis
BACKGROUND

The implementation of HIV-protease inhibitor drugs (HIV-PI’s) into combination therapy regimens has been central to the development of improved care and outcomes for HIV/AIDS patients. These agents have proven highly effective in providing a sustained suppression of viral load, and have dramatically reduced the incidence and severity of opportunistic infection in HIV/AIDS patients(1-4). However, these agents have also been implicated in the development of a variety of drug toxicities, including hepatic injury, metabolic complications, insulin resistance and lipodystrophies. In addition, a few recent reports have suggested that this important drug class is associated with cardiovascular toxicities themselves, including atherogenesis, vasculopathies, and cardiac structural and/or functional changes and some suggestion of myocardial infarctions.(5-14) Whether these are direct drug toxicities or indirect interactions with HIV pathogenesis remains to be defined, since retroviral pathogenesis itself has been associated with cardiovascular dysfunction and hypertriglyceridemia in both animals and humans.(15-17). Moreover there have also been a few recent reports suggesting that individual HIV-PI’s might have specific toxicity profiles,(18; 19) and therapy could be modulated depending on patient risk factors.

Given the importance of the HIV-PI drug class and the widespread and chronic use of these agents in an expanding patient population, further understanding of this potential drug toxicity is imperative.
Over the last decade, the importance of the vascular endothelium for maintenance of cardiovascular health has become evident and early injury and/or dysfunction has been associated with many cardiovascular disease states. (20-25). Recent clinical reports have suggested that HIV-PI drugs may have negative effects on endothelial function in HIV patients (5-14). In addition, a potential role for direct endothelial toxicity has also been supported by a recent report that Ritonavir can cause direct toxicities to endothelial cells in vitro. (26) However, these clinical findings have not been universal and the direct toxicities of other HIV-PI agents have not been reported.

Here we investigated a role for direct endothelial toxicity induced by 3 HIV-PI’s Saquinavir, Indinavir and Ritonavir. In initial studies we employed functional assessments using isolated animal vascular tissues, followed by mechanistic investigations using murine endothelial cells in culture at clinically relevant concentrations. Our focus in these studies included oxidant related mechanisms since others and we have demonstrated that reactive oxygen and/or nitrogen species may contribute to endothelial dysfunction and injury in a wide array of disease settings(27-33) including HAART related lipodystrophies.
METHODS

Isolated vascular function

Functional studies using rat thoracic aorta segments were conducted similar to methods previously described (27). Six male Sprague Dawley rats were used for functional studies and of 6-8 rings obtained per animal, half were employed as control and the other half as HIV-PI treated. Following 1 hour of equilibration in oxygenated Krebs’ buffer, the vessel segments were exposed to either 5µM Indinavir, 5µM Ritonavir, 5µM Saquinavir or vehicle for 2 hr. Segments were then precontracted with phenylephrine (2.0µM, approximately 80% of maximal contraction). Cumulative relaxant responses to acetylcholine (ACH, 1x10^{-9}-2x10^{-4} M, endothelium dependent and NO mediated vasorelaxation) or sodium nitroprusside (SNP, 1x10^{-9}-2x10^{-4}, endothelium independent) were recorded. After washing (10min), maximal contractile response to 125 mM KCl was recorded at the end of the studies for each segment.

Intracellular Reactive Oxygen/Nitrogen Species

The intracellular detection of reactive oxygen and reactive nitrogen species was conducted using 5(6)-chloromethy-2, 7-dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probes, Oregon), as previously described (34). This cell permeable dye, DCF-DA diffuses into cells and is hydrolyzed by intracellular esterases to polar 6-carboxy-2’, 7’-dichlorodihydrofluorescein. This nonfluorescent fluorescein analog is trapped in cells and can be oxidized to highly fluorescent 6-carboxy-2’, 7’-dichlorofluorescein by intracellular oxidants.
(including hydrogen peroxide, superoxide anion, hydroxyl radical), or reactive nitrogen species (peroxynitrite) emits fluorescence at 515nm. Thus this method provides appropriate detection of the major endothelial cell oxidant species.

Mouse arterial endothelial cells (passage 16-20) were cultured to 80% confluence and then exposed to each of protease inhibitors (or vehicle) for 2 or 24 hrs. Control (vehicle treated) cells for each time point were also included. Following dye loading cells were incubated in the dark for 30 minutes, washed with protein free medium and fluorescent images were immediately captured with Olympus CMAD-2 camera system using a 25% neutral density filter. The captured images, 5-10 per slide (more than 100 cells investigated per treatment group and time point), were then analyzed using research image analysis software (ImagePro Plus, Media Cybernetics, Silver Spring, MD, USA).

**Cellular Apoptosis vs. Necrosis**

Cells were grown to confluence on 12 well glass slides and then treated with protease inhibitors at 5µM for 2 hrs and mechanisms of cell death were investigated using fluorescence microscopy via a commercially available assay kit (Annexin V-Cy3/6-Carboxyfluorescein diacetate kit, Sigma, St. Louis, MO). After a 10 minute incubation in double labeling solution the cells were placed on ice and fluorescent images were captured at WB and IR fluorescence using the Optronics Magnafire imaging system and analyzed using research image analysis software (ImagePro Plus; Media Cybernetics, Silver Spring, MD, USA). Late stage apoptosis was identified by positive staining of nucleus by propidium
iodium (PI) and that of phosphatylserine by annexin V. Necrosis was identified by positive staining of PI only. Cells that were labeled with both annexin V and PI were defined as apoptotic cells, whereas those that only exhibited PI fluorescence were defined as necrotic cells. Percentage of cells that demonstrated either feature of fluorescent labeling was then calculated.

**Cellular NO production**

The cellular production of nitric oxide was conducted using 2-(3,6-dihydroxy-4,5-diamino-9H-xanthen-9-yl)-benzoic acid (DAF-2) as previously described (35). DAF-2 is a sensitive fluorescent indicator commonly used for the detection of nitric oxide (NO). It reacts with NO in the presence of oxygen to yield the highly fluorescent triazolofluorescein (DAF-2T). Fluorescence is monitored using excitation and emission wavelengths of 485 and 538 nM, respectively, at a detection limit for NO of 2-5 nM. Mouse arterial endothelial cells (passage 16-20) were cultured to 80% confluence and then exposed to protease inhibitor (5µM or vehicle) for 24 hrs. Control (vehicle treated) cells were also included. Following dye loading cells were incubated in the dark for 30 minutes, washed with protein free medium, then exposed to Ach (30µM) and fluorescent images were immediately captured with Olympus CMAD-2 camera system using a 25% neutral density filter. The captured images, 5-10 per slide (more than 100 cells investigated per treatment group and time point), were then analyzed using research image analysis software (ImagePro Plus, Media Cybernetics, Silver Spring, MD, USA).
**Statistical Analyses**

All data are represented as mean ± SE. Cumulative concentration responses (Figure 1b and c) were curve fitted to the 4-parameter logistic equation using Graphpad analysis software as previously described (). Statistical evaluations were performed using Sigmastat software (Jandel Scientific Inc., San Rafeal, CA). Comparisons among treatment groups were performed using student’s t tests or analysis of variance where appropriate. In all cases, p<0.05 was deemed statistically significant.
RESULTS:

Shown in Figure 4.1 are effects of HIV-PI’s Indinavir, Ritonavir or Saquinavir on isolated vascular segments (n=6-10). Following incubations with drug or vehicle contractile and relaxant responses were measured. No significant change in phenylephrine induced (alpha agonist contractile response) or KCl induced (nonspecific smooth muscle depolarization response) was observed following HIV-PI treatment. Despite unaltered vascular contractile properties significant alteration in acetylcholine induced vasorelaxation was observed with incubation with Ritonavir and Saquinavir, but not Indinavir (Figure 1b). Curve fitting of the cumulative concentration exposures provided statistically significant changes in acetylcholine Emax and EC50 values (Figure 3.1b inset panels, p<0.05), for Ritonavir and Saquinavir respectively, with no changes observed with Indinavir. In contrast to diminished acetylcholine responses no significant change in vasorelaxant response to the endothelium independent and ‘spontaneous’ nitric oxide liberating agent nitroprusside (Figure 4.2).

Given evidence of decreased NO signal induced by HIV-PI’s at clinically relevant concentrations in rat aortas, we investigated potential involvement of reactive oxidant/nitrogen species using DCF fluorescence live cell imaging. HIV-PI’s caused time and concentration dependent increases in intracellular ROS/RNS production relative to control (Figure 4.3). This was quenched by the addition of the general sulfhydryl antioxidant N-acetylcysteine at 100µM, demonstrating the specificity of the oxidant derived fluorescence signal. Moreover, it was noted each drug showed and individual time profile of oxidant production. Saquinavir
and Ritoanvir demonstrated an early induction of oxidant production (45 minutes and 2Hrs), while Indonavir showed a delayed response with no significant increase at the earlier timepoints, but significant increase at the 24hr timepoint.

In light of apparent endothelial toxicities from our vascular studies, we also evaluated potential mechanisms of endothelial cell death following incubation with HIV-PI. At 2 hrs of drug incubation significant proportions of the treated cells were positively stained for the early phase of apoptosis (Annexin-V positive), with minimal evidence of necrosis (Figure 4.4). Later time points demonstrated a greater degree of necrosis positive cells (data not shown).

Since we observed endothelium dependant relaxant response impairment in the HIV-PI treated aortas in a relatively short time, we then attempted to investigate if there was a change in the cellular production of NO in endothelial cells.

Incubation of endothelial cells with the HIV-PI Saquinavir resulted in a decreased NO response as measured by DAF-2. (figure 4.5)
DISCUSSION:

While introduction of HIV-PI's and HAART therapy have proven to be highly effective in decreasing morbidity and mortality in HIV/AIDS patients, it is now well recognized that HIV/AIDS patients are at increased risk for a wide variety of cardiac and vascular disorders, including cardiomyopathies, myocarditis, and vasculopathies.(5-17). In addition altered metabolic characteristics such as lipid mobilization changes (e.g. hypertriglyceridemia), known risk factors for coronary artery disease have been characterized in this population, even before the introduction of HIV protease inhibitor drugs (pre-1995). Despite a study that demonstrated that HAART therapy is associated with a reduction in cardiac complications, (36) many studies have implicated HIV-PI drugs as contributors to the observed cardiovascular abnormalities in this patient population. Given the importance of HIV-PI agents as a critical component to successful HAART therapy and the necessity for chronic use the mechanistic understanding of their cardiovascular toxicities is critical for improved long term therapy.

Recent studies have suggested that the HIV-PI’s have clinical effects that include apparent impairment in vascular endothelial function (a hallmark feature of atherogenesis and coronary artery disease)(5). These vascular changes may be explained by either direct drug toxicity to endothelium in vivo or indirect effects related to other metabolic or pathogenic mechanisms (hyperlipidemia, altered cytokines, insulin resistance). Moreover some recent reports also suggest that individual HIV-PI’s might have varying potentials for producing vascular
complications. Given these complexities in vivo we tested the hypothesis that HIV-PI’s have direct actions on vasculature.

Using isolated vascular segments we observed selective impairment of endothelium dependent and NO mediated vascular relaxation, with no apparent alteration in contractile tone or response to an exogenous NO donor. It was interesting to note that, at the timepoint studied, (2hrs) only Ritonavir and Saquinavir produced altered endothelial function, while indonavir incubation had no apparent effect. Saquinavir produced a decrease in maximal relaxation, Ritonavir shifted the curve to the right. These data represent evidence for a direct action of HIV-PI’s on vasculature and are consistent with selective endothelial cell toxicity. These observations also concur with one clinical report demonstrating impaired brachial artery flow response following HIV-PI use in patients. (5) Given our initial findings at the intact vessel level we then conducted additional mechanistic studies using isolated murine endothelial cells. Our studies thus complement a previous report by Chen et al. (37) who examined the effects of Ritonavir on isolated endothelial cells with respect to viability (marker enzyme release) and DNA damage.

Given the observation that Endothelium dependant NO responses were impared in the HIV-PI treated aort's we then investigated the effect of HIV-PI incubation on NO production, in response to AcH, by murine endothelial cells in culture. In this experiment, we used only tested the effects of saquinavir, and observed a decrease of approximately 30% in Ach driven NO production as compared to control, further supporting the aortal incubation experiment data.
To investigate potential mechanisms of decreased available NO, we then evaluated the effects of HIV-PIs with respect to intracellular oxidant production using live cell imaging. Increased reactive oxygen species are often seen in various settings of endothelial dysfunction, and in previous investigations, we have demonstrated that this cell type is an avid producer of reactive oxygen and nitrogen species when stressed (27; 29; 38)). Moreover, the diversion of NO into RNS production could explain the decreased available NO seen in this setting. We observed significant increases in reactive species (as measured by the nonspecific detector of reactive nitrogen and oxygen species, DCF) at 45mins, 2 and 24 hours post HIV-PI incubation at 5 and 10µM. Of note is that under the experimental conditions employed the incubation medium containing drug is removed at the time of oxidant detection (e.g. cells are washed and loaded with dye), thus direct chemical oxidant production from HIV-PI is not an operable mechanism of our observations. As part of these experiments we employed the general antioxidant NAC to quench the observed DCF signal. In initial studies we have demonstrated that 1mM NAC can completely quench DCF signal (demonstrating oxidant mediated signal production in our conditions rather than fluorescence self-catalysis). We then found that 100µM NAC significantly abrogated the oxidant signal in the HIV-PI treated cells at both times. Moreover, it was noted each drug showed an individual time profile of oxidant production. Saquinavir and Ritoanvir demonstrated and early induction of oxidant production (45 minutes and 2Hrs), while Indonavir showed a delayed response with no significant increase at the earlier timepoints, but significant increase at the 24hr
timepoint. This is consistent with our isolated vessel function data in which only Saquinavir and Ritonavir induced endothelial dysfunction at 2hrs, while Indinavir did not have any effect, further suggesting that all HIV-PI’s do not act in a similar manner.

Although at this time the source of intracellular oxidants is unclear potential sites may include disruption of the mitochondrial electron transport chain or activation of various oxidase enzymes, and given the different time profiles, each HIV-PI might follow different pathways of oxidant production.

We then evaluated potential mechanisms of cell death (e.g. early apoptosis vs. necrosis), and found that as early as 2 hours during drug exposure significant increases in apoptosis marker was detected in approximately 30% of the cells exposed to Saquinavir and Indonavir. In contrast, the incidence of necrosis was relatively low (<5%) . We are unable to demonstrate data on Ritonavir since we did not find enough surviving cells to produce any summarized data. Our finding that apoptosis predominates for Saquinavir and Indonavir are in contrast to those of Chen et al. who did not detect evidence of apoptosis at, but this may be related to the times investigated (early measures in this study vs. 24 and 48 hours by Chen) the endpoint investigated (Annexin-V staining vs. DNA damage) and/or the specific drug investigated (Saquinavir and Indonavir vs. Ritonavir).

Although the precise mechanism by which HIV-PI’s causes endothelial cell apoptosis remains to be defined, intracellular oxidant increases might be playing a role in the phenomena. It is interesting to note that reduced serum glutathione
has been described in HIV+ patients and that supplementation has been linked to increased CD4+ counts and possible survival benefits(39-43). It is possible that antioxidant status may play a role in increased risk for HIV-PI clinical drug toxicities as well. It would be of clinical relevance to see if the initial observations of selective endothelial and NO impairment observed in isolated vessels could be prevented / abrogated by antioxidant supplementation. Moreover, we have shown an apparent NO impairment in vessels in face of incubation with HIV-PI's. Though this data is supported by clinical observations of endothelial dysfunction in patients receiving HIV-PI therapy, no studies have been performed evaluating the expression and function of the NOS isoforms in this setting. Along with increased oxidants shunting availability of NO to peroxynitrite and other reactive nitrogen species chemistries, investigation of NOS isoforms themselves in presence of protease inhibitors is also warranted.

Many previous studies have documented that various components of the HIV virus as well as activated cytokines are toxic to endothelial cells (tat protein, GP120, TNF, others) (44-46). Here we provide additional evidence that HIV-PI drugs themselves may cause direct toxicity to this important cell type. Furthermore we also present evidence that all HIV-PI drugs might not act in the same way in terms of endothelial toxicity, and further investigation of this phenomena might be beneficial in designing therapy for HIV/AIDS patients. This drug class, while undoubtedly beneficial to the HIV+ population, may have unique toxicities to this cell type (in light of our isolated vascular experiments)
and while the mechanisms of endothelial toxicity are not completely defined they may be blunted by appropriate antioxidant use. These findings also suggest that the risk of atherosclerosis associated with HIV-PI's may not be exclusively related to hyperlipidemia but also a more vulnerable vascular surface since apoptotic endothelial cells and surface irregularities are prone to plaque formation and thrombosis. This is consistent with observation of abnormal coronary vasculature from HIV/AIDS patients receiving HAART, (47) and further support a significant role for endothelium as an important effector of HIV/AIDS and/or HIV-PI related cardiovascular complications.
REFERENCE:


42. Aukrust P, Svardal AM, Muller F, Lunden B, Berge RK, Ueland PM, Froland SS: Increased levels of oxidized glutathione in CD4+ lymphocytes associated with disturbed intracellular redox balance in human immunodeficiency virus type 1 infection. *Blood* 86:258-267, 1995


FIGURE LEGENDS

Figure 4.1. Saquinavir and ritonavir but not indinavir, inhibited the endothelium-dependent relaxation response to acetylcholine. Segments were incubated with HIV protease inhibitors for two hours and then exposed to PE followed by accumulative increment of ACH in the presence of HIV protease inhibitors (See details in Methods section). Shown are absolute percentage relaxation responses. CTRL, control; INDO, indinavir; RITO, ritonavir; SAQ, saquinavir. Insert a: EC50; Insert b: maximal relaxation. * p<0.05 as compared to the control, n=6-9.

Figure 4.2. The endothelium-independent relaxation response to sodium nitroprusside was not significantly affected by HIV protease inhibitors. Accumulative response curves are shown; Insert a: maximal relaxation; Insert b: EC50. (n=6-9).

Figure 4.3. HIV protease inhibitors time dependently increased DCF fluorescence in cultured endothelial cells. Top panel: representative pictures. Middle right Panel: Grouped data. NAC: N-acetylcysteine (20 mM). Data show that SAQ and RITO, but not INDO, caused a significant increase of DCF fluorescent signal following two hour incubation and this increase was blocked by antioxidant, NAC. * p<0.05 (n=30-90 cells). Middle Left Panel: Grouped data of DCF fluorescence intensity. Note that each of the protease inhibitors caused a significant increase of DCF fluorescent intensity following 24-hour incubation and this increase was
blocked by NAC. * p<0.05 (n=25-64 cells). Bottom Panel; Time course of DCF fluorescence in the presence and absence of HIV protease inhibitors. Note that DCF fluorescence signal followed a differential time course for each protease inhibitor (n=15-92 cells.)

Fig 4.4. Induction of apoptosis by HIV-PI’s. Cells were incubated with HIV-PI’s (5 µM) for two hours. Upper Panels: Composite image of FITC-conjugated annexin V (green) and Propidium Iodide (red) co-labeling of cells treated to HIV-PI’s. Note the nuclear condensation depicted by Propidium Iodide staining. INDI pictures at 2(early) and 24(late) hrs. all others at 2hrs. Lower Panel: Percentage of apoptotic and necrotic cells.

Fig 4.5. Effect of HIV-PI Saquinariv on NO release: Cells were incubated with HIV-PI Saquinariv (5 µM) for 24hrs and NO release in response to Ach measured by DAF-2 was shown to be reduced.
Figure 4.1. Saquinavir and ritonavir but not indinavir, inhibited the endothelium-dependent relaxation response to acetylcholine.
Figure 4.2. The Endothelium-Independent Relaxation Response
Figure 4.3 HIV Protease Inhibitors Time Dependently Increased DCF Fluorescence In Cultured Endothelial Cells
Figure 4.4. Induction Of Apoptosis By HIV-PI's.
Figure 4.5. Effect Of HIV-PI Saquinavir On NO Release
CHAPTER 5

VASCULAR ENDOTHELIAL TOXICITY INDUCED BY HIV PROTEASE INHIBITOR: EVIDENCE OF OXIDANT RELATED DYSFUNCTION AND APOPTOSIS.

This chapter has been submitted to *Cardiovascular Toxicology* and is presented in the style appropriate to the journal.
ABSTRACT:

HIV-protease inhibitor drugs (HIV-PI’s) are critical for HAART efficacy but several recent reports have suggested that metabolic and/or cardiovascular toxicities are associated with these drugs. Given the importance of the HIV-PI drug class and the widespread and chronic use of these agents in an expanding patient population, further understanding of this potential drug toxicity is imperative. Here we investigated a role for direct endothelial toxicity induced by Saquinavir (SAQ), the first HIV-PI drug marketed in the US and still an important component of HAART therapies. In initial studies using isolated vascular tissues we observed selective impairment of endothelium dependent vasodilation with no effect on contractile responses. Subsequent studies using human endothelial cells in culture at clinically relevant concentrations (5 and 10µM, 2-48 hrs) demonstrated concentration dependent increases in cell death, mainly via apoptosis rather than necrosis (determined via Annexin-V positive membrane labeling). Live cell imaging also demonstrated increased intracellular oxidant production (as measured by DCF fluorescence), which could be abrogated by incubation with the antioxidant N-acetylcysteine (NAC). NAC also prevented SAQ induced apoptotic cell death. These data demonstrate that SAQ has direct toxicological effects on endothelial cells, and that the toxicity apparently involves apoptotic pathway activation via reactive oxygen and/or nitrogen species.
INTRODUCTION:

The implementation of HIV-protease inhibitor drugs (HIV-PI's) into combination therapy regimens has been central to the development of improved care and outcomes for HIV/AIDS patients. These agents have proven highly effective in providing a sustained suppression of viral load, and have dramatically reduced the incidence and severity of opportunistic infection in HIV/AIDS patients. (1-5) However, these agents have also been implicated in the development of a variety of drug toxicities, including hepatic injury, metabolic complications, insulin resistance and lipodystrophies. (17-20) In addition, a few recent reports have suggested that this important drug class is associated with cardiovascular toxicities themselves, including atherogenesis, vasculopathies, and cardiac structural and/or functional changes and some suggestion of myocardial infarctions. (10-16,21-30) Whether these are direct drug toxicities or indirect interactions with HIV pathogenesis remains to be defined, since retroviral pathogenesis itself has been associated with cardiovascular dysfunction and hypertriglyceridemia in both animals and humans. (6-9,46) Given the importance of the HIV-PI drug class and the widespread and chronic use of these agents in an expanding patient population, further understanding of this potential drug toxicity is imperative.

Over the last decade, the importance of the vascular endothelium for maintenance of cardiovascular health has become evident and early injury and/or dysfunction has been associated with many cardiovascular disease states. (35-40) A few recent clinical reports have suggested that HIV-PI drugs may have negative effects on endothelial function in HIV patients. (21,24-26,28) In addition, a potential role for direct endothelial
toxicity has also been supported by a recent report that Ritonavir can cause direct toxicities to endothelial cells in vitro. (30) However, these clinical findings have not been universal (31) and the direct toxicities of other HIV-PI agents have not been reported. Here we investigated a role for direct endothelial toxicity induced by Saquinavir, the first HIV-PI drug marketed in the US and still an important component of HAART therapies. In initial studies we employed functional assessments using isolated animal vascular tissues, followed by mechanistic investigations using human endothelial cells in culture at clinically relevant concentrations. Our focus in these studies included oxidant related mechanisms since others and we have demonstrated that reactive oxygen and/or nitrogen species may contribute to endothelial dysfunction and injury in a wide array of disease settings (31-35), including HAART related lipodystrophies.
METHODS:

*Isolated vascular function*

Functional studies using rat thoracic aorta segments were conducted similar to methods previously described (43). Four male Sprague Dawley rats were used for functional studies and of 6-8 rings obtained per animal, half were employed as control and the other half as Saquinavir treated. Following 1 hour of equilibration in oxygenated Krebs’ buffer, the vessel segments were exposed to Saquinavir (5µM) or vehicle for 2 hr. Segments were then precontracted with phenylephrine (2.0µM, approximately 80% of maximal contraction). Cumulative relaxant responses to acetylcholine (ACH, 1x10^-9 - 2x10^-4 M, endothelium dependent and NO mediated vasorelaxation) or sodium nitroprusside (SNP, 1x10^-9 - 2x10^-4, endothelium independent) were recorded. After washing (10min), maximal contractile response to 125 mM KCl was recorded at the end of the studies for each segment. In all aspects of these studies, Saquinavir was used in pure drug form, which was obtained from Hoffman-La Roche Inc.

*Human endothelial cell culture experiments*

Human umbilical vein endothelial cells (HUVECS) were obtained from Clonetics, Walkersville, MD were grown to confluence. Culture media consisted of endothelial growth medium, EGM-2 (Clonetics, Walkersville, MD) containing 0.5% serum.

*General Cell Viability Assay*

Endothelial cells (5x10^4) were seeded in UV transparent 96-well plates, grown to confluence for 24 hours, then treated overnight in medium alone or medium containing
saquinavir (0 to 10µM) for 24 or 48 hours. Cells were then gently washed and fixed in 5% buffered formalin, and stained with crystal violet as a marker of cell viability, as previously described (48). Crystal violet signal was then assayed spectrophotometrically at 590nm (Spectramax Plus, Molecular Devices, Sunnyvale, CA). Preliminary experiments verified the linear response of the violet intensity with respect to viable endothelial cell number (approximate detection limit 1000 viable cells), intra- and inter-day variability were each less than 5%.

**Cellular Apoptosis vs. Necrosis**

Cells were grown to confluence on 12 well glass slides and then treated with Saquinavir at 5 and 10uM for 2 hrs and mechanisms of cell death were investigated using fluorescence microscopy via a commercially available assay kit (Annexin V-Cy3/6- Carboxyfluorescein diacetate kit, Sigma, St. Louis, MO). After a 10 minute incubation in double labeling solution the cells were placed on ice and fluorescent images were captured at WB and IR fluorescence using the Optronics Magnafire imaging system and analyzed using research image analysis software (ImagePro Plus; Media Cybernetics, Silver Spring, MD, USA). Using this method live cells stain green due to externalization of phosphatidylserine while necrotic cells stain red due to permeability to propidium iodide. Early apoptotic cells stain both green and red.

**Intracellular Reactive Oxidant Species**

The intracellular detection of reactive oxygen and reactive nitrogen species was conducted using 5(6)-chloromethy-2, 7-dichlorodihydrofluorescein diacetate (DCF-DA,
Molecular Probes, Oregon), as previously described (47). This cell permeable dye, DCF-DA diffuses into cells and is hydrolyzed by intracellular esterases to polar 6-carboxy-2', 7'-dichlorodihydrofluorescein. This nonfluorescent fluorescein analog is trapped in cells and can be oxidized to highly fluorescent 6-carboxy-2', 7'-dichlorofluorescein by intracellular oxidants (including hydrogen peroxide, superoxide anion, hydroxyl radical), or reactive nitrogen species (peroxynitrite) (46) emits fluorescence at 515nm. Thus this method provides appropriate detection of the major endothelial cell oxidant species. Endothelial cells were grown to 80% confluence on 6-mm glass cover slips and then treated with Saquinavir for 2 or 24 hrs. Control (vehicle treated) cells for each time point were also included. Following dye loading cells were incubated in the dark for 30 minutes, washed with protein free medium and fluorescent images were immediately captured with Olympus CMAD-2 camera system using a 25% neutral density filter. The captured images, 5-10 per slide (more than 100 cells investigated per treatment group and time point), were then analyzed using research image analysis software (ImagePro Plus, Media Cybernetics, Silver Spring, MD, USA).

**Statistical Analyses**

All data are represented as mean ± SE. Cumulative concentration responses (Figure 1b and c) were curve fitted to the 4-parameter logistic equation using Graphpad analysis software as previously described (). Statistical evaluations were performed using Sigmastat software (Jandel Scientific Inc., San Rafeal, CA). Comparisons among treatment groups were performed using student’s t tests or analysis of variance where appropriate. In all cases, p<0.05 was deemed statistically significant.
RESULTS:

Shown in Figure 5.1 are effects of Saquinavir on isolated vascular segments (n=6-10). Following incubations with drug or vehicle contractile and relaxant responses were measured. No significant change in phenylephrine induced (alpha agonist contractile response) or KCl induced (nonspecific smooth muscle depolarization response) was observed following saquinavir treatment (Figure 5.1a). Despite unaltered vascular contractile properties significant alteration in acetylcholine induced vasorelaxation was observed (Figure 5.1b). Curve fitting of the cumulative concentration exposures provided statistically significant changes in acetylcholine Emax and EC50 values (Figure 5.1b inset panels, p<0.05). In contrast to diminished acetylcholine responses no significant change in vasorelaxant response to the endothelium independent and ‘spontaneous’ nitric oxide liberating agent nitroprusside (Figure 5.1c).

In light of apparent endothelial toxicities from our vascular studies, we evaluated effects of Saquinavir on general cell survival in vitro. Using a general staining method that detects remaining adherent cells (see methods) we found that incubation of human endothelial cells with Saquinavir at 10µM, but not 5µM, for 24 or 48 hours caused a significant decrease in cell survival (Figure 2).

We investigated potential mechanisms of endothelial cell death following incubation with Saquinavir. At 2 hrs of drug incubation significant proportions of the treated cells were positively stained for the early phase of apoptosis (Annexin-V positive), with minimal
evidence of necrosis (Figure 5.3). Later time points demonstrated a greater degree of necrosis positive cells (data not shown).

Given of evidence of apoptosis induced by Saquinavir at clinically relevant concentrations we investigated potential involvement of reactive oxidant species using DCF fluorescence live cell imaging. Saquinavir caused time and concentration dependent increases in intracellular oxidant production relative to control (Figure 5.4). This was quenched by the addition of the general sulfhydryl antioxidant N-acetylcysteine at 100µM, demonstrating the specificity of the oxidant derived fluorescence signal.

We then tested the hypothesis that NAC could protect against Saquinavir induced endothelial cell death, measuring general cell survival at 24 and 48 hrs and early cell death mechanisms at 2 hrs (data shown in Figure 5). Incubation of cells with the sulfhydryl antioxidant (100µM) completely prevented Saquinavir induced cell loss at 24 or 48 hours (Figure 5.5a). In parallel experiments, we found that NAC abrogated the presence of apoptotic cells, but had no impact on the very low prevalence of necrosis at 2 hrs (Figure 5.5b).
DISCUSSION:

It is now well recognized that HIV/AIDS patients are at increased risk for a wide variety of cardiac and vascular disorders, including cardiomyopathies, myocarditis, and vasculopathies. (29,48) In addition altered metabolic characteristics such as lipid mobilization changes (e.g. hypertriglyceridemia), known risk factors for coronary artery disease have been characterized in this population, even before the introduction of HIV protease inhibitor drugs (pre-1995). (6-9) Despite a study that demonstrated that HAART therapy is associated with a reduction in cardiac complications, (47) many studies have implicated HIV-PI drugs as contributors to the observed cardiovascular abnormalities in this patient population. Given the importance of HIV-PI agents as a critical component to successful HAART therapy and the necessity for chronic use the mechanistic understanding of their cardiovascular toxicities is critical for improved long term therapy.

Recent studies have suggested that the HIV-PI's have clinical effects that include apparent impairment in vascular endothelial function (a hallmark feature of atherogenesis and coronary artery disease). These vascular changes may be explained by either direct drug toxicity to endothelium in vivo or indirect effects related to other metabolic or pathogenic mechanisms (hyperlipidemia, altered cytokines, insulin resistance). Give these complexities in vivo we tested the hypothesis that HIV-PI's have direct actions on vasculature, employing Saquinavir as the model agent. This agent was the first to be launched in the US and remains widely used; its inhibition of the HIV protease is similar to others in the drug class. Additional advantages of this agent were
the ability to avoid complicated co-solvent mixtures for in vitro experimentation and the absence of active metabolites.

Using isolated vascular segments we observed selective impairment of endothelium dependent and NO mediated vascular relaxation, with no apparent alteration in contractile tone or response to an exogenous NO donor. These data represent evidence for a direct action of HIV-PI’s on vasculature and are consistent with selective endothelial cell toxicity. These observations also concur with one clinical report demonstrating impaired brachial artery flow response following HIV-PI use in patients. (26) Given our initial findings at the intact vessel level we then conducted additional mechanistic studies using isolated human endothelial cells. Our studies thus complement a previous report by Chen et al. (30) who examined the effects of Ritonavir on isolated endothelial cells with respect to viability (marker enzyme release) and DNA damage.

Using a general live/dead assay for adherent endothelial cells we detected significant loss of cell viability at clinically relevant concentrations (less than 20µM) after 24 and 48 hours of incubation. Under the conditions employed we observed an approximate Ki for cell killing of approximately 10µM (see Figure 5.2). This concentration is within the range of typical peak plasma concentrations in patients receiving therapy (at a recommended dose of 1200mg thrice daily, peak plasma concentrations range from 2-13µM) (32). We then evaluated potential mechanisms of cell death (e.g. early apoptosis vs. necrosis), and found that as early as 2 hours during drug exposure significant
increases in apoptosis marker was detected in approximately 30% of the cells. In contrast, the incidence of necrosis was relatively low (<5%) and not related to drug concentration or exposure time. Our finding that apoptosis predominates for Saquinavir are in contrast to those of Chen et al. who did not detect evidence of apoptosis at, but this may be related to the times investigated (early measures in this study vs. 24 and 48 hours by Chen) the endpoint investigated (Annexin-V staining vs. DNA damage) and/or the specific drug investigated (Saquinavir vs. Ritonavir).

Increased reactive oxygen species are often seen in various settings of endothelial dysfunction, and in previous investigations, we have demonstrated that this cell type is an avid producer of reactive oxygen and nitrogen species when stressed (35). We then therefore evaluated the effects Saquinavir with respect to intracellular oxidant production using live cell imaging. We observed significant increases in reactive species (as measured by the nonspecific detector of reactive nitrogen and oxygen species, DCF) at 2 and 24 hours post Saquinavir incubation at 5 and 10µM. Of note is that under the experimental conditions employed the incubation medium containing drug is removed at the time of oxidant detection (e.g. cells are washed and loaded with dye), thus direct chemical oxidant production from Saquinavir is not an operable mechanism of our observations. As part of these experiments we employed the general antioxidant NAC to quench the observed DCF signal. In initial studies we have demonstrated that 1mM NAC can completely quench DCF signal (demonstrating oxidant mediated signal production in our conditions rather than fluorescence self-catalysis). We then found that 100µM NAC significantly abrogated the oxidant signal in the Saquinavir treated cells at
both times and Saquinavir concentrations. Although at this time the source of intracellular oxidants is unclear potential sites may include disruption of the mitochondrial electron transport chain (see other chapters in this issue) or activation of various oxidase enzymes.

Given the observation that 100µM NAC could significantly reduce the extent of Saquinavir induced oxidant prevalence but not have effects on control cells we evaluated the effects of this combined treatment (Saquinavir plus NAC 100µM) with respect to cell survival, apoptosis and necrosis. We found that the addition of NAC prevented Saquinavir induced endothelial cell death at 24 and 48 hours and abrogated the prevalence of apoptotic cells at 2 hours with no impact on necrosis. These data are therefore consistent with a mechanism of rapid (<2h) Saquinavir induced oxidant production, leading to initiation of apoptotic death sequences. Further characterization of the pathways involved in this process and targets involved in initiation are clearly warranted.

Although the precise mechanism by which Saquinavir causes endothelial cell apoptosis remains to be defined, all the cellular effects of at least this protease inhibitor appear to be overcome by the addition of a common antioxidant. It is interesting to note that reduced serum glutathione has been described in HIV+ patients and that supplementation has been linked to increased CD4+ counts and possible survival benefits (41,42). It is possible that antioxidant status may play a role in increased risk for HIV-PI clinical drug toxicities as well. It would be of clinical relevance to see if the
initial observations of selective endothelial and NO impairment observed in isolated vessels could be prevented / abrogated by antioxidant supplementation. (studies are underway in our laboratory at present). Moreover, since we have shown an apparent NO impairment in vessels in face of incubation with Saquinavir. Though this data is supported by clinical observations of endothelial dysfunction in patients receiving HIV-PI therapy, no studies have been performed evaluating the expression and function of the NOS isoforms in this setting. Along with increased oxidants shunting availability of NO to peroxynitrite and other reactive nitrogen species chemistries, investigation of NOS isoforms themselves in presence of protease inhibitors is also warranted.

Many previous studies have documented that various components of the HIV virus as well as activated cytokines are toxic to endothelial cells (tat protein, GP120, TNF, others) (29). Here we provide additional evidence that HIV-PI drugs themselves may cause direct toxicity to this important cell type. This drug class, while undoubtedly beneficial to the HIV+ population, may have unique toxicities to this cell type (in light of our isolated vascular experiments) and while the mechanisms of endothelial toxicity are not completely defined they may be blunted by appropriate antioxidant use. These findings also suggest that the risk of atherosclerosis associated with HIV-PI's may not be exclusively related to hyperlipidemia but also a more vulnerable vascular surface since apoptotic endothelial cells and surface irregularities are prone to plaque formation and thrombosis.
This is consistent with observation of abnormal coronary vasculature from HIV/AIDS patients receiving HAART, (6) and further support a significant role for endothelium as an important effector of HIV/AIDS and/or HIV-PI related cardiovascular complications.
REFERENCES:


dysfunction in HIV-positive patients under highly active active antiretroviral therapy. *AIDS*. **17**(5): 765-768.


FIGURE LEGENDS:
Figure 5.1: Endothelial dysfunction in isolated vascular tissue. Aortic segments were incubated with either Saquinavir 5µM or vehicle (KREBS) for 2hrs. Panel 5.1a: Contractile responses. No significant changes in maximal Phenylephrine or 125 mM KCL induced contractions. Fig 1b&c: Relaxant responses presented as percent relaxation of phenylephrine precontraction (80% maximal contraction). Panel 1b: change in vascular endothelium-dependant relaxant response to acetylcholine (ACH 1nM to 1µM) obtained from control (open circles) or Saquinavir (closed circles). Statistically significant changes in EC50 and Emax seen (p<0.005): n=6-10. Panel 1c: endothelium-independent relaxant response to nitric oxide donor sodium nitroprusside (SNP) showed no significant differences

Figure 5.2: Concentration dependent endothelial cytotoxicity induced by Saquinavir. Endothelial cells were incubated with Saquinavir (0,5 or 10 µM) for 24 or 48 hrs. Data represented as percentage of control at 24hrs. Cells were stained with crystal violet as a marker of cell survival and assayed spectrophotometrically at 590nm. Saquinavir at 10µM but not 5µM caused marked decreases in surviving endothelial cells at both time points. Values are mean ±SEM, N = triplicate experiments. * Denotes statistically significant difference from vehicle treated control (p<0.005)

Figure 5.3: Concentration dependent increases in endothelial apoptosis induced by Saquinavir. Endothelial cells were incubated with 0,5 or 10µM Saquinavir for 2hours and the mechanism of cell death investigated using a commercially available assay kit (Annexin V-Cy3/6-Carboxyfluorescein diacetate kit). Apoptotic cells stain green while
necrotic cells stain red. Filled circles represent apoptotic cell and filled circles represent necrotic cell. Data represented as percentage of total cells, N = triplicate experiments. * Denotes statistically significant difference from vehicle treated control (p<0.005)

Figure 5.4: Saquinavir induced increase in intracellular oxidant production.
Increased time and concentration dependent oxidant signal on incubation with Saquinavir (filled bars) as measured by DCF fluorescence live cell imaging, inhibited by co-incubation with 100µM N-acetyl cysteine (NAC, open bars). Data presented as relative fluorescence units (RFU), average of at least 100 cells/treatment ±SEM. * denotes statistically significant difference from corresponding only Saquinavir treated time-point. (p<0.005)

Figure 5.5: NAC protects against Saquinavir induced cell death. Panel 5a: Saquinavir (filled bars) induced cell death by crystal violet assay at 24 and 48 hrs prevented by co-incubation with 100µM NAC (open bars). Data represented as percentage of control at 24hrs, mean ± SEM. * denotes statistically significant difference from corresponding only NAC treated time-point. (p<0.005)
Panel 5b: Saquinavir (10µM) induced early apoptosis (2hr time-point) prevented by NAC. Panel 5c: No change in necrosis on incubation with NAC. Saquinavir only filled bars, NAC co-incubation open bars. Data represented as percentage of total cells, N = triplicate experiments. * Denotes statistically significant difference from corresponding only Saquinavir treated time-point (p<0.005).
Figure 5.1: Endothelial dysfunction in isolated vascular tissue
Figure 5.2: Concentration dependent endothelial cytotoxicity induced by Saquinavir
Figure 5.3: Concentration dependent increases in endothelial apoptosis
Figure 5.4: Saquinavir induced increase in intracellular oxidant production
Figure 5.5: NAC protects against Saquinavir induced cell death
CHAPTER 6

Characterization Of Diabetic Cardiomyopathy In A Mouse Model Of Diabetes

This chapter has been prepared for submission to *Diabetes* and is presented in the style appropriate to the journal.
**ABSTRACT**

**Objective:** Type I diabetes mellitus is complicated by severe progressive cardiovascular diseases, including hypertension, congestive heart failure and coronary artery disease. Diabetes is also a major risk factor for the development of arrhythmias. Mechanistic studies are often difficult to conduct in this patient population and can be confounded by co-existing variables. Time-dependencies of clinically relevant parameters of cardiac performance in a mouse model would be uniquely useful (for transgenic studies, etc), but have not been previously investigated. We tested the hypothesis that the STZ diabetic mouse model, time mimics events observed in Type I non-atherogenic cardiomyopathy in a functionally relevant and linear manner.

**Methods:** Hyperglycemia was induced in male CF-1 mice weighing 16-18g with a single dose of streptozotocin (STZ, 150 mg/kg i.p. prepared daily in citrate buffer pH 4.5 for maximal stability). Animals were studied longitudinally at 0, 1week and 5 weeks post-STZ (n=18 each time point), for assessment of both diastolic and systolic performance by non-invasive echocardiography, as well as electrocardiographically for conduction abnormalities.

**Results:** Significant and rapid hyperglycemia was observed at 1week post-STZ and persisted throughout the 5 week study (avg 1w 254±33, 5w 308.±41. mg/dl). Statistically significant impairment in LV fractional shortening was observed at both 1 week and 5 weeks (down ~20%). We also observed and early reduction in the E wave (Control 102.99±3.59, 1 week 88.43±5.17) and E/A ratios (control 3.53±0.25, 1week 3.04±0.24) at 1 week, but at 5 weeks the diastolic parameters were back to
control. ECGs from control and diabetic mice showed a significant decrease in the P-wave amplitude, P- duration however was unaltered. We also observed a consistent time dependant increase atrio-ventricular conduction time as shown by the increased QRS complex duration (Control 20.0±0.4ms, 1 week diabetic 22.39±0.53, 5 week STZ 25.16±0.84ms, p<0.005). Further, we observed an increase in the QT interval, which persisted after heart rate correction, (QTcF, Control 126.4±2.3, 5w STZ 134.53±1.57).

Conclusions: This study suggests that the STZ mouse model is appropriate for the mechanistic study of Type I diabetic cardiomyopathy, and provides time-dependent, clinically relevant assessments of cardiac performance (systolic, diastolic and electrocardiographic) as a foundation for further mechanistic studies.
INTRODUCTION

Type I diabetes mellitus is complicated by several progressive cardiovascular diseases, including hypertension, congestive heart failure and coronary artery disease, as over 75% of all diabetic patients die from cardiovascular events. (1-3). Many of these complications may be secondary to errors in lipid metabolism and attendant atherosclerosis. However, a non-atherogenic cardiomyopathy in Type I diabetics has been recognized for over 20 years (4). This unique form of cardiac disease occurs in roughly 30% of all Type I patients, and presents as early diastolic abnormalities followed by later decreases in left ventricular ejection fraction, overt failure and death (5). While this syndrome is well recognized, the mechanisms involved are poorly understood and specific therapeutic strategies in this patient population are currently undefined (6).

Mechanistic studies are often difficult to conduct in this patient population and can be confounded by co-existing variables (e.g., diet, activity level, genetic predispositions, etc). A relevant animal model would be valuable, particularly for experimental therapeutics. We have previously described cardiac abnormalities in the streptozotocin rat model of diabetes, but time-dependencies of clinically relevant parameters of cardiac performance in a mouse model would be uniquely useful (for transgenic studies, etc), but have not been previously investigated. Here we tested the hypothesis that this model mimics events observed in Type I non-atherogenic cardiomyopathy. In initial studies, we developed reliable and convenient methods for assessment of both diastolic and systolic performance in this model using non-invasive echocardiography. Our inclusion of diastolic performance assessments was
based on the recent studies in humans and animals demonstrating that diastolic abnormalities may precede systolic changes in many forms of cardiomyopathy (including diabetes related cardiac failure). We additionally investigated a role for cardiac hypertrophy and/or fibrosis during diabetic progression.
METHODS

Study Design

All aspects of our animal use were in accordance with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee. Hyperglycemia was induced in Pathogen-free male CF-1 mice (age 6 weeks, n=18, Harlan Laboratories, Indianapolis, IN) weighing 16-18g with a single dose of streptozotocin (STZ, 150 mg/kg i.p. prepared daily in citrate buffer pH 4.5 for maximal stability) or vehicle control. Animals were housed in a sterile cage rack system with HEPA filtered air circulation (approximately 50 air changes/hour, Allentown Caging Inc., Allentown, PA).

Animals were studied longitudinally at 0, 1week and 5 weeks post-STZ (n=18 each time point). Eighteen age-matched mice were treated with vehicle and studied at 0, 1week and 5 weeks, to evaluate control parameters for this time dependency study. Statistical differences in these control groups (0 vs. 1week vs 5weeks) were not observed for any parameter evaluated, therefore these observations were pooled and served as control values. In all figures shown these control values are represented at time 0. Mice were sacrificed at the 5week time point.

Blood glucose

Blood glucose was determined at each time point with a Glucometer Encore (Bayer) clinical blood glucose monitor. Intraperitoneal glucose tolerance tests (IPGTT) were also performed at each time point. 200mg/kg glucose solution was administered i.p. and blood glucose values were measured at 0, 60 and 120 minutes post injection. Area under the curve (AUC) shows glucose handling over time.
Assessment of cardiac function

At 0, 1 and 5 weeks post-STZ, in vivo cardiovascular function was determined using a Sonos 1000 echocardiography unit (Hewlett-Packard, Andover, MA), as previously described (7). Mice were anesthetized by halothane inhalation (0.25–1.5% halothane in 95/5% O₂/CO₂) and normothermia was maintained by heating pad. In separate studies, systolic blood pressure was determined by tail cuff monitor (Model 289Z, IITC Life Sciences, Woodland Hills, CA). A 7.5 MHz pediatric probe (optimized and dedicated to rodent studies) placed in the parasternal, short axis orientation recorded LV systolic and diastolic internal dimensions. Three loops of M-mode data were captured for each animal, and data were averaged from at least 5 beat cycles/loop. Parameters were determined using the American Society for Echocardiography leading-edge technique in blinded fashion. These parameters allowed the determination of LV fractional shortening (FS) by the equation: 

\[ FS = \left( \frac{LVID_d - LVID_s}{LVID_d} \right) \times 100\% \]

where LVID refers to the LV internal dimension at diastole (d) and systole (s). Ascending aortic flow waveforms were recorded using a continuous wave doppler flow probe oriented in a short axis, suprasternal manner. Peak aortic flow and velocity-time integrals (VTI) were calculated from these waveforms. After sacrifice, aortic root cross-sectional area was measured and cardiac output (CO) was calculated by the equation: 

\[ CO = \text{Heart rate} \times \text{VTI} \times \text{aortic cross-sectional area} \]

Autopsy measurements of aortic root cross-sectional area were conducted due to their convenience and high degree of accuracy for rodent studies. We observed no significant age- or diabetes related changes in left ventricular outflow dimensions at any time point studied (Day 0 and 5 week controls
and 1 and 5 week diabetic mice). Transmitral flow waveforms were recorded using continuous wave doppler oriented in the parasternal, long axis. Peak transmitral (E wave) flow was determined, as well as E wave acceleration slope and deceleration slope, as measures of diastolic ventricular function. Where E and A waveforms were baseline resolved, E wave slopes were extrapolated from the upper 2/3 of the waveform (from apex toward baseline). Intra- and inter-observer variability for these measurements were 3% and 5% respectively. All systolic and diastolic parameters were analyzed by a blinded observer.

**Electrocardiography**

Anesthesia induction with halothane (Halocarbon Labs, River Edge, NJ) was achieved by placing the animals in an isolation chamber with a mixture of 95% oxygen and the anesthetic. Inhalation anesthesia was maintained using a modified low-flow vaporizer with a nose cone (Bickford Vapomatic, A.M. Bickford Inc., Wales Center, NY) at a minimally effective concentration of halothane.

Animals were placed on a heated gel pack (Instant Heat Pads from Harvard Apparatus, South Natick, Massachusetts) designed to maintain normothermia during data acquisition. Recordings were made with adhesive electrodes attached to all four paws (BIOPAC Systems, Inc, Santa Barbara, CA) with the mice in a supine position. Electrocardiograms were digitally recorded using a physiologic data acquisition system (MP100, Biopac Systems), with a sampling rate of 2000 Hz. The ECGs were signal averaged (from Lead I) over 150–200 beats per acquisition for determination of the P wave duration, P–R interval, and QT interval as previously described (8). P wave duration (a measure intra-atrial conduction) was measured
from the onset of the P wave to the point where the P wave returns to the baseline. The P–R interval (representing intra-atrial and atrio-ventricular conduction) was measured from the P wave onset to the onset of the QRS complex (representing ventricular depolarization). The end of QRS could not be measured accurately, and therefore the QRS duration has not been reported. The QT interval (representing the duration of ventricular depolarization and re-polarization) was measured from the onset of the QRS complex to the end of the T wave (intersection with the baseline). The average intra-observer variability for all parameters measured was < 2%. Since the QT interval is highly dependent on heart rate, the appropriateness of classical QT correction factors was tested. The average RR interval was determined from a 30 second sample. Rate corrected QT interval, QTc, was calculated using the methods of Bazett (QTc = QT/RR1/2) and Fridericia (QTc = QT/RR1/3). The slope of the QTc vs. RR relationship was determined for each correction method; the method resulting in a slope closest to zero was determined to be most appropriate for rate correction.

**Cardiac histology**

Animals were sacrificed with 100mg/kg i.p. pentobarbital sodium (Abbott Laboratories, Chicago, IL). The apical portion of the heart was equatorially bisected just distal to the mitral valve and immersed in 10% formalin. Tissues were paraffin embedded and blocked according to standard procedures. Five micron sections were evaluated using standard protocols for hematoxylin/eosin staining to determine LV cross-sectional areas and Masson’s Trichrome stain for fibrosis, as we have previously described (9).
**Image capture and digital image analysis**

Cardiac images were captured using a Polaroid digital camera (Polaroid Corporation, Cambridge, MA) and transferred into research-based digital image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD). Equatorial LV cross-sectional areas were determined by subtracting LV lumenal cross-sectional area from total LV area following digital spatial calibration. The extent of fibrosis in the LV was determined by color segmentation analysis. Multiple left ventricular images were captured from each tissue section under identical lighting and optical settings as to encompass 60% of total left ventricular area. Images were then segmented to eliminate background and nuclear counterstain from analysis. Percentage of total LV area demonstrating blue staining (indicative of extracellular matrix) was determined. Intra- and inter-observer variability (5 cardiac images, 3 blinded observers) were each less than 2% for this technique.

**Statistical analysis**

Parameters between diabetic and control groups were evaluated for significance by one-way ANOVA, with Student-Newman-Keuls post-hoc analysis for all functional data and Student t-tests for all histological data. In all cases, results are expressed as mean ± sem and significance was defined as p<0.05.
RESULTS

Blood glucose concentrations and IPGTT were determined during STZ progression and are shown in Figure 6.1. As expected, significant and rapid hyperglycemia was observed at 1 week post-STZ and persisted throughout the 5 week study (Figure 6.1, Upper Panel). Further we also observed an impairment in glucose handling as measured by AUC of blood glucose after bolus glucose administration over time (Control $3349.83\pm403.98$, 1w $10695.0\pm2536.33$, 5w $9254.50\pm2717.42$ mg/dl). (Figure 6.1, Lower Panel). Basal blood glucose values went from $117.00\pm36.32$ mg/dl at control to $254.23\pm33.96$ mg/dl at one week and remained elevated ($308.94\pm41.3$ mg/dl) at 5 weeks+.

In the duration of the study, systolic blood pressures as measured by tail cuff manometry remained unchanged (control $110.00\pm2.61$, 1 week $108.00\pm4.88$, 5 weeks $105.0\pm3.46$ mm Hg, p =NS, figure 6.2). Heart rate as measured by ECG showed a downward trend especially at 1 week, but was not significantly different (control $479.32\pm14.16$ bpm, 1 week $413.11\pm39.95$ bpm, 5 weeks $449.65\pm14.74$ bpm, figure 6.2). Further, body weight as determined at each time point showed a significant decrease only at 5 weeks (Control $30.2\pm0.41$ g, 1 week $31.16\pm0.38$ g, 5 weeks $26.4\pm0.83$ g).

High quality cardiac ultrasound imaging was achieved under light inhalation anesthesia. Representative M-mode, Doppler aortic flow and transmitral flow images from Control and Diabetic (5 week post-STZ) animals are shown in Figure 6.3. LV wall motion as well as aortic and transmitral flow velocities were clearly visualized at
all time points studied. Cardiac performance was determined in control groups at 0, 1 and 5 weeks post-STZ. No significant age related effect was observed in any of the cardiac performance indicators, and therefore these controls were combined for subsequent statistical analyses.

Cardiac performance parameters derived from M-Mode images are shown in Figure 6.4. Statistically significant impairment in LV fractional shortening was observed at both 1 week and 5 weeks. Increase in left ventricular inner dimension (LVID) at systole, indicative of LV dilation, was also observed at 1 and 5 weeks post-STZ.

Aortic Doppler flow velocity analysis revealed no evidence cardiac output or stroke volume changes in the STZ model (Figure 6.5). Following sacrifice, left ventricular outflow tract diameter was determined to allow calculation of cardiac output. No differences in left ventricular outflow diameter were detected among treatment groups (average area mm$^2$). Individual values were used to calculate cardiac output. Though there was a trend towards decreased CO, it did not reach significance (p>0.05). The velocity-time integral, an indirect measure of ventricular stroke volume, was also not significantly decreased. Peak aortic flow was unchanged at any time point studied (figure 6.5).

In previous studies, we have demonstrated transmitral flow parameters to be sensitive, age and HR independent indicators of diastolic function ((10; 11). In the mouse STZ model, we observed and early reduction in the E wave (Control 102.99±3.59, 1 week 88.43±5.17) and E/A ratios (control 3.53±0.25, 1 week 3.04±0.24) at 1 week, but at 5 weeks the diastolic parameters were back to control. Representative photomicrographs of cross-sections and digital imaging parameters
from STZ and control hearts are shown in Figure 6.7. Estimated LV masses (from M-mode images) and cross-sectional tissue areas were not statistically different at any time point, whereas a statistically significant increase in LV lumenal area was observed at 5 weeks of diabetes compared to control (indicative of LV dilation). LV fibrosis was detected by Masson’s trichrome stain, assessed by digital imaging and expressed as percent of total LV cross-sectional tissue area. No significant elevations in extra-cellular matrix prevalence were observed in diabetic hearts compared to control at any time point during the study (Figure 6.7).

Representative ECGs from control and diabetic mice are shown in figure 6.8. As can be observed from the figure there was a significant decrease in the P-wave amplitude, (data graphically represented in figure 6.9), suggesting an impaired conduction through the atria, P-wave duration however was unaltered. We also observed a consistent time dependant increase ventricular depolarization time as shown by the increased QRS complex duration (Control 20.0±0.4ms, 1 week diabetic 22.39±0.53, 5 week STZ 25.16±0.84ms, p<0.005). Further, we observed an increase in the QT interval at 5 weeks (control 62.43±1.06ms, 5w diabetic 68.46±0.99, data not shown), which persisted after heart rate correction, as shown in figure 9 (QTcF, Control 126.4±2.3, 5 Week STZ 134.53±1.57) suggesting an impaired ventricular repolarization at 5 weeks.
DISCUSSION

Though diabetes mellitus is defined as a metabolic disorder, a majority of the morbidity and mortality associated with this disease state is directly attributable to cardiovascular causes (12). Diabetes mellitus is an independent risk factor for a variety of cardiovascular diseases, including hypertension, atrial fibrillation and arrhythmia, cardiomyopathy and heart failure (13). The cause of accelerated cardiovascular disease in diabetes is multifactorial, and although atherosclerotic events (secondary to the loss of metabolic control)(2) have a major influence on morbidity and mortality in diabetes, interestingly, a subset of diabetic patients develops a specific cardiomyopathy in the absence of clinically detectable atherosclerosis and/or coronary artery disease (5; 14-16).

In general, this unique form of cardiomyopathy presents with early reductions in diastolic performance (increased LV end diastolic pressure, myocardial stiffening, prolonged iso-voluminic relaxation times), followed by progressive reductions in systolic function (increased LV end systolic dimension and volume, decreased LV ejection fractions) in the absence of microvascular ischemia(5; 14-16).

Several hypotheses have been suggested to explain the specific pathogenesis behind this non-ischemic cardiomyopathy. Alterations in calcium homeostasis through decreases in sarcoplasmic reticular calcium transport, decreases in cardiac myosin ATP-ase activity, decreases in NA+K+ ATP-ase and Ca+2 ATP-ase activity, shifts in cardiac myosin heavy chain isoforms (V1 to V3), alterations in sarcoplasmic proteins (myosin light chain 2 and troponin I), increased sympathetic activity have been demonstrated and might play a role in depressing cardiac contractility (17-21).
Further, some studies have also shown changes in the endothelial function of the coronary network in the diabetic heart, suggesting decreased microcirculatory function might be involved (22). In addition, it has been shown that the diabetic heart is exposed to increased oxidative stress due to elevated glucose levels, diabetic neuropathy, increased sympathetic activity, increased renin-angiotensin system activity and myocardial ischemia/functional hypoxia (23; 24). The resulting increase in reactive oxygen and nitrogen species can in turn lead to altered signal transduction, abnormal gene expression and activation of apoptotic pathways, possibly causing myocardial cell death, which could play a critical role (25).

However, the initiating events in this unique diabetic cardiomyopathy are unknown, and its participation in the development of more progressive cardiovascular disease states is undefined. Thus far no efforts have been made to specifically address this unique cardiac phenomenon therapeutically.

First recognized in 1963, the STZ rodent model has historically been valuable for basic insights of the consequences of hyperglycemia in vivo (26). Some advantages include a high degree of reproducibility and convenient species size and cost. While some investigators have been critical of the STZ rodent as an appropriate model for diabetes, it may be particularly relevant in studying the Type I disease state and/or consequences of loss of glucose control (27). In particular, since this mouse model is devoid of severe hyper-lipidemia and atherosclerotic events (typical in Type II cardiac etiologies), it may be most appropriate in modeling aspects of this non-atherogenic Type I cardiomyopathy. While 30% of otherwise asymptomatic Type I patients suffer this specific cardiomyopathy, the relevance of the STZ mouse to this
phenomenon has not yet been established in a rigorous and time-dependant manner. Here we used clinically relevant echocardiographic indicators of systolic and diastolic cardiac performance, as well as ECGs to test the hypothesis that the STZ mouse model is appropriate for the mechanistic study of this Type I diabetic cardiomyopathy.

Echocardiography is an emerging technology in the study of rodent cardiac performance. This approach provides the advantages of non-invasive in vivo assessment of clinically relevant cardiac and vascular structure and performance indicators. This provides the opportunity to study time-dependent changes in the same rodent and allows for serial studies during disease progression and/or during experimental therapies.

An important consideration in rodent echocardiography is choice of anesthetic regimen, since many widely used anesthetics (e.g., ketamine/xylazine, pentobarbital) have significant cardio-depressant effects, most notably reduction of HR. In our laboratory we have recently found that light anesthesia via halothane inhalation provided more reliable and physiologically relevant cardiac performance parameters in normal mice (8). Thus, here we employed light inhalation halothane anesthesia in an effort to minimize anesthesia related influences. This approach has afforded important advantages, including high quality imaging, high methodological reproducibility (intra- and inter-animal variability less than 8%), as well as sensitive detection of time-dependent changes in resting diastolic and systolic performance.

Probe alignment is a particularly important consideration for M-mode echocardiography, as the perpendicularity of the short axis plane with the ventricular
walls is essential to validate the mathematical assumptions and equations used to derive fractional shortening, cardiac dimension, and LV mass parameters. The alignment of the imaging plane benefits greatly from the application of 2-D imaging (at level of papillary muscles) prior to M-mode capture, as employed in these studies. However, when conducted with an appreciation for these limitations, M-mode echocardiography remains a reproducible and convenient method for the measurement of cardiac structure and contractile events, and an important aspect of current clinical assessments of cardiac performance.

Single dose STZ induced rapid and significant changes in glucose control in the STZ mouse, as determined by plasma glucose and IPGTT measurements throughout the study time course. Our blood sugar values are a little lower than other published values at similar time points, though still substantially higher than control. This might be a function of the lower STZ dose used in the study (150mg/kg rather than 200-250mg/kg normally utilized) producing a milder diabetic state (28). It was our intention to create a less severe model of diabetes, to more closely represent the clinical state, wherein uncontrolled blood glucose levels of 500+mg/dl are exceedingly rare. The lower blood sugars, in addition to the slight reduction in AUC after IPGTT test at 5 weeks as compared to 1 week, suggests that the mice still have some pancreatic function, though severely reduced.

In accordance to our belief that this is a less severe model of the diabetic state, we did not observe a significant reduction in heart rate with time, unlike other studies (7). However we did observe a significant decrease in body weight with time as previously described in this model.
We observed changes in multiple indicators of systolic function late in diabetic progression. These included increases in LV inner dimensions, as measured by echocardiographic (LVIDs) and histological parameters (LV lumen area at sacrifice) indicative of LV dilation, as well as a significant reduction in percent LV fractional shortening. Although the observed changes in LV fractional shortening may seem relatively mild (20% reduction at 5 weeks), it is important to note that the observed values of 15% fractional shortening are consistent with moderate to severe systolic failure in mice. While fractional shortening is a load dependent parameter, blood pressure was not significantly altered during the time-course of diabetes. This data, combined with the Doppler aortic flow data demonstrating no significant changes in maximal aortic flow velocities in diabetic vs. control mice, may suggest that the observed impairments in basal systolic function are apparently largely independent of afterload conditions in this setting.

These late structural changes and systolic impairments are highly consistent with the clinical presentation of Type I cardiomyopathy, and suggest the value of the STZ model in this setting.

Although reliable indicators of systolic performance (fractional shortening, ejection fraction, cardiac output) have been fairly well characterized by the cardiology community, the selection of reliable and robust indicators of diastolic performance is far less standardized (29).

An important clinical advance has been the evaluation of transmitral flow velocities, including Peak E velocity, Peak A velocity, E/A ratio, diastases interval, and acceleration and deceleration slopes of the E wave. Peak E velocity, A velocity and
therefore E/A ratios have been shown before by our group to be dependant of HR. However in the absence of dramatic HR changes in our diabetic animals we employed these parameters to investigate diastolic functional changes. We found an early (1 week) decrease in E peak velocity, which translated to a significant decrease in the E/A ratio, suggesting that the passive ventricular filling due to ventricular dilation in these mice is impaired early in disease progression. However this impairment was not evident at 5 weeks suggesting some compensatory mechanism might have taken over.

Interestingly, the time courses of diastolic and systolic dysfunction are distinct in this animal model. Significant reduction in diastolic parameters preceded alterations in systolic parameters. This initial change in cardiac performance during diastole is consistent with our previous investigations in a rat model of diabetes, and is similar to many clinical reports. Therefore, this small animal preparation is apparently well suited for further investigations regarding mechanisms of diastolic versus systolic abnormalities during diabetes related cardiac dysfunction.

A controversy exists in the current literature as to the obligatory role of LV fibrosis deposition in the myocardial stiffening observed during Type I cardiomyopathy. For this reason we assessed LV mass and cross-sectional area (LV hypertrophy is a common etiology of LV diastolic dysfunction and fibrosis deposition during the progression of cardiomyopathy. In previous studies we developed a reliable and unbiased analysis system to determine the extent of interstitial fibrosis in cardiac muscle. No increases in LV size or extent of fibrosis were observed, suggesting that fibrosis deposition and/or LV hypertrophy are not obligatory events
in the initiation and/or progression of diabetic cardiomyopathy. These findings are in contrast to studies in other genetic diabetes models which suggested that increased perivascular fibrosis, (possibly due to increased PKCβ2 expression), plays a role in the development of diabetic cardiomyopathy (30; 31), but are similar to those previously shown by our group in a diabetic rat model (7).

Our findings of striking decrement in diastolic (early) and systolic (later) dysfunction in the absence of significant increases in LV wall mass or extracellular matrix, suggest that hypertrophy and/or fibrosis are not driving mechanisms in this setting. Other metabolic and/or biochemical events may be operable and warrant further investigation (i.e., impairment in energetics and/or calcium handling).

Arrhythmia and sudden cardiac death are prevalent and serious cardiac complications of diabetes that are difficult to control therapeutically in diabetic (and non-diabetic) patients (2; 4-6; 11; 12). For example, QTc interval prolongation is an independent predictor for all-cause and cardiovascular mortality in Type I diabetics, carrying greater relative risk for cardiovascular mortality than either smoking or hypertension in this patient population (11). In addition, many of the currently employed pharmacological approaches for arrhythmia control have limited efficacies, life-threatening toxicities, and can influence glycemic control.

Although cardiac conduction abnormalities in diabetes are well described, to date no studies have detailed the time course of electrical changes using clinically relevant ECG methodologies, especially with respect to early and initiating events. ECG waveforms were acquired under light inhalation anesthesia, maintaining physiological heart rates to provide for more reliable and physiologically relevant
cardiac performance assessments (7; 16). Diabetes (1 and 5 week post-STZ) was associated with the absence of normal P-wave morphology, consistent with atrial flutter or fibrillation. Q-T intervals were also elongated at 5 weeks of diabetes. A time dependant widening of the QRS complex was also observed, which could be indicative of ventricular repolarization deficits. Interestingly while Q-T elongation and P wave changes were observed later in the course of diabetes, QRS changes were evident earlier, at the first time-point looked at. Q-Tc prolongation can predispose the heart to arrhythmia (torsades de pointes), and has been described as an independent risk factor for mortality in Type I diabetic patients(10; 11). These observations are highly consistent with findings in diabetic patients, as diabetes is an independent risk factor for both atrial and ventricular arrhythmias, and suggest that the STZ-treated mouse is appropriate for the mechanistic study of diabetic cardiac conduction abnormalities.
CONCLUSION

Using the STZ model of Type I diabetes, we have demonstrated that rodent echocardiography can be a useful, clinically relevant tool for the study of diabetic cardiomyopathy, as sensitive changes in systolic and diastolic performance (and their interdependencies). We observed significant early impairments in diastolic function, followed by persistent decreases in systolic performance. These changes parallel the clinical presentation of Type I diabetic non-atherogenic cardiomyopathy, and this animal preparation may have particular value in modeling this specific clinical phenomenon. We further employed this model to test the obligatory role of fibrosis in the development of diabetic cardiomyopathy—no evidence of increased fibrosis or hypertrophy was observed.

Finally, we also conducted a ECG analysis on the mice at the same timepoints and found evidence for both atrial and ventricular conduction impairments, which is consistent with the increased clinical risk for both atrial and ventricular arrhythmias in diabetes.

This study suggests that the STZ mouse model is appropriate for the mechanistic study of Type I diabetic cardiomyopathy, and provides time-dependent, clinically relevant assessments of cardiac performance as a foundation for such studies.
REFERENCE:

FIGURE LEGENDS:
Figure 6.1: Glucose control is moderately impaired in streptozotocin mice, in a time dependant manner. Top panel: STZ induced hyperglycemia is evident at 1 week and and persists through out the period of study. Lower Panel (right): IPGTT. Glucose disposal is altered in STZ mice. Lower panel (left): Area under the curve (AUC) of IPGTT shows a significant disruption of glucose disposal in STZ mice.
Figure 6.2: General characteristics of STZ mice. Top Panel: No significant change was seen in blood pressure values. Middle Panel: A trend towards decreased heart rates, but no significant difference observed. Bottom Panel: Significant difference in body weight seen at 5 week post-STZ.
Figure 6.3:Representative images of control and diabetic echocardiographic parameters. M-mode and continuos wave Doppler images captured under light anesthesia. Representative images from control and 5w diabetic animals.
Figure 6.4:Impaired contractility and left ventricular dilation in diabetes. Panel A: No significant change seen in left ventricular diastolic diameter. Panel B: Significant dilatation of left ventricular systolic diameter was observed at both 1 and 5 weeks. Panel C: Significant difference of seen in fractional shortening percentage at all time points. Panel D: Significant correlation between fractional shortening and hyperglycemia was observed (p<0.05)
Figure 6.5: Aortic flow parameters. Top panel: No significant change in velocity time intervals of aortic flow. Middle Panel: Cardiac output was not significantly different. Bottom panel: No difference in stoke volume.
Figure 6.6: Left ventricular diastolic functional impairment. Top panel: E/A ratio, measure of ventricular diastolic filling was significantly reduced at earlier timepoint. Middle Panel: E wave, measure of passive diastolic ventricular filling was significantly reduced only at one week. Bottom Panel: A trend towards increase but no significant change in A wave, measure of ventricular filling due to atrial contraction. (p=NS).
Figure 6.7: Morphological analysis of diabetic heart. Panel A: No significant increase in heart weight (hypertrophy) in diabetic 5 week animals. Panel B: No evidence of increase in left ventricular wall area. Panel C: significant increase in left ventricular lumen area, suggestive of ventricular dilated cardiomyopathy. Panel D: No change in fibrosis was evident (p=NS).
Figure 6.8: Representative signal-averaged ECGs from control and diabetic mice. Four lead ECG’s were collected in anesthetized mice at 0, 1 and 5 week following treatment with STZ or vehicle control. Waveforms were signal averaged over 300 beats. Representative signal-averaged waveforms from control and diabetic mice at showing altered P-wave morphology, abnormal S-T segment shape and QRS broadening at advanced stages of diabetic cardiomyopathy.
Figure 6.9: Cardiac electrophysiological parameters. Average data from control and diabetic animals at 0, 1 and 5 week post-STZ. At 5weeks of diabetes, P-waves were undetectable in many of the waveforms studied, and these
intervals were not calculated. Q-Tc represents Q-T interval corrected for heart rate by Fridericia’s method \[Q-Tc = Q-T/(R-R interval)^{1/3}\]. In control animals, no age-dependent effects (0 vs. 1w vs. 5w) were observed in any parameter studied. *, p<0.05 vs. pooled control.
Figure 6.1: Glucose control is moderately impaired in streptozotocin mice, in a time dependant manner.
Figure 6.2: General characteristics of STZ mice
Figure 6.3: Representative images of control and diabetic echocardiographic parameters.
Figure 6.4: Impaired contractility and left ventricular dilation in diabetes
Figure 6.5: Aortic flow parameters

- VTI (cm)
- CO (mL/min)
- SV (mL)
Figure 6.6: Left ventricular diastolic functional impairment
Figure 6.7: Morphological analysis of diabetic heart
Figure 6.8: Representative signal-averaged ECGs from control and diabetic mice
Figure 6.9: Cardiac electrophysiological parameters
CHAPTER 7

Mechanistic Investigation Of Diabetic Cardiomyopathy

This chapter has been prepared for submission to Diabetes and is presented in the style appropriate to the journal.
ABSTRACT

Objective: Type I diabetes mellitus is complicated by severe progressive cardiovascular diseases, including hypertension, congestive heart failure and coronary artery disease. Diabetes is also a major risk factor for the development of arrhythmias. In the previous chapter, we demonstrated that our mouse model mimicked functional events observed in Type I non-atherogenic cardiomyopathy. We had a unique opportunity to carry out histochemical analysis that could then be correlated to diastolytic, systolic, conduction data on each individual animal and thus attempt to investigate the mechanisms involved in deteriorating cardiac function.

Methods: Hyperglycemia was induced in male CF-1 mice weighing 16-18g with a single dose of streptozotocin (STZ, 150 mg/kg i.p. prepared daily in citrate buffer pH 4.5 for maximal stability). Animals were sacrificed with 100mg/kg, and the apical portion of the heart was equatorially bisected just distal to the mitral valve and immersed in 10% formalin. Immunohistochemistry for protein 3-nitrotyrosine(3NT), Nitric Oxide Synthase-2 (NOS2), Cyclooxygenase-2 (COX2), Xanthine Oxidase (XO), Glucose transporter (GLUT4) and C-reactive protein (CRP), Platelet Endothelial Cell Adhesion Molecule (PECAM), Connexin40 (Cx40) and Connexin43 (Cx43) was then carried out.

Results: We saw protein 3NT levels increase dramatically (48%), but no concurrent increase in NOS2 or COX2 were observed. Specific induction of XO (44% increase), and increased C-RP (25%), was observed, as well as a significant reduction in number of PECAM stained microvessels (26%). Though there was no increase in Cx40 or Cx43 content, we also saw striking increases (60-70 fold) in mid-myocyte
staining for cardiac Cx43, suggesting alteration of tightly regulated gap junctions in diabetes.

**Conclusions:** We found evidence supporting a role for increased reactive nitrogen species, through the induction of a specific oxidant enzyme, XO, and not NOS, thus supporting the use of antioxidant therapies in diabetic cardiomyopathy. Furthermore we found evidence of increased myocardial CRP, which might be of interest as a potential pathophysiological agent as well as a marker of inflammation and cardiovascular risk. We observed a significant reduction in myocardial microvessel density, suggesting an explanation for the increased ischemic risk in diabetes, even in the absence of overt macrovascular coronary artery disease. And finally we show that cardiac ventricular Cx43 distribution is altered during diabetic cardiomyopathy, and suggest that these alterations may mediate some of the electrophysiological abnormalities associated with this condition.
INTRODUCTION

Type I diabetes mellitus is complicated by severe progressive cardiovascular diseases, including hypertension, congestive heart failure and coronary artery disease, as over 75% of all diabetic patients die from cardiovascular events. (1-3) Many of these complications may be secondary to errors in lipid metabolism and attendant atherosclerosis. However, a non-atherogenic cardiomyopathy in Type I diabetics has been recognized for over 20 years (4). This unique form of cardiac disease occurs in roughly 30% of all Type I patients, and presents as early diastolic abnormalities followed by later decreases in left ventricular ejection fraction, overt failure and death (5). While this syndrome is well recognized, the mechanisms involved are poorly understood and specific therapeutic strategies in this patient population are currently undefined (6).

Diabetes is also a major risk factor for the development of arrhythmias. 20% of both type 1 and 2 diabetic patients show QT prolongation (7). A prolonged QT interval is considered an indicator of an increased risk of malignant ventricular arrhythmias and/or sudden death. Furthermore diabetes is also a primary risk factor for the development of atrial fibrillation (8).

In the previous chapter, we demonstrated that our mouse model mimicked functional events observed in Type I non-atherogenic cardiomyopathy. We saw early diastolic dysfunction and sustained systolic dysfunction, as well as conduction abnormalities consisting of prolonged QT intervals and ablation of P waves on the electrocardiogram, consistent with clinical human data, suggesting this is a good model to investigate mechanisms of dysfunction. Since we carefully cataloged each
mouse as an individual patient, we now had the unique opportunity to carry out histochemical analysis that could then be correlated to diastolic, systolic, conduction data on each individual animal. So here we attempted to investigate the mechanisms involved in deteriorating cardiac function.
METHODS

Study Design

All aspects of our animal use were in accordance with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee. Hyperglycemia was induced in Pathogen-free male CF-1 mice (age 6 weeks, n=18, Harlan Laboratories, Indianapolis, IN) weighing 16-18g with a single dose of streptozotocin (STZ, 150 mg/kg i.p. prepared daily in citrate buffer pH 4.5 for maximal stability) or vehicle control. Animals were housed in a sterile cage rack system with HEPA filtered air circulation (approximately 50 air changes/hour, Allentown Caging Inc., Allentown, PA).

Animals were studied longitudinally at 0 and 5 weeks post-STZ (n=18 each time point). Eighteen age-matched mice were treated with vehicle and studied at either 0 or 5 weeks, to evaluate control parameters for this time dependency study. Statistical differences in these control groups (0 vs. 5weeks) were not observed for any parameter evaluated, therefore these observations were pooled and served as control values. In all figures shown these control value are represented at time 0.

Blood glucose was determined at each time point with a Glucometer Encore (Ames) clinical blood glucose monitor.

Cardiac histology

Animals were sacrificed with 100mg/kg i.p. pentobarbital sodium (Abbott Laboratories, Chicago, IL). The apical portion of the heart was equatorially bisected just distal to the mitral valve and immersed in 10% formalin. Tissues were paraffin embedded and blocked according to standard procedures.
**Image capture and digital image analysis**

Cardiac images were captured using a Polaroid digital camera (Polaroid Corporation, Cambridge, MA) and transferred into research-based digital image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD).

Cross-sectional areas of each heart were visualized with an Olympus BX-40 microscope (400x magnification for protein 3-nitrotyrosine(3NT), Nitric Oxide Synthase-2 (NOS2), Cyclooxygenase-2 (COX2), Xanthine Oxidase (XO), Glucose transporter (GLUT4) and C-reactive protein (CRP), 800x magnification for Platelet Endothelial Cell Adhesion Molecule (PECAM), Connexin40 (Cx40) and Connexin43 (Cx43); Olympus Inc., New York, NY) and captured using an Insight QE digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were then analyzed for extent of diaminobenzidine signal in each tissue using research-based digital image analysis software (Image Pro Plus 4.0; Media Cybernetics, Silver Spring, MD), as previously described (9).

At this magnification individual cells can be identified and individually analyzed. 5-7 images were captured from random locations around each ventricular cross section, under identical lighting and optical settings, so as to encompass the >70% of the cross-section available. Images were then segmented to eliminate background and nuclear counterstain from analysis. Extent of immunoreactivity was determined by measuring optical density of diaminobenzidine signal in each image. Integrated optical densities were determined for each image as a measure of staining intensity, and values were then averaged for each heart to provide a measure of prevalence.
at each time point. Intra- and inter-observer variability for this procedure was each less than 2%.

In addition to IOD analysis for total content of PECAM staining, we also assessed the number of microvessels per tissue mm$^2$ by a digital imaging approach. Briefly, color segmentation was used to delineate positive endothelial cell (microvessel) staining from the rest of the tissue. Positive objects were filtered based on size and aspect ratios to separate microvessels from single cells and/or inappropriate staining. The final count was then checked by the user to ensure that vessels were counted appropriately. Results are expressed as microvessels per unit area of tissue. Intra-observer variability (one blinded observer, 50 myocytes, 3 trials) and inter-observer variability (two blinded observers, 50 myocytes each) for this approach were less than 3 and 5%, respectively.

Similarly, in additional to total protein content, we assessed the distributional changes in cardiac Cx40 and Cx43 in control, over the same time course and in the same histological sections. A digital imaging approach was developed to assess Cx40 and Cx43 staining intensities at the myocyte short axes (Cx43 localizes to myocyte gap junctional regions under normal conditions to facilitate inter-myocyte signaling (10-15) versus the mid-myocyte region (the area of the myocyte that did not have abundant Cx43 presence under control conditions). In preliminary experiments, the mean distance from the short axis edge that completely encompassed Cx43 staining at the inter-myocyte junctions was determined for each control heart. The 95% confidence interval (mean length + two standard deviations) for this value was determined to be 7.5 microns from each short axis edge. This
value was used to define the “short axis” staining for Cx43; all staining that was detected outside of this control confidence interval, but still contained within the myocyte, was defined as “mid-myocyte” staining for Cx43. Integrated optical densities of Cx43 staining were determined for each of these myocyte regions, by methods identical to those described above. Only longitudinally oriented cardiac myocytes (over 300 individual myocytes studied) were assessed for Cx43 distributions by this digital imaging approach. Intra-observer variability (one blinded observer, 50 myocytes, 3 trials) and inter-observer variability (three blinded observers, 50 myocytes each) for this approach were less than 1 and 3%, respectively.

**Statistical analysis**

Parameters between diabetic and control groups were evaluated for significance by one-way ANOVA, with Student-Newman-Keuls post-hoc analysis, for all functional data and Student t-tests for all histological data. Significant correlations were assessed using Spearman's non-parametric correlation analysis. A total of 30-35 data points were used for each regression analysis. In all cases, results are expressed as mean ± sem and significance was defined as p<0.05.
RESULTS

Cardiac immunohistochemistry

Following functional analyses, hearts were collected and prepared for the histochemical studies of left ventricular protein content and distribution by immunohistochemistry.

Shown in Figure 7.1 are representative photomicrographs from left ventricular crossections stained for protein 3NT, a stable biomarker of reactive oxygen and nitrogen species. We saw a significant increase (~48%) in the total protein 3NT content in the 5week diabetic tissues as compared to age-matched controls as seen in the graph. Further, we observed a significant inverse correlation between the cardiac protein 3NT content and the functional deficiency (fractional shortening) as plotted per animal (p=0.0004, r = -0.8).

Since we saw protein 3NT levels increase dramatically, we investigated NOS2, an inducible NOS isoform as shown in figure 7.2, we saw no significant changes.

We then probed the cardiac tissues for presence of inducible oxidase, as shown in figure 7.3. There was no significant change in cardiac COX-2 levels in the diabetic animals at 5 week, but we saw a significant increase in XO levels (44% increase over age matched controls), thus suggesting that an increase in oxidant enzymes, not NOS, might be responsible for increased oxidant stress in this setting.

We also looked at GLUT4 levels in the diabetic myocardium, which had been shown to be changed in other studies, however, we observed no significant changes in GLUT4 levels as shown in figure 7.4. Also shown in figure 7.4 are protein CRP levels, a marker of inflammation that has recently been demonstrated to be of
significance in various cardiovascular settings. We saw a significant increase in CRP levels (25% increase over control), suggesting a role for inflammation in this setting. Since microvascular complications have been demonstrated in diabetes in the retina and kidney, we looked at microvasculature in the myocardium as shown in figure 7.5. Anti-PECAM (also called CD31, a specific endothelial cell marker) antibody was used to demarcate capillaries. Total PECAM staining (indicative of total endothelial cells) was not different between the diabetic and control groups. However, further investigation, using an image analysis technique to count the number of microvessels (based in size and aspect ratios) showed a significant (26%, p < 0.05) decrease in the number of microvessels in the diabetic myocardium as compared to age matched control mice. Further, this decrease showed a significant correlation to the contractile dysfunction seen in the diabetic mice. (p < 0.05, r = 0.54).

Figure 6 and 7 show representative photomicrographs from left ventricular cross-sections stained for Cx40 (Figure 6, top panels) and Cx43 (Figure 7.7, top panel). Connexin staining patterns were aligned with myocardial architecture at myocyte-myocyte junctions, with more prevalent staining observed for Cx43 compared to Cx40 (consistent with literature reports that Cx43 is the predominant isoform in adult ventricular tissue (10; 11). Cx40 content was unchanged in diabetic hearts relative to control hearts, with no detectable alterations in Cx40 content or distribution (Figure 7.6, p = NS by integrated optical density analysis). Although Cx43 levels showed no significant change to in total connexin content, we saw a significant change in the distribution. In control hearts, Cx43 staining was localized to the myocyte short axis region, in discrete bands perpendicular to the myocyte long axis. In contrast, Cx43
localization became highly disorganized in diabetic hearts, as Cx43 short axis bands became wider and less linear, and increased staining prevalence in the mid-myocyte regions was observed. We developed a digital image analysis approach to quantify the relative changes in cardiac Cx43 distribution during the progression of diabetic cardiomyopathy in this model, determining the staining intensities for Cx43 in the short axis regions and the mid-myocyte regions for individual cardiac myocytes from each mouse heart cross-section. Over 300 myocytes were assessed using this digital imaging approach. We observed no significant increases in Cx43 prevalence in the short axis regions but interestingly, we also saw striking increases (60-70 fold) in mid-myocyte staining for cardiac Cx43. When expressed as a percentage of total Cx43 prevalence, mid-myocyte staining increased from approximately 20% of total staining in controls to over 30% post-STZ.
DISCUSSION

A significant complication of diabetes mellitus is the development of cardiovascular disease, including dilated and hypertrophic cardiomyopathies, congestive heart failure, arrhythmias and sudden cardiac death. The risk of developing heart failure is 2.4 times higher for men with diabetes, and 5.1 times higher for women with diabetes, as compared to control populations, and 75% of all diabetics die from cardiovascular disease. (1; 2) While the mechanisms underlying these cardiac events are complex, these complications are largely attributed to the high prevalence of coronary artery disease (secondary to the loss of metabolic control) in this patient population (1; 2).

Interestingly, a subset of diabetic patients develop a specific cardiomyopathy in the absence of clinically detectable atherosclerosis and/or coronary artery disease [(5; 6; 12-15). In general, this unique form of cardiomyopathy presents with early reductions in diastolic performance and cardiac conduction abnormalities, followed by progressive impairment in systolic function, all developing in the absence of macrovascular ischemia [(12-1415]. The initiating events in this unique diabetic cardiomyopathy are unknown, and its participation in the development of more progressive cardiovascular disease states is undefined and thus far no efforts have been made to specifically address this unique cardiac phenomenon therapeutically.

In the previous chapter we demonstrated that the mouse STZ model mimics this specific form of nonischemic cardiomyopathy, presenting with time dependent abnormalities in left ventricular contractility and relaxation, as well as
electrocardiographic abnormalities. Thus, it appeared that this model is appropriate for further study of diabetes related electrophysiological abnormalities. 

Hyperglycemia is known to induce increase in oxidants, primarily though mitochondrial dysregulation (16). Reactive nitrogen species (RNS) are a family of biologically relevant oxidants derived from the interaction of nitrogen based intermediates (e.g. nitric oxide) with reactive oxygen species (superoxide anion, hydroxyl radical, hydrogen peroxide) (17). RNS can have profound cellular effects and toxicities due to the distinct reactivites of RNS relative to their reactive oxygen precursors (18). These reactivites include the avid capacity to cause nitration of tyrosine residues, resulting in the stable formation of 3-nitrotyrosine residues (19). Protein-3NT formation has been demonstrated to be a potent structural and functional post-translational protein modification, and has been observed in a wide array of acute and chronic cardiovascular disease states, including diabetes. Further, RNS formation and attendant protein nitration have been shown to modify the function of multiple proteins that depend on tyrosine phosphorylation for their activity (9; 20-24). We therefore looked for evidence of increased protein 3NT in our diabetic cardiac tissues, and saw a dramatic increase. We also had the opportunity to correlate 3NT staining to the in vivo functional deficiency in the same mouse, and observed a highly statistically significant correlation, thus suggesting that oxidative damage might be involved in the development of diabetic dysfunction. Since we observed increased 3NT, we looked at enzymes involved in the formation of inducible precursors, NOS2 and oxidases (COX-2 and XO). However, there was no significant alteration in NOS2 levels. COX-2 induction occurs in a wide variety of
inflammatory cardiac settings, and COX-2 inhibition has been shown to have therapeutic value (25; 26). Hence, we investigated COX-2 levels in the diabetic myocardium, but we did not see any significant changes. Finally we also looked at XO, another inducible oxidase, that has been shown to be upregulated and involved in superoxide production, and vascular endothelial dysfunction in diabetes (27). Further, there have also been studies suggesting that plasma XO levels are upregulated in both animal models and humans (28; 29), but there have been no reports on the myocardium. Here, we show that there is local myocardial induction of XO. Further, it appears that the increased reactive nitrogen species seen in diabetes is due to increased superoxide and not NOS upregulation. This ties in well with the endothelial dysfunction seen in diabetes too, increased reactive oxygen species formation by oxidases would divert NO from physiological functions into production of reactive nitrogen species, resulting in loss of vasodilatory capacity. (27) Recently, there has been interest in the regulation of glucose transporters in diabetic cardiomyopathy. It has also been shown non-diabetic, but hypertrophic hearts show insulin resistance due to glucose transporter deficiencies (30; 31), which might increase their susceptibility to ischemia and myocardial damage (32). The myocardium expresses two isoforms of glucose transporters, GLUT1 and GLUT4, but GLUT4 is the predominant isoform. Insulin stimulates glucose transport and translocates both GLUT4 and GLUT1 from an intracellular site to the cell surface (33). Thus it is easy to understand the importance of GLUTs to cardiomyopathy and insulin resistant states. However, some reports have suggested that GLUT4 levels are reduced in type one diabetic models (34; 35). Hence we investigated GLUT4
levels in our diabetic mice, to probe for metabolic modifications of cardiomyopathy. However, we did not see any significant changes.

Since we observed increases in 3NT and XO, both of which are upregulated in inflammatory states, we looked investigated C-reactive protein (CRP) levels. CRP is a marker of inflammations and has been shown to be a good independent predictor of cardiovascular risk (36; 37). Serum CRP levels have also been shown to be elevated in diabetic patients. (38).

However, since diabetes predisposes the patient to cardiovascular damage, and CRP has been shown to be a good marker for cardiovascular risk, the relationship between diabetes and serum CRP is complicated. Though CRP was initially considered a biomarker with no physiological action, recent reports suggest that CRP might have independent pathophysiological actions, including acting as a chemoattractant for monocytes, attenuating nitric oxide production and endothelial progenitor cell survival, differentiation, and function (39; 40).

We therefore investigated CRP levels in the myocardium, and observed and increased staining for CRP in the diabetic mice. While studies of CRP actions have concentrated on the endothelium, and the development of atherogenesis and coronary artery disease, it might be interesting to investigate the direct effects of CRP on cardiomyocytes, and also to investigate the relationship between hyperglycemia and possible cardiomyocyte CRP production.

While the cardiac macrovascular effects of diabetes are well recognized, diabetic microvascular studies have generally focused on diabetic retinopathy. A few studies have however shown that there are cardiac microvascular functional abnormalities...
(deficits, unlike overproliferation in retina) in diabetes both clinically and in animal models (6; 41-43) and it has been suggested that these microvascular abnormalities might contribute to the myocardial functional deficits through episodes of silent local myocardial ischemia, in times of increased myocardial demand. The cardiac microvasculature consists of vessels with diameters of <500µm in humans, a heterogeneous mix of small arterioles, capillaries and post-capillary venules, responsible for the distribution of blood, water and solutes to the working cardiomyocytes. This network cannot be visualized angiographically, and normally requires use of intra coronary Doppler catheters, contrast-enhanced MRI, or other advanced imaging in-vivo. (44). However, post-sacrifice imaging is far less complicated.

We developed an imaging method, using a specific endothelial cell marker PECAM, to delineate endothelial cells. The staining was then sorted based on shape and aspect ratios, to filter out individual cells, large vessels and other inappropriate staining, resulting then in a count of only microvessels, which we defined as staining with an aspect ratio of 1:1 to 3:1 and size less than 100µm (as determined on an initial control set).

Though there was no change in the total endothelial cell staining, there was a significant decrease in the number of microvessels found in the diabetic myocardium as compared to the control myocardium, which also correlated to the myocardial dysfunction. This is especially interesting when we consider the increased CRP staining that we observed in the same mice. A recent report suggested that CRP can attenuate endothelial progenitor cell survival and function. Endothelial progenitor
cells are major contributors to adult post-natal neo-vascularization, especially post-ischemia, and may contribute as much as 25% of endothelial cells in newly formed blood vessels (45; 46). This is even more interesting in view of a recent report suggesting that endothelial progenitor cells might be dysfunctional in diabetes (47). Further investigation is needed. 

Other than contractile deficits we also observed a slowing of conduction in the diabetic hearts. Clinically arrhythmia and sudden cardiac death are prevalent and serious cardiac complications of diabetes, that are difficult to control therapeutically in diabetic (and non-diabetic) patients [(1; 2; 5; 6; 12-15). For example, QTc interval prolongation is an independent predictor for all-cause cardiovascular mortality in Type I diabetics, carrying greater relative risk for cardiovascular mortality than either smoking or hypertension in this patient population (12; 13). Many of the currently employed pharmacological approaches for arrhythmia control have limited efficacies, life-threatening toxicities, and can influence glycemic control. 

Connexins are integral protein components of intercellular gap junctions, and are involved in the dynamic regulation of channel conductance (48; 49). Multiple isoforms of connexins exist and are differentially expressed across multiple organ systems, including the heart (10; 11). Connexin isoform 43 (Cx43) is the predominant isoform in the cardiac left ventricle, expressed throughout the working myocardium, while connexin isoform 40 (Cx40) is expressed selectively in the conducting left ventricular myocardium (10; 11). These connexin isoforms provide the electrical connections that are essential for the coordinated excitation of the myocardial syncytium, and are aligned predominantly along the myocyte short axes,
providing directed pathways for impulse conduction. Therefore, both the content and
the intracellular distribution of connexin isoforms are likely to be important for normal
cardiac excitation and contraction. Despite a large number of reports describing the
biological significance of connexin isoforms for normal physiological function in the
heart (isolated cell studies and murine transgenic studies), fewer reports have
described the role for connexins in cardiac disease states, and none have studied
the content and distribution of cardiac connexin isoforms in a relevant model of
diabetic cardiomyopathy. Here we established the time course of ECG changes in
the mouse STZ model of Type I diabetic cardiomyopathy, and tested the hypothesis
that alterations in connexin isoform content and distribution may play a functional
role in these changes.

Following functional analyses, hearts were prepared for immunohistochemical
studies to determine the cardiac content and distributional changes in Cx43 and
Cx40. In control tissues, Cx43 was the predominant isoform detected in the left
ventricle, consistent with previous literature reports. We observed no alteration in
Cx40 or Cx43 content.

However, since these studies were performed using in situ methods, we had the
opportunity to assess both the content and distribution of Cx43 isoforms throughout
the myocardium. In spite of no changes in connexin content, we observed striking
qualitative evidence of altered Cx43 distributions, as Cx43 staining appeared to
migrate from its strict stepwise alignment with individual myocyte short axis
connections to the mid-myocyte regions. We developed an imaging approach to
assess these distributional changes quantitatively, using Cx43 staining in control
mice to define the short axes distributions expected in normal healthy myocardium, and establishing 95% confidence intervals for control Cx43 distributions in myocytes that exhibited longitudinal alignment in our cross-sections. We defined Cx43 intramyocyte staining that fell outside this interval as “mid-myocyte” staining. Cx43 which has been distributed to the mid-myocyte regions is unlikely to mediate normal cardiac conduction, since this staining was virtually absent in control myocytes, and since these proteins would not provide myocyte-to-myocyte connections through the intercalated disk regions of the myocyte short axes. Using this approach, we found that in control hearts, about 20% of total Cx43 staining was found in the mid-myocyte region (as defined by our preliminary studies); however, by 5 weeks post-STZ, over 30% of Cx43 was localized to the mid-myocyte region. This corresponded to a >60% increase in mid-myocyte Cx43 content (as determined by integrated optical density analysis). In another chapter of this thesis, we looked at connexin distribution across the atria in a canine model of atrial fibrillation, and demonstrated changes in the content and distribution of Cx40 the major atrial connexin, but not Cx43 (predominant ventricular Cx) Thus, altered myocyte connexin distribution may be a general and important characteristic in various settings of cardiac arrhythmia.

These data provide the first evidence that cardiac connexins are altered during diabetic cardiomyopathy, and suggest that these alterations may mediate some of the electrophysiological abnormalities associated with this condition. These alterations developed in the absence of significant cardiac structural remodeling, as this mouse model does not develop overt ventricular hypertrophy or increased fibrotic deposition in our hands (preceding chapter).
CONCLUSIONS

Using the STZ model of Type I diabetes, we have demonstrated that rodent echocardiography can be a useful, clinically relevant tool for the study of diabetic cardiomyopathy, as sensitive changes in systolic and diastolic performance were detected. We also conducted ECG analysis on the mice at the same time points and found evidence for both atrial and ventricular conduction impairments, which is consistent with the increased clinical risk for both atrial and ventricular arrhythmias in diabetes.

This study suggested that the STZ mouse model is appropriate for the mechanistic study of Type I diabetic cardiomyopathy and provides time-dependent, clinically relevant assessments of cardiac performance as a foundation for such studies. We therefore conducted mechanistic investigations in the cardiac tissues, and found evidence supporting a role for increased reactive nitrogen species, through the induction of a specific oxidant enzyme, XO, and not NOS, thus supporting the use of antioxidant therapies in diabetic cardiomyopathy. Furthermore we found evidence of increased myocardial CRP, which might be of interest as a potential pathophysiological agent as well as a marker of inflammation and cardiovascular risk. In keeping with the reported clinical loss of coronary reserve in diabetes, we observed a significant reduction in myocardial microvessel density, suggesting an explanation for the increased ischemic risk in diabetes, even in the absence of overt macrovascular coronary artery disease.
Finally we show that cardiac ventricular Cx43 distribution is altered during diabetic cardiomyopathy, and suggest that these alterations may mediate some of the electrophysiological abnormalities associated with this condition.
REFERENCE:


FIGURE LEGENDS:

Figure 7.1: Diabetic Hearts demonstrate increased protein 3NT staining that correlated with contractile dysfunction. Left ventricles from diabetic mice assessed for 3NT prevalence. Upper Panel: Representative photomicrographs, brown staining demonstrates protein nitration. Middle panel: Digital image analysis was employed to quantify extent of 3NT immunoprevaence. Protein 3NT was statistically elevated in diabetic hearts as compared to age matched controls (n=18 per group). Lower Panel: Statistically significant correlation between protein 3NT staining and loss of contractile function in individual mice.

Figure 7.2: NOS2 levels are not significantly changed in diabetic hearts. Upper Panel: Representative photomicrographs, brown staining demonstrates NOS2 protein. Lower panel: Digital image analysis was employed to quantify extent of NOS2 immunoprevaence. Protein NOS2 was not statistically changed in diabetic hearts as compared to age matched controls (n=18 per group).

Figure 7.3: Selective elevation of inducible oxidases in diabetic hearts. Upper panel: Digital image analysis was employed to quantify extent of COX 2 immunoprevaence. Protein COX 2 was not statistically changed in diabetic hearts as compared to age matched controls (n=18 per group). Lower panel: Digital image analysis was employed to quantify extent of XO immunoprevaence. Protein XO was statistically elevated in diabetic hearts as compared to age matched controls (n=18 per group).

Figure 7.4: Inflammation markers, not glucose transporters are significantly changes in diabetes. Upper panel: Digital image analysis was employed to quantify extent of GLUT4 immunoprevaence. Protein GLUT4 was not statistically changed in diabetic hearts as compared to age matched controls (n=18 per group). Lower panel: Digital image analysis was employed to quantify extent of CRP immunoprevaence. Protein CRP was statistically elevated in diabetic hearts as compared to age matched controls (n=18 per group).
Figure 7.5: Diabetic Hearts demonstrate decreased number of microvessels that correlated with contractile dysfunction. Left ventricles from diabetic mice assessed for microvessel prevalence. Upper Panel (right): Representative photomicrographs, brown staining demonstrates PECAM. Upper Panel (left): Digital image analysis was employed to quantify extent of PECAM immunoprevaence. PECAM content was not statistically changed in diabetic hearts as compared to age matched controls (n=18 per group). Lower Panel (right): Number of microvessels per mm$^2$ of tissue is statistically reduced in diabetes. Lower Panel (left): Statistically significant correlation between reduction in microvessel density and loss of contractile function in individual mice.

Figure 7.6: Connexin40 content and distribution is unaltered in diabetic ventricles. Upper Panel: Representative photomicrographs, brown staining demonstrates Cx40. Middle panel: Digital image analysis was employed to quantify extent of Cx40 immunoprevaence. Protein Cx40 was not statistically elevated in diabetic hearts as compared to age matched controls (n=18 per group). Lower Panel: Digital image analysis for Cx40 staining in short axis versus mid-myocyte regions. The average total Cx40 staining that exhibited mid-myocyte localization as expressed as a percentage of total myocyte staining (mid-myocyte IOD/total myocyte IOD) is shown in the lower panel. No significant difference was observed.

Figure 7.7: Connexin43 distribution but not content is altered in diabetic ventricles. Upper Panel: Representative photomicrographs, brown staining demonstrates Cx43. Middle panel: Digital image analysis was employed to quantify extent of Cx43 immunoprevaence. Protein Cx43 was not statistically elevated in diabetic hearts as compared to age matched controls (n=18 per group). Lower Panel: Digital image analysis for Cx43 staining in short axis versus mid-myocyte regions. The average total Cx43 staining that exhibited mid-
myocyte localization as expressed as a percentage of total myocyte staining (mid-myocyte IOD/total myocyte IOD) is shown in the lower panel. Significant elevation of mid-myocyte IOD was observed.
Figure 7.1: Diabetic Hearts demonstrate increased protein 3NT staining that correlated with contractile dysfunction

\[ r = -0.80 \]
Figure 7.2: NOS2 levels are not significantly changed in diabetic hearts.
Figure 7.3: Selective elevation of inducible oxidases in diabetic hearts
GLUT4 PREVALENCE (CARDIAC OD)

CTRL  STZ

CRP PREVALENCE (CARDIAC OD)

CTRL  STZ

Figure 7.4: Inflammation markers, not glucose transporters are significantly changes in diabetes.
Figure 7.5: Diabetic Hearts demonstrate decreased number of microvessels that correlated with contractile dysfunction
7.6: Connexin40 content and distribution is unaltered in diabetic ventricles
Figure 7.7: Connexin43 distribution but not content is altered in diabetic ventricles
CHAPTER 8

Mechanistic Investigations In A Canine Models Of Atrial Remodeling Due To Chronic Atrial Dilatation

This chapter has been prepared for submission to *Circulation Research* and is presented in the style appropriate to the journal.
ABSTRACT

Objective: Mitral Regurgitation (MR) is a common medical problem. A significant complication of MR is the increased incidence of sudden death, shown to be anywhere between 0.9-2% \(^1\,^2\), probably due to an increased incidence of atrial and ventricular arrhythmias. Mitral valve disease, in particular MR has a reported incidence of ~20-50% in patients with AF, and is one of the most common underlying co-morbidities associated with AF. Surgical correction of the valve rarely eliminates the arrhythmia \(^3\,^4\), with approximately 75% of the patients continuing to have AF after surgery. Here we investigated a model of AF susceptibility induced by mitral regurgitation in a canine model over time, to isolate the mechanisms of dysfunction.

Methods: Iatrogenic Mitral regurgitation was induced in 11 male dogs (20-40 lbs) by surgically dissecting the chordae tendinae under fluoroscopic guidance in the lab of Dr. R Hamlin. The dogs were studied at 1 (n=5) and 6 (n=6) months post MR induction. Equal numbers of age-matched sham control dogs were included.

Results: The average degree of MR in our dogs was mild to moderate. The inducibility of AF in these dogs by right atrial pacing showed a marked increase in the MR vs control dogs at both time-points (3/6 MR dogs at 1 month and 3/5 at 6 months). These studies showed the MR dog model to closely resemble clinical MR, with similar changes in atrial dilatation, P wave durations and K\(^+\) current changes, previously unknown in MR. Immunohistochemistry however only showed regional increases in fibrosis, but not to the magnitude that would be required to produce the global conduction changes required to affect P wave duration (a whole atrial phenomena). We however also found data suggesting that the atria as a whole, is
subjected to increased oxidant stress and chronic globalized inflammation and infiltration. Furthermore we also saw changes in Cx content and distribution; Cx40 showed changes in both content and distribution, Cx 43 on the other hand showed no changes in distribution. We show that merely increased fibrosis is not sufficient to explain the mechanical and electrical abnormalities seen in MR, but rather, oxidative stress and inflammatory mechanisms might also play important roles by modulating various components, and heterogenous distribution of connexin protein might be involved in development of arrhythmia substrate.
INTRODUCTION

Mitral Regurgitation (MR) is a common medical problem, with about 500,000 discharge diagnoses every year in the United States and though the actual incidence is unknown. Upto 2% prevalence in general adult population is suggested \(^1\), Patients with severe MR however have a poor prognosis, with an annual mortality rate of 5% without surgical intervention \(^7\), and patients with cardiomyopathy and MR have a significantly higher mortality than in cardiomyopathy without MR.

Causes of MR include degenerative (20-70%), ischemic (13-30%) and endocarditis (10-12%) etiologies \(^8\). In the absence of disease, MR is thought to occur due the progressive dilation of the fibrous mitral annulus, with consequent loss of overlap of mitral leaflets resulting in a central regurgitant jet – functional insufficiency. In ischemia the mechanisms are more complex and might include damage to the papillary muscle coordinating the mitral valve apparatus, muscle elongation and left ventricular damage in addition to annulus elongation resulting in MR. Acute MR is also often seen after myocardial infarction or infective endocarditis both of which can result in rupture of the valve, papillary muscle and chordae tendinae. Severe MR after myocardial infarction has an even worse prognosis with only a 50% survival in 5 years.

Progression of MR is a vicious cycle as MR exacerbates the volume overload of an already dilated ventricle resulting in further progression of annular dilation, increases in ventricular wall tension with worsening MR and increased failure.

A major complication of MR is the increased incidence of sudden death, shown to be anywhere between 0.9-2% \(^1\)\(^2\), probably due to an increased incidence of atrial and
ventricular arrhythmias. In fact Mitral valve disease, in particular MR has a reported incidence of ~20-50% in patients with AF, and is one of the most common underlying co-morbidities associated with AF, probably due to the chronic dilation that the left atrium is subjected to. The atrial substrate in MR is distinct from that in lone AF. In particular, atrial structural remodeling is more prominent in patients with MR. This MR-altered atrial substrate promotes the development and maintenance of AF. Further, MR-induced structural and mechanical alterations are associated with increased stroke, independent of AF. In patients with pre-existing MR, new onset AF significantly worsens mitral regurgitation, and results in further hemodynamic consequences. Understanding the atrial substrate in MR should permit the development of novel therapies targeted to this subpopulation of AF patients.

Surgical correction of the valve rarely eliminates the arrhythmia ³-⁵), with approximately 75% of the patients continuing to have AF after surgery. In fact several groups are now recommending performing concurrent AF surgery whilst replacing the mitral valve, including using cryoablation, radiofrequency, or surgical incisions ³,⁴,⁹.

While the existing clinical data suggests that increased atrial dilation due to MR is a predisposing factor for development of AF, the mechanisms remain undefined, and therapeutic options to surgery are not optimized.

Furthermore, while various animal models for AF have been developed including canine and goat models, these have generally used a protocol of rapid atrial pacing to produce a susceptibility to AF. Here we investigated a model of AF susceptibility
induced by mitral regurgitation in a canine model over time, to isolate the mechanisms of dysfunction.
METHODS

Canine MR Model And Study Design

Iatrogenic MR was induced in 11 male dogs (20-40 lbs) by surgically dissecting the chordae tendinae under fluoroscopic guidance in the lab of Dr. R Hamlin. Before the production of mitral regurgitation, dogs received physical exams and submax exercise studies in which oxygen consumption and respiratory exchange quotients were measured to ascertain normal cardiovascular status.

The dogs were studied at 1 (n=5) and 6 (n=6) months post MR induction. Equal numbers of age-matched sham control dogs were included.

Myocardial structure and function were monitored serially by 2D and 2D-directed M-mode echocardiography, electrocardiography, and thoracic radiography. Left atrial dimension was evaluated by echocardiography.

Electrophysiology

In-vivo atrial electrophysiologic remodeling processes during MR were measured by a combination of atrial effective refractory periods (ERPs), AF inducibility, and P wave duration (atrial depolarization). Electrophysiological parameters were measured non-invasively during light butorphanol sedation.

Ionic current remodeling

These studies were performed in the laboratory of Dr. Cynthia Carnes. Action Potentials were measured using the amphotericin-perforated patch whole cell recording technique. Only cells with clear striations and sharp margins are used. Ion currents were measured at physiologic temperature (35°C) using a series of test potentials from –30 mV to + 50 mV at three second intervals. Holding potential of –
60 mV was used to inactivate inward sodium current. The bath solution \( (T = 35^\circ C) \) contained 134 mM NaCl; 1 mM MgCl₂; 5 mM KCl; 5 mM HEPES; 1 mM CaCl₂; 5 mM d-glucose, pH of 7.4 achieved with NaOH. Nifedipine (2 mM) was added to block L-type calcium current and to isolate the calcium independent component of \( I_{\text{to}} \). The pipet solution contained 130 mM KCl; 5 mM MgCl₂; 5 mM HEPES; 5 mM EGTA; pH adjusted to 7.2 with KOH. Pipets were backfilled with Amphotericin B containing pipet solution. \( I_{\text{to}} \) current was measured as the difference between the peak transient outward current and the steady-state current measured at the end of the step. \( I_{\text{K-sus}} \) was measured as the steady-state current.

**Histology and Immunohistochemistry**

Left atrial Free Wall (FW) and Atrial Appendages (AA) from MR and control dogs were paraffin embedded and blocked by standard procedures. General morphology and extent of fibrosis deposition were assessed using Masson’s Trichrome stain (cytoplasm, red; collagen, blue; nuclei, black) with a kit based approach (Sigma Chemical).

Myocyte hypertrophy measurements were conducted on H&E stained slides, a minimum of 5 images per tissue sample. Number of nuclei (blue)/view counted, tissue area/view determined by Image Pro, and cellular area calculated as tissue area/nucleus. Intra-observer error <5%, Inter-observer error<14%

5µ sections were also assessed for evidence of 3-Nitotyrosine (3NT), Nitric Oxide Synthase (NOS) 2, Myleperoxidase (MPO), β Tubulin, Connexin 40 and 43 (Cx40,
Cx43), using commercially available antibodies. Simultaneous detection of mast cells (Toluidine blue) and eosinophils (Vital red) was also performed. Staining controls included antibodies preadsorbed with purified antigen (addition of antigen eliminated positive staining in each case, demonstrating antibody specificity) or equal concentration of host IgG. Diaminobenzidine (0.06% w/v) was used to provide visualization of immunoreactivity, with methyl green counterstaining.

**Digital Image Analysis**

Cross-sectional areas of each heart were visualized with an Olympus BX-40 microscope (400x magnification for 3NT, β Tubulin, and Myleperoxidase (MPO), 800x magnification for myocyte hypertrophy, Cx40 and Cx43; Olympus Inc., New York, NY) and captured using an Insight QE digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were then analyzed for extent of diaminobenzidine signal in each tissue using research-based digital image analysis software (Image Pro Plus 4.0; Media Cybernetics, Silver Spring, MD), as previously described.

At this magnification individual cells can be identified and individually analyzed. 5-7 images were captured from random locations around each atrial cross section, under identical lighting and optical settings, so as to encompass >70% of the cross-section available. Images were then segmented to eliminate background and nuclear counterstain from analysis. Extent of immunoreactivity was determined by measuring optical density of diaminobenzidine signal in each image. Integrated optical densities were determined for each image as a measure of staining intensity, and values were then averaged for each heart to provide a measure of prevalence at each time point. Intra- and inter-observer variability for this procedure was each less than 2%.

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In parallel studies, we assessed the distributional changes in cardiac Cx40 and Cx43 in control and MR Dogs, over the same time course and in the same histological sections as described above. A digital imaging approach was developed to assess Cx40 and Cx43 staining intensities at the myocyte short axes (Cx43 localizes to myocyte gap junctional regions under normal conditions to facilitate inter-myocyte signaling \textsuperscript{10-15} versus the mid-myocyte region (the area of the myocyte that did not have abundant Cx43 presence under control conditions). In preliminary experiments, the mean distance from the short axis edge that completely encompassed Cx43 staining at the inter-myocyte junctions was determined for each control heart. The 95% confidence interval (mean length + two standard deviations) for this value was determined to be 10.5 microns from each short axis edge. This value was used to define the “short axis” staining for Cx43; all staining that was detected outside of this control confidence interval, but still contained within the myocyte, was defined as “mid-myocyte” staining for Cx43. Integrated optical densities of Cx43 staining were determined for each of these myocyte regions, by methods identical to those described above. Only longitudinally oriented cardiac myocytes (over 300 individual myocytes studied) were assessed for Cx43 distributions by this digital imaging approach. Intra-observer variability (one blinded observer, 50 myocytes, 3 trials) and inter-observer variability (three blinded observers, 50 myocytes each) for this approach were less than 1 and 3%, respectively.
**Western Blot Analysis**

Whole cell homogenates were prepared from 1month atrial tissues, (3 MR and 3 Control) 70µg protein loaded per lane, bands isolated using SDS-PAGE. Total protein contents per band were determined by FastBlot (GenoTech Inc. St. Louis, MO), then probed for Kv channel content (anti-Kv channel antibodies against Kv4.3, Kvβ1.2 and KCHIP2, kindly provided by Dr. Jeanne Nerbonne). The blot was blocked with 10% non-fat dry milk solution and then probed for protein-3NT formation using a polyclonal anti-3NT antibody (Upstate Biotech, 1:1000 dilution), followed by anti-rabbit horseradish peroxidase labeled secondary. After antibody incubation, the blot was developed with ECL Western blotting detection reagent (Amersham Biosciences). Immunoblots were imaged and analyzed with digital image-analysis software (LabWorks Image Analysis Software, Laboratory Technologies Corporation, LABTECH, Upland, CA). Images were background corrected, and integrated optical intensity of each band was determined. Nitrated tyrosine per protein was then obtained as a ration of IOD of nitration signal to IOD of protein signal per K channel as previously described 16

**Statistical Analysis**

All data presented represent 5-6 individual animals per group. Statistical comparisons were made by one-way ANOVA’s with Student-Newman-Keuls post hoc tests. P<0.05 denoted statistical significance.
RESULTS

The degree of MR in our preliminary study was graded using the method of Sellers by contrast angiography. The average degree of MR in our dogs was mild to moderate (average: grade I). The degree of left ventricular impairment was modest after 6 months of MR in these dogs. The average LV end-diastolic internal diameter (LVIDD) increased from 4.3 cm to 4.9 cm while the average LV end-systolic internal diameter (LVIDS) increased from 2.8 cm to 3.3 cm. The average left ventricular fractional shortening was unchanged from 33.1% at baseline to 33.9% at termination of the study. However, the inducibility of AF in these dogs by right atrial pacing showed a marked increase in the MR vs. control dogs at both time-points (3/6 MR dogs at 1 month and 3/5 at 6 months). None of the control dogs showed evidence of inducible atrial arrhythmias at either time point.

Atrial Structural Remodeling

Left atrial dimension (LAD) was evaluated by echocardiography as shown in Figure 8.1A (Data obtained by Drs. R. Hamlin and C. Carnes). Echocardiography demonstrated an increase in LAD end diastolic diameter at 1 month, but not at 6 months. However, as shown in figure 8.1B, Left Atrial Transport Function as measured by Peak A wave velocity showed a trend toward a significant MR-induced reduction (average ~18%). The Peak E/A ratio showed a significant reduction as compared to control at 6 months.
Ventricular parameters were also studied, (data not shown). The degree of left ventricular impairment was modest after 6 months of MR in these dogs. The average LV end-diastolic internal diameter (LVIDD) increased from 4.3 cm to 4.9 cm while the average LV end-systolic internal diameter (LVIDS) increased from 2.8 cm to 3.3 cm. The average left ventricular fractional shortening was unchanged from 33.1% at baseline to 33.9% at termination of the study. None of the dogs had clinical symptoms of heart failure (pulmonary edema, exercise intolerance). Thus, this model is one of mild chronic MR and not of overt heart failure.

**Electrophysiology**

Heart rate was unchanged after the production of MR (102 ± 10 bpm at baseline compared to 93 ± 13 after MR). However, MR resulted in P wave prolongation. As shown in the figure 8.2A, ECG recorded at baseline and after 6 months of MR in the same animal resulted in prolongation of P wave duration from 37 msec to 52 msec. There is a significant prolongation of the P wave duration after 6 months of MR from 51 to 58 msec (p<0.05), as shown in figure 2B. (Data obtained from Dr. C Carnes).

**Atrial ionic K+ currents after Chronic MR**

$I_{to}$ current was measured as the difference between the peak transient outward current and the steady-state current measured at the end of the step. $I_{K-sus}$ was measured as the steady-state current. Mean (SE) $I_{to}$ data is shown in figure 8.3 and demonstrates selective modulation of $I_{to}$ in MR over time. Mitral regurgitation causes biphasic regulation of $I_{to}$ (p<0.05), with a reduction after one month and an increase after 6 months.
**Histochemical Studies: Cellular Hypertrophy and Intercellular Fibrosis**

Representative images of cardiac myocytes (CTRL and 6month MR) are shown in figure 8.4a. We observed a significant increase (~47%) in myocyte area as determined by image analysis techniques in MR dogs at six months. We did not perform a similar analysis at one month.

Figure 8.4b shows representative images of fibrosis in Control and 6 month MR dogs. We observed a significant increase in fibrosis in left atrial free wall (LFW) at one month, which appeared to be swamped out by age-associated increase in fibrosis at 6 months. In the left atrial appendage in contrast there was no significant difference at 1 month, but a significant increase at 6months.

**Histochemical Studies: β Tubulin**

There was trend towards higher prevalence of microtubules as seen by total β tubulin content at 6 months in the LFW, and a significant increase in β tubulin content in the left atrial appendage, as shown in figure 8.5.

**Histochemical Studies: Protein 3NT**

Figure 8.6 shows protein 3NT immunoprevelance in control and MR dog. Protein 3-NT is a reliable biological marker of reactive oxygen and nitrogen species. We saw a significant increase in protein nitration at 1 month in the left free wall, and significant increases at both time points in the left atrial appendage.
**Histochemical Studies: Nitric Oxide Synthase 2 (NOS2)**

We then investigated NOS-2 as a potential source for the increased protein nitration observed. Figure 8.7 demonstrates increased NOS-2 prevalence at both sites at both time-points suggesting increased immune activation.

**Histochemical Studies: Immune cell infiltration**

Immune cell infiltration has been shown to be involved in cardiac dysfunction setting. Here we carried out simultaneous detection of mast cells (by toluidine blue) and eosinophils (by vital red) and observed a significant increase in mast cell infiltration at 1 month which abated by 6 months in both left free wall and atrial appendage as shown in figure 8.8a. Minimal counts of Eosinophils were noted (data not shown).

Figure 8.8b shows a sustained increase in infiltration of MPO+ neutrophils at both 1 and 6 months in both sites studied. Thus we observed a discrete profile of immune infiltration over time.

**Histochemical Studies: COX-2**

We also looked for COX-2, a pro-oxidant enzyme known to be upregulated in cardiac myocytes during stress. There was no significant increase in the COX-2 expression, rather a surprising significant decrease in expression at 1 month in MR dogs.

**Histochemical Studies: Cx40 and Cx43**

The immunoprevalence of Cx40 is shown in figure 8.10a. As shown in the graphs, Cx40 content shows a trend towards an increase that reached statistical significance at 6 months in the left free wall, whereas the left atrial appendage showed an increase in Cx40 prevalence only at 1 month.
Figure 8.10b shows Cx43 prevalence by immunohistochemistry. We observed a significant decrease in Cx43 only in the left atrial free wall at the 1-month time point with no other significant changes.

Since the Cx isoform distribution has even more relevance than connexin content, we then investigated the distribution of Cx40 and Cx43, in individual myocytes. Figure 8.11a shows Cx40 distribution through the middle of the myocyte as a percentage of total connexin in the myocyte. In control animals in the free wall, this % was minimal (1.1±0.6% at 1 month, and 2.7±2.0% at 6 months) but increased dramatically in the MR dogs to (21.9±5.1% at 1 month and 16.4±4.0% at 6 months). Similarly in the left atrial appendage the middle Cx40% increased from 8.5±3.1% at one month control and 8.4±4.3 at 6 month control to 25.4±4.9 at 1 month MR and 23.2±3.8 at 6 month MR.

Distribution of Cx43 on the other hand showed no changes at either time point in either site.

**Western Blots**

Total protein content of Kv channel proteins was not significantly different in studies performed in either our lab, or those of Dr J. Nerbonne (data not shown). In preliminary nitration studies, however (n=3 MR and n=3 sham control) we observed a trend towards increased nitration of Kv4.3 (p=0.18) and Kvβ1.2 (p=0.20). There was no change in nitration of KCHIP2.
DISCUSSION

The degree of MR in this study was mild to moderate as graded by using the method of Sellers by contrast angiography\textsuperscript{17}. The dogs showed a preliminary increase in left ventricular shortening fraction, as previously reported by Dr Hamlin's group, consistent with the emergence of a low resistance pathway into the left atrium with the development of MR. With chronic MR, however we observed modest left ventricular dysfunction (increase in LV internal diameter at systole and diastole), with fractional shortening not significantly different from baseline values. Heart rate was also unchanged after the production of MR (102 ± 10 bpm at baseline compared to 93 ± 13 after MR). None of the dogs had clinical symptoms of heart failure (pulmonary edema, exercise intolerance). The model therefore is consistent with that of mild chronic MR and not overt heart failure.

In contrast we observed a significant change in the atrial structure and contractile function. Left atrial dimension as determined by echocardiography showed a significant increase at 6 months, and there was a corresponding decrease in contractile function as measured by A/E ratios. Ventricular filling occurs in two waves, the initial relaxation of the ventricle causes passive filling, which can be observed by echocardiography as the E wave, and the remainder is pushed into the ventricle by atrial contraction, seen as the A wave. In the MR dogs at 6 months there was a trend towards a significant MR induced reduction in peak A wave velocity (average ~18%). When the A wave velocity is normalized to the peak E wave velocity, to account for heart rate dependant changes in A wave velocities, there was a significant reduction in the MR dogs, showing a decreased atrial contractile
function. In MR patients clinically too, left atrial dilatation occurs and is a strong predictor of atrial fibrillation. Regurgitant jets and dilatation result in increased wall stress and mechanical load on the cardiomyocytes, which could modulate function, and indeed Myocyte disarray has been reported in MR and AF (varnava). Stretch induced fibroblast activation with resulting increase in fibrosis has also been proposed to be a major factor in regional conduction delays, thus providing a substrate for re-entry and atrial fibrillation. MR has been shown to be associated with increased fibrosis, which in turn has been linked to increased risk of arrhythmias and sudden death. In this study the MR dogs were shown to have a greater vulnerability to AF, (AF could be induced in 50% of MR dogs, none of control). We therefore investigated these hearts for underlying changes in structure and protein expression that could explain the increased vulnerability to AF with chronic MR. Furthermore we also noted an increase in the P-wave duration which suggests slower intra-atrial wave propagation. We therefore conducted immunohistochemical studies at the myocyte level to look for structural changes. In a preliminary study, we looked at left atrial free wall samples for cellular hypertrophy at only six-month tissues. Consistent with previous animal model and clinical reports, we observed increased cellular hypertrophy in the MR dogs suggesting changes at the myocyte level. We then investigated interstitial fibrosis. Surprisingly we saw only regional changes in intercellular fibrosis. Given that global conduction differences would have been required to produce the P-wave changes that we observed at the same time points, the change in fibrosis, seems to be a contributing but not the only factor at play.
A recent field that is gaining attention in cardiac disease milieu is the intracellular scaffolding of myocytes, the cytoskeleton which along with providing structural support and compartmentalization is also the embedding matrix for ion channels. Changes in the cytoskeleton can therefore affect conduction, as well as changing the contractility of the cell by affecting cellular stiffness. Microtubules are a major component of cardiac myocyte cytoskeleton, consisting of hollow protein cylinders of $\alpha$ and $\beta$ tubulin heterodimers, aligned along the longitudinal axis of the cardiac myocyte. Disruption of the myotubular structure has been linked to various pathological conditions, like ischemia, \cite{23} and proliferation has been linked to decreased myocardial contractility due to hypertrophy \cite{24}. We therefore carried out preliminary investigations of $\beta$ tubulin total content, in the six-month dogs and observed a trend towards increase in the left free wall with a significant increase in the left atrial appendage. Further investigation into the cytoskeleton modification (or intracellular stiffness) in addition to interstitial fibrosis (extracellular stiffness) might be of value.

In addition to modulating conductivity and contractility, fibroblasts are also known to increase oxidant stress. Oxidant stress has also been shown to be involved in many models of cardiac dysfunction, \cite{16,25-28} including a related model of AF produced by rapid atrial pacing. We therefore investigated protein 3-nitrotyrosine content, a stable biomarker of reactive oxygen and nitrogen species. As expected we saw a significant increase in protein 3NT in the atrial appendage at both 1 and 6 months. In the free wall, we saw a significant increase at one month, but not at six months.
However we also noted a significant age related increase in 3NT in the free wall even in controls, which might have swamped out any potential differences.

Other than fibroblasts, inflammatory reactions are important sources of reactive species and have been studied in relation to cardiac disease. We therefore investigated evidence of inflammation and infiltration. As shown in figure 8.7, we observed a striking increase in NOS-2, or inducible nitric oxide synthase all across the atrium, in both sites studied and at both time points, suggesting a generalized chronic inflammation of the atria due to MR. This increase in NOS-2 could account for the increase in reactive nitrogen species as seen by increased protein nitration.

Further, other than a 2003 study suggesting the presence of infiltrates in MR dogs, immune cell infiltration in MR is not well defined. No studies to date, have attempted to quantify interstitial immune cell presence in MR. We conducted specific histological studies probing for leukocytes that mediate non-specific immunity, and that are known to participate in the formation of reactive oxygen and nitrogen species. Shown in Figure 8.8 are representative images from cardiac tissues stained with specific probes for mast cells, neutrophils, and eosinophils. Cell counts were determined for each treatment group and expressed per cross-sectional area. Interestingly, we saw distinct time profiles of individual immune cells. Mast cells showed a dramatic acute increase at one month, and were back to baseline levels at 6 months, while MPO+ cells (neutrophils) showed a sustained increase through to 6 months. Mast cells are central mediators of inflammatory reactions and release histamine, proteases, chemotactic factors and cytokines TNF-α and IL-4, that can modulate adhesion molecules on endothelial cells. Activated endothelial cells
express the intercellular adhesion molecule (ICAM-1), endothelial-leukocyte adhesion molecule (ELAM-1) and vascular cell adhesion molecule (VCAM-1) on their cell surface, and thus can draw more immune cells, prolonging the inflammatory reaction. Neutrophils, on the other hand are short-lived as cardiac immune infiltrates, and have potent reactive nitrogen species formation capabilities, through a variety of nitrogen containing (NO, NO\textsuperscript{2}\textsuperscript{-}, etc) and reactive oxygen (O\textsuperscript{2}\textsuperscript{-}, H\textsubscript{2}O\textsubscript{2}, OH\textsuperscript{-}, HOCl, etc) precursors. These data suggest that a steady state infiltration of neutrophils occurs during both time points and at all sites in the atrium, thus perhaps responsible for RNS formation observed. We did not observe any significant amounts of eosinophils at any time point, or in any site.

While the factors involved in the recruitment and/or activation of these immune cells in the heart remain unknown, the selective infiltration of immune cells, and their protracted residence in cardiac muscle interstitium, has the potential to modulate cardiac function. Further studies to define the role of immune cell trafficking and activation in cardiac muscle are needed.

Another source of reactive oxygen species that has special importance in the myocardium is COX-2, which has been shown to be up in inflammatory conditions in the myocardium, as well as possibly modulated through cardiac fibroblasts\textsuperscript{29,30}. We therefore also looked immunohistochemically for COX-2 immunoprevalence. However other than a contra-intuitive drop in COX-2 prevalence at one month in only the left free wall, we did not see any significant changes, therefore leading us to conclude that COX-2 might not play an important role in this setting.
Taken together, these studies showed the MR dog model to closely resemble clinical MR, with similar changes in atrial dilatation and P wave durations. Biphasic $K^+$ current changes were observed. Immunohistochemistry however only showed regional increases in fibrosis, but not to the magnitude that would be required to produce the global conduction changes required to affect P wave duration (a whole atrial phenomena). We also found data suggesting that the atrium as a whole is subjected to increased oxidant stress and a chronic globalized inflammation and infiltration. In this setting, it is then warranted to suggest that along with focal fibrosis, changes might be occurring at the individual myocyte level. One alternative explanation is altered cell-to-cell conduction, which could be affected by altered gap junctional protein (connexin) expression. Gap junctions are clusters of transmembrane channels that link plasma membranes of neighboring cells forming low resistance pathways for conduction of action potentials, and thus are responsible for orchestrating contraction. Each channel consists of 2 hexamers of hemichannels (connexons) and each connexon is a hexamer of connexin subunits. There are 20 connexin (Cx) subunits identified so far. The most abundant human atrial Cx is Cx40, with smaller amounts of co-localized Cx43, while predominant Cx in the ventricle is Cx43, with Cx45 being present at low abundance throughout the heart $^{12,31}$. The intracellular location of Cx appears to be highly controlled in normal myocytes, forming a step like distribution pattern in the ventricle and only localized to short axis ends of atrial myocytes. Alterations of Cx localization and content are now well-established features in cardiac disease settings, e.g. ischemia and cardiac infarcts result in decrease in Cx43 content and distribution. However, there is
controversy in the literature about changes in atrial gap junction proteins in AF, with some reports suggesting that Cx40 is elevated in patients susceptible to post-operative AF \(^{32}\) while other studies suggest that Cx40 is down regulated in AF patients \(^{33,34}\), while yet another study \(^{35}\) suggested that Cx43 was significantly down regulated while Cx40 expression varied (down in AA, up in FW). Animal studies have also given conflicting data, chronic atrial fibrillation produced by pacing in goat resulting in decreased content and heterogeneous distribution of Cx40, while a rat model showed altered distributions of Cx43.\(^{34,36}\)

However the human studies included a diverse population of AF patients, and studied Cx distributions at varied sites (right/left atrium, appendage/free wall) and as has been seen in our study there appear to be site differences in dysfunction. Furthermore, the animal studies were performed in a rapid atrial pacing model which has already been shown to have a distinct etiology from chronic dilatation induced AF as seen in the MR model. Our model showed heterogenous changes in connexin distribution. As seen in figure 8.10, Cx40 showed a trend towards increase in the left free wall and was significantly increased at 6months, while in the left atrial appendage showed an increased expression at 1month, but not significantly increased at 6months.

Cx43 on the other hand was only significantly decreased in the left free wall at one month but not at six months, and showed no significant difference at either time point in the appendage.
Since these studies were performed using *in situ* methods, we had the opportunity to assess both the content and distribution of Cx isoforms throughout the myocardium as shown in figure 8.11. We observed striking qualitative evidence of altered Cx40 distributions, as Cx40 staining appeared to migrate from its strict alignment with individual myocyte short axis connections to the mid-myocyte regions. We developed an imaging approach to assess these distributional changes quantitatively, using Cx40 staining in control dogs to define the short axes distributions expected in normal healthy myocardium, and establishing 95% confidence intervals for control Cx40 distributions in myocytes that exhibited longitudinal alignment in our cross-sections. We defined Cx40 intramyocyte staining that fell outside this interval as “mid-myocyte” staining. Cx40, which has been distributed to the mid-myocyte regions, is unlikely to mediate normal cardiac conduction, since this staining was virtually absent in control myocytes, and since these proteins would not provide myocyte-to-myocyte connections through the intercalated disk regions of the myocyte short axes. Using this approach, we found that in control hearts, less than 10% of total Cx40 staining was found in the mid-myocyte region (as defined by our preliminary studies); however, by in both appendage and free wall this increased to ~25% at both 1 and 6 month time points. (as determined by integrated optical density analysis) . Similar analyses carried on with Cx43 showed no significant increases in midline Cx43 content, suggesting that distribution of Cx40, which is the main Cx isoform, expressed in the atrium is of particular importance.
Finally, since we saw changes in K-currents as measured by Ito, we conducted preliminary experiments into the content of various K+ channels. The western blot analyses was run in the laboratory of Dr. J. Nerbonne, and no significant difference was observed in the protein contents of Kv4.3, Kvβ1.2 or KCHIP2 (data not shown). However, as we observed evidence for increased oxidative stress and RNS in these animals, and Ito has been shown to be sensitive to redox status (evidence in Dr. Carnes Lab), we tested the hypothesis that increased nitration of Kv4.3, Kvβ1.2 or KCHIP2 might be seen. As seen in figure 12, we observed a trend towards increased nitration of Kv4.3 and Kvβ1.2, with no change in nitration status of KCHIP2. However as the total n was small (3 animals/group) we do not have a definitive answer yet.

In conclusion, we have demonstrated the MR model mimics the functional and electrical parameters of clinical MR. This model is a milder, chronic model of AF substrate and might mimic chronic human AF more closely than the more common rapid atrial pacing model (which is more of an acute model). Furthermore we also show that merely increased fibrosis is not sufficient to explain the mechanical and electrical abnormalities seen in MR, but rather, oxidative stress and inflammatory mechanisms might also play important roles by modulating various components. Of particular importance is the effect of oxidant stress on various ion channels. We show preliminary evidence on increased nitration of specific K+ channels. Furthermore we also show evidence of dysregulated Cx expression and distribution. Cx40, the most abundant connexin in the atrium, shows heterogeneous expression and dislocation intracellularly to the midline axis, instead of the normal
short axis end distribution pattern. This could result in dispersion of the electrical signal and provide substrate for increased vulnerability to AF.

Finally the resistance of connexon channels is modulated in part by connexin protein phosphorylation status at both serine and tyrosine sites. In other studies in our lab (mihm M, in review), we show evidence of increased tyrosine nitration of connexins in pathological conditions. Future directions would include an investigation of redox status of connexins in MR dogs.
REFERENCE:


FIGURE LEGENDS

Figure 8.1: Echocardiographic parameters at baseline and after production of chronic MR. Top Left Panel: Echocardiogram in the same dog at baseline and after production of chronic MR. Note the significant MR-induced left atrial (LA) dilatation, with modest dilatation of the left ventricle (LV). (IVS) interventricular septum; (RA) right atrium; (RV) right ventricle. Bottom Left Panel: Averaged LAD-ED (left atrial diameter – end diastole) and LAD-ES (left atrial diameter – end systole), represented as increase from basal control. (p<0.05)
Top Right Panel: Peak A wave velocity is reduced in chronic MR. Baseline echocardiogram and following 6 weeks of MR in the same animal. Bottom Left Panel: Peak A wave velocity/Peak E wave velocity

Figure 8.2: MR results in P wave prolongation. Top Panel: ECG recorded at baseline, prior to the production of MR. P wave duration is 37 msec. ECG recorded in the same animal after 6 months of MR. P wave duration is prolonged to 52 msec.

Figure 8.3: I_{to} current density-voltage relationship (mean ± SE). Mitral regurgitation causes biphasic regulation of I_{to} (p<0.05), with a reduction after one month and an increase after 6 months.

Figure 8.4: MR causes increases in Hypertrophy and Fibrosis. Top Left Panel: Representative pictures of control and MR 6 month left free wall (H&E stain, 800X magnification) Bottom Right Panel: Averaged values for 6 month dogs, p<0.05. Top Right Panel: Representative photomicrographs trichrome staining in control and 1 month MR left free wall. Bottom Right Panel: Averaged 1and 6 month left atrial free wall and appendage data, p<0.05.
Figure 8.5: Tubulin prevalence in control and 6 month MR dogs. Top panel: left free wall. Bottom Panel: left atrial appendage, p<0.05.

Figure 8.6: Protein 3NT in control and MR dogs at 1 and 6 months. Top panel: Representative photomicrographs. Middle Panel: left free wall. Bottom Panel: left atrial appendage, p<0.05.

Figure 8.7 NOS-2 prevalence in control and MR dogs at 1 and 6 month. Top panel: left free wall. Bottom Panel: left atrial appendage, p<0.05.

Figure 8.8 MR causes increases in Infiltration of immune cells. Top Left Panel: Representative pictures of control and MR 6 month left free wall (Mast cell stain, 400X magnification) Bottom Right Panel: Averaged values for Control and MR dogs at 1 and 6 month dogs, p<0.05. Top Right Panel: Representative photomicrographs of MPO+ staining in 6 month control and MR dogs left free wall. Bottom Right Panel: Averaged 1 and 6 month left atrial free wall and appendage data, p<0.05.

Figure 8.9: COX-2 prevalence in control and MR dogs at 1 and 6 month. Top panel: left free wall. Bottom Panel: left atrial appendage, p<0.05.

Figure 8.10 MR causes selective heterogenous increases in Connexins. Top Panel: Representative pictures of control and MR 6 month left free wall (800X magnification) Bottom Right Panel: Averaged Cx40 values for Control and MR dogs at 1 and 6 month dogs, p<0.05. Bottom Right Panel: Averaged 1 and 6 month left atrial free wall and appendage Cx43 IOD data, p<0.05.
Figure 8.11 MR disrupts Cx40 but not Cx43 distribution. Right Panel: Averaged middle myocyte Cx40 values for Control and MR dogs at 1 and 6 month dogs, p<0.05. Right Panel: Averaged 1 and 6 month left atrial free wall and appendage middle myocyte Cx43 IOD data, p=NS

Figure 8.12 MR causes selective increases in nitration of K-channel proteins. Top Panel: Representative pictures of western blots for protein 3NT. Lower panel: averaged protein nitration, (corrected to total K-channel protein by Fast Blot).
Figure 8.1: Echocardiographic parameters at baseline and after production of chronic MR
Figure 8.2: MR results in P wave prolongation
Figure 8.3: Mitral regurgitation causes biphasic regulation of $I_{to}$
Figure 8.4: MR causes increases in Hypertrophy and Fibrosis
Figure 8.5: Tubulin prevalence in control and 6 month MR dogs
Figure 8.6: Protein 3NT in control and MR dogs at 1 and 6 months.
Figure 8.7 NOS-2 prevalence in control and MR dogs at 1 and 6 month
Figure 8.8 MR causes increases in Infiltration of immune cells
Figure 8.9: COX-2 prevalence in control and MR dogs at 1 and 6 month
Figure 8.10 MR causes selective heterogenous increases in Connexins.
Figure 8.11 MR disrupts Cx40 but not Cx43 distribution.
Figure 8.12 MR causes selective increases in nitration of K-channel proteins.
APPENDIX A

Differential Mechanism Of Substrate Remodeling In Two Models Of Atrial Fibrillation:
Rapid Atrial Pacing Induced Atrial Fibrillation vs. Chronic Atrial Dilatation Induced By Mitral Regurgitation
ABSTRACT

Atrial Fibrillation (AF) is the most common sustained arrhythmia in the United States currently affecting about 2 million Americans. While AF occasionally occurs in the absence of other cardiovascular disease (termed lone AF), it is more commonly seen in the presence of definite risk factors. Mitral Regurgitation (MR, present in 1-2% of general adult population) in particular is a common primary risk factor for development of AF. About 50% of patients with mitral valve disease will develop AF. Data from animal models suggest that chronic AF developing as a result of MR is different from lone AF arising in the absence of detectable structural disease. We had unique access to animal (dog) models of both rapid pacing induced AF and surgically induced MR resulting in AF. Hence we tested the hypothesis that these two models had differing underlying mechanisms of structural remodeling, resulting in development of substrate for AF. We did not see a major increase in fibrosis in the paced AF dogs, only the right atrial free wall showed a significant increase, while the MR dogs showed significant increase in 3 of 4 sites studied. In contrast to fibrosis we saw similar increases in cellular hypertrophy in both models, with increases in the left free wall, and decrease in cell size in the right free wall. There was no significant increase in mast cells or eosinophils in either model at the time point studied. However we saw a significant increase in MPO+ cells (neutrophils) in both the paced and MR induced AF models. Finally we conducted a preliminary study to determine if the total plasma antioxidant capacities were affected in the models. Rapid atrial pacing resulted trend towards decreased plasma antioxidant capacity,
suggesting perhaps oxidative events in the atria had depleted the antioxidant capacity.

In conclusion, it appears that discrete mechanism might be in play in the two different models of AF. Rapid pacing induced AF, results in acute changes in cellular hypertrophy, increased specific immune cell infiltration, and possibly oxidant production. On the other hand, fibrosis along with cellular hypertrophy and immune cell infiltration appear to be involved in the long term remodeling associated with MR.
INTRODUCTION

Atrial Fibrillation (AF) is the most common sustained arrhythmia in the United States currently affecting about 2 million Americans. While AF occasionally occurs in the absence of other cardiovascular disease (termed lone AF), it is more commonly seen in the presence of definite risk factors, namely, increased age, male gender, coronary artery disease, valvular disease, chronic obstructive pulmonary artery disease, heart failure and after heart disease or heart surgery.\(^1\)-\(^3\)

Mitral Regurgitation (MR, present in 1-2% of general adult population) in particular is a common primary risk factor for development of AF\(^1\). About 50% of patients with mitral valve disease will develop AF\(^3\). Surgical correction of the valve rarely eliminates the arrhythmia\(^4\)-\(^6\), with approximately 75% of the patients continuing to have AF after surgery. In fact several groups are now recommending performing concurrent AF surgery while replacing the mitral valve, including using cryoablation, radiofrequency, or surgical incisions\(^7\)\(^,\)\(^4\). Thus it appears that MR results in an underlying long-term change in atrial structural and electrophysiological properties that predispose to AF, and persist even after correction of MR, leading to sustained chronic AF. Since mechanisms of AF development are difficult to study in vivo in human populations, many animal models have been developed, primarily involving larger animals, sheep and dog models, being most common.

Data from animal models suggest that chronic AF developing as a result of MR is different from lone AF arising in the absence of detectable structural disease. Though electrophysiological remodeling is associated with both types of AF, they differ in specifics. In lone AF, an acute incident of AF, resulting in
electrophysiological remodeling leading to increased vulnerability to chronic AF, modeled by several days of pacing induced AF in animal, there is a significant reduction in duration of atrial refractory period (ERP). Furthermore, there is also a loss in normal rate adaptation of ERP to faster stimulation rates, which thus can create a positive feedback loop, ultimately resulting in conversion of unstable, self terminating AF in stable chronic AF.  

In chronic surgically induced MR models on the other hand, left atrial dilatation, prolongation of atrial ERP, and greater inducibility of AF is seen. The changes in ERPs are thus in opposite directions in the two models of AF. This suggests that there might be mechanisms of remodeling, though ultimately both resulting in increased vulnerability to AF. We had unique access to animal (dog) models of both pacing induced AF and surgically induced MR resulting in AF. Hence we tested the hypothesis that these two models had differing underlying mechanisms of structural remodeling, resulting in development of substrate for AF.
METHODS

Canine Pacing Induced AF Model And Study Design

Adult male beagles (n=6, 10.7±0.7) were used for the study. All procedures were approved by the Ohio State University Institutional Animal Care and Use committee. Rapid atrial pacing protocol was as described before. Briefly, animals were anesthetized with butorphanol tartrate (0.1 mg/kg) and acepromazine maleate (0.1 mg/kg); anesthesia was maintained with halothane 1% to 2%. During anesthesia two bipolar electrodes were implanted transvenously into the right atria under fluoroscopic guidance. One electrode was placed in the right midlateral free wall for effective refractory period determination. The other azygous vein and the cranial vena cava for pacing. The animals were allowed to rest 2-4 days before initiation of pacing. The right atrium was paced at 400bmp for 48 hours, allowed to recover for a minimum of 2 days or until ERP returned to 90% of normal, and then subjected to a second bout of 48 hour pacing. At the end of the 2nd pacing session the study was terminated and the animals euthanized. Sham controls (n=5) were also included and received identical handling.

Canine MR Model And Study Design

Iatrogenic Mitral regurgitation was induced in 6 male dogs (20-40 lbs) by surgically dissecting the chordae tendinae under fluoroscopic guidance in the lab of Dr. R Hamlin. Animals were anesthetized with butorphanol tartrate (0.1 mg/kg) and acepromazine maleate (0.1 mg/kg) anesthesia was maintained with isoflurane. LV angiography was performed to determine the severity of MR, which was noted to be mild to moderate. Before the production of mitral regurgitation, dogs received
physical exams and submax exercise studies in which oxygen consumption and respiratory exchange quotients were measured to ascertain normal cardiovascular status. The dogs were studied at 6 (n=6) months post MR induction, after which the study was terminated and the animals euthanized. Equal numbers of age-matched sham surgical control dogs were included.

**Tissue Collection**

At the time of euthanasia, tissue was rapidly collected from the 11 paced AF + 6 sham controls (labeled PACED and SHAM), and 6MR + 6 sham controls (labeled MR and CTRL) at four different sites, the left Atrial Appendage (AA), Left atrial Free Wall (FW), Right AA and Right FW, to provide a comprehensive look at across the heart, and account for regional variations. The tissue was blotted dry and formalin fixed for 48hrs.

**Histology and Immunohistochemistry**

Tissues were paraffin embedded and blocked by standard procedures. General morphology and extent of fibrosis deposition were assessed using Masson’s Trichrome stain (cytoplasm, red; collagen, blue; nuclei, black) with a kit based approach (Sigma Chemical).

Myocyte Hypertrophy measurements were conducted on H&E stained slides, a minimum of 5 images per tissue sample. Number of nuclei (blue)/view counted, tissue area/view determined by image pro, and cellular area calculated as tissue area/nucleus. Intra-observer error <5%, Inter-observer error<14%

5µ sections were also assessed for evidence of Myleperoxidase (MPO), using commercially available antibodies. Simultaneous detection of mast cells (astra blue)
and eosinophils (vital red) was also performed. Staining controls included equal concentration of host IgG. Diaminobenzidine (0.06% w/v) was used to provide visualization of immunoreactivity, with methyl green counterstaining.

**Digital Image Analysis**

Cross-sectional areas of each heart were visualized with an Olympus BX-40 microscope (400x magnification for MPO and fibrosis, 800x magnification for myocyte hypertrophy, Olympus Inc., New York, NY) and captured using an Insight QE digital camera (Diagnostic Instruments, Sterling Heights, MI, 1290 × 960 resolution). At this magnification individual cells can be identified and individually analyzed. 5-7 images were captured from random locations around each ventricular cross section, under identical lighting and optical settings, so as to encompass the >70% of the cross-section available. Images were then segmented to eliminate background and nuclear counterstain from analysis. Extent of fibrosis was determined as amount of blue stain to tissue area per images. Mast cells and MPO positive cells were hand counted in each image. Inter-observer error was determined to be <7% for the counts.

**Total Antioxidant Capacity Assay**

Since this method is sensitive to both reactive oxygen and reactive nitrogen species, it provides a broad assessment of antioxidant capacity of a given tissue sample. The assay fluorometrically measures the capacity of samples (plasma/cardiac homogenates) to quench free radicals. Free radicals are produced by decomposition of 2,2'-diazobis(2-amidinopropane) dihydrochloride (AAPH), and convert dichlorofluorescein-acetate (DCFH-DA; a free radical probe), to a highly fluorescent
dichlorofluorescein (DCF). DCF production was measured fluorometrically 
(excitation 480nm, emission 526nm) over time (~1hour). The DCF production 
 fluorescence) curve can be shifted to the right with the addition of 10µM of trolox. 
Fluorescence curves can be progressively shifted to the right (increase in onset 
time) with increasing concentrations of trolox. 
10µM of trolox (captures 2 peroxyl radicals) was used an internal standard when 
comparing different samples.

Protein content of plasma samples from the dogs were assessed by BCA protein 
assay and equal protein concentrations of plasma used to perform assay. The 
intra- and inter- assay coefficients of variation of this assay are <5% and <10% 
respectively.

**Statistical Analysis**

All data presented represent 5-6 individual animals per group. Statistical 
comparisons were made by one-way ANOVA’s with Student-Newman-Keuls post 
hoc tests. P<0.05 denoted statistical significance.
RESULTS

Atrial pacing resulted in the expected decrease in ERP values (~50%, p<0.005) as published (c carnes). The degree of MR in our study was graded using the method of Sellers by contrast angiography. The average degree of MR in our dogs was mild to moderate (average: grade I). The degree of left ventricular impairment was modest after 6 months of MR in these dogs. However the inducibility of AF in these dogs by right atrial pacing showed a marked increase in the MR vs control dogs (3/5 at 6 months). None of the control dogs showed evidence of inducible atrial arrhythmias at either time point.

Histochemical Studies: Intracellular Fibrosis

Figure A1.1 shows relative presence of fibrosis in paced and MR dogs as compared to respective controls. Fibrosis is represented as a percent of total tissue. As can be seen in the figure there is a more consistent increase in fibrosis in the MR dogs (right panel) as compared to the paced dogs (left panel). In the paced dogs, only the right free wall showed evidence of fibrosis, whereas in the MR dogs, 3 of 4 sites showed increased fibrosis.

Histochemical Studies: Cellular Hypertrophy

Cellular hypertrophy experiments were carried out in the left and right free wall of the four groups. We observed a similar pattern in both paced and MR induced dogs, with significant increase in myocyte hypertrophy in the left atrium (~25% for paced, ~47% for MR) in myocyte area as determined by image analysis techniques, and reduction in cell area in the right free wall.

Histochemical Studies: Immune cell infiltration
Immune cell infiltration has been shown to be involved in cardiac dysfunction setting. Here we carried out simultaneous detection of mast cells (by astra blue) and eosinophils (by vital red). We saw no observable infiltration of eosinophils at any site, and the number of mast cells was not significantly increased at any site as shown in figure A1.3.

Figure A1.4 shows infiltration of MPO+ neutrophils. We saw evidence of increased neutrophils only in three of four sites in the paced AF tissues, with the most dramatic increases in the right atrial. In the MR all four sites showed significant increases in neutrophil counts with the most dramatic increases in the atrial appendage.

**Total antioxidant capacity assay**

Finally, we also investigated the total plasma antioxidant capacities of the paced and MR dogs. As seen in figure A1.5 there was no significant change in the total plasma antioxidant capacities in the two models. However the paced AF dogs showed a trend towards reduced plasma total antioxidant capacity (p = 0.0569).
DISCUSSION

AF is the most common arrhythmia in the United States. However, the etiologies of AF development are varied. AF occasionally develops in the absence of structural cardiac abnormality, but is most often associated with various cardiovascular abnormalities, including coronary artery disease, valvular disease, chronic obstructive pulmonary artery disease, and heart failure and after heart disease or heart surgery \(^1,3\). Treatment strategies however are limited and not perfected, with many arrhythmic drugs having substantial side effects and even predisposing to arrhythmia themselves.

Both an episode of acute AF and chronic dilatation caused by mitral regurgitation results in increased vulnerability to sustained chronic AF. But there appear to be differences in the electrical remodeling seen in the two settings, as seen with the help of pacing induced AF animal models and animal models of MR. \(^8-11,14\)

Thus it appears plausible that different mechanism of remodeling occur in these similar, but distinct settings. Here we investigated structural and oxidative mechanisms in these two setting to determine their differential importance, if any.

While MR induced dilatation, effects primarily the left atria, and similarly the paced AF model was paced through the left atrium, atrial fibrillation can arise due to mischarging cells anywhere in the atrium. To obtain a comprehensive understanding of events occurring in the heart as a whole, therefore, we investigated four different sites, the atrial free wall and appendage in both the right atrium and the left atria.

We did not see a major increase in fibrosis in the paced AF dogs, only the right atrial free wall showed a significant increase, while the MR dogs showed significant
increase in 3 of 4 sites studied. While acute dilatation can result in shortening of the atrial ERP, MR results in chronic left atrial dilatation and increase in wall stress, which in turn can result in increased fibrosis. Further, due to the direction of the regurgitant jet, different sections of the atria could experience different stress, resulting in differential fibrosis formation, thus resulting in a discontinuous myocardium, and differential conduction velocities at different sites, contributing to AF. Thus it appears that fibrosis has more of a role to play in chronic dilatation settings. However, it must be noted that as the paced AF dogs were euthanised immediately after the second pacing period there might not have been enough time for the production of substantial fibrosis, for although procollagen RNA increases have been shown soon after myocardial damage, fibrosis formation is a dynamic process that can continue for upto 3 months after damage has occurred.

In contrast to fibrosis we saw similar increases in cellular hypertrophy in both models, with increases in the left free wall, and decrease in cell size in the right free wall. While the increase in size in the left free wall was expected the decrease in the right atrium is surprising.

There was no significant increase in mast cells or eosinophils in either model at the time point studied. However we saw a significant increase in MPO+ cells (neutrophils) in both the paced and MR induced AF models. Thus a specific immune cell infiltration appears to be involved in the atrial remodeling process. Neutrophils, are short-lived as cardiac immune infiltrates, and have potent reactive nitrogen species formation capabilities, though a variety of nitrogen containing (NO, NO$_2^-$, etc) and reactive oxygen (O$_2^-$, H$_2$O$_2$, OH$, HOCl$, etc) precursors, and could
contribute to remodeling though oxidative mechanisms. Even though neutrophils increased in both models, they showed a differential distribution with neutrophil concentrations being much higher in the right atrium of the paced dogs, while in the MR dogs, the neutrophils were more concentrated in the appendages of both the right and left atrium and less so in the free walls.

Finally we conducted a preliminary study to determine if the total plasma antioxidant capacities were affected in the models. Rapid atrial pacing resulted trend towards decreased plasma antioxidant capacity, suggesting perhaps oxidative events in the atria had depleted the antioxidant capacity.

In conclusion, it appears that discrete mechanism might be in play in the two different models of AF. Rapid pacing induced AF, results in acute changes in cellular hypertrophy, increased specific immune cell infiltration, and possibly oxidant production. On the other hand, fibrosis along with cellular hypertrophy and immune cell infiltration appear to be involved in the long term remodeling associated with MR. Thus, the data suggest that AF mechanism are discrete in the two settings and thus therapeutic strategies for AF must take into account the varying etiologies of AF.
REFERENCE:


FIGURE LEGENDS

Figure A.1: Fibrotic changes in Rapid atrial pacing and mitral regurgitation models.

Right panel: Fibrosis in sham and paced dogs, expressed as percentage of total tissue. Left panel: Fibrosis in sham surgical control and mitral regurgitation dogs. A-left free wall, B-left atrial appendage, C-right free wall, D-right atrial appendage.

Figure A.2: Hypertrophy in rapid atrial pacing and mitral regurgitation models. Right panel: Hypertrophy in sham and paced dogs, expressed as total tissue/total number of nuclei. Left panel: Hypertrophy in sham surgical control and mitral regurgitation dogs. A-left free wall, B-right free wall.

Figure A.3: Immune cell infiltration in rapid atrial pacing and mitral regurgitation models – Mast Cells. Right panel: Mast cell infiltration in sham and paced dogs, expressed as number of cells/µ² of tissue. Left panel Mast cell infiltration in sham surgical control and mitral regurgitation dogs. A-left free wall, B-left atrial appendage, C-right free wall, D-right atrial appendage. (p=NS)

Figure A.4: Immune cell infiltration in rapid atrial pacing and mitral regurgitation models – Neutrophils. Right panel: Neutrophils infiltration in sham and paced dogs, expressed as number of cells/µ² of tissue. Left panel Neutrophils infiltration in sham surgical control and mitral regurgitation dogs.
infiltration in sham surgical control and mitral regurgitation dogs. A-left free wall, B-left atrial appendage, C-right free wall, D-right atrial appendage.

Figure A.5: Plasma antioxidant capacity. No significant difference, but a trend towards decreased antioxidant values in rapid atrial pacing dogs (p=0.0569).
FIGURE A.1: Fibrotic changes in Rapid Atrial Pacing and Mitral Regurgitation models
FIGURE A.2: Hypertrophy in Rapid Atrial Pacing and Mitral Regurgitation models.
FIGURE A.3: Immune cell infiltration in Rapid Atrial Pacing and Mitral Regurgitation – Mast cells
FIGURE A.4: Immune cell infiltration in in Rapid Atrial Pacing and Mitral Regurgitation models – Neutrophils
FIGURE A.5 Plasma antioxidant capacity
APPENDIX B

Investigation Of Efficacy Of ALT-711, A Novel Cross-Linker Breaker For Mitral Regurgitation Induced Atrial Fibrillation
ABSTRACT

Advanced glycation end products (AGEs), are products of non-enzymatic glycation and oxidation of proteins and lipids. They have been shown to accumulate and form crosslinks on long-lived vascular and myocardial structural proteins in normal aging e.g. collagen. Cross-linking results in increased stability of fibrosis by decreasing collagen breakdown. Structural factors decreasing compliance (fibrosis) have been implicated in atrial fibrillation (AF). We tested the hypothesis that specifically targeting fibrosis stability with ALT-711 might have therapeutic value in AF. To do so, we used a dog model of mitral regurgitation (MR) induced AF. MR was surgically induced in 12 dogs and after 6 months of MR, n=6 dogs received one dose of ALT-711. Tissue samples from left atrial appendage and left atrial free wall were also isolated and immediately formalin fixed for immunohistochemistry.

Electrophysiological experiments failed to show a significant effect of ALT-711 on MR induced changes in Ito and Iksus. MR induced site-specific increases in myocyte hypertrophy, interstitial fibrosis, myotubular content, and oxidant stress. However, there does not seem to be a corresponding increase in AGE formation. While ALT-711 succeeded in reducing fibrosis formation and also resulted in lower AGE staining, there was no effect on hypertrophy and oxidant stress markers. In conclusion, ALT-711 does not seem to be of major therapeutic value in this setting. Furthermore, decreases in fibrosis failed to reduce vulnerability to arrhythmia, suggesting that increased fibrosis is not enough to explain the development of arrhythmias in chronic atrial dilatation models.
INTRODUCTION

Advanced glycation end products (AGEs), are products of non-enzymatic glycation and oxidation of proteins and lipids. They have been shown to accumulate and form crosslinks on long-lived proteins like vascular and myocardial structural proteins in normal aging \(^1\). This accumulation and crosslinking is accelerated in diabetes and situations of inflammation and increased oxidant stress. AGE crosslinking has also been shown to impair normal function of proteins, cell and organs \(^2,3\). In the myocardium, AGE crosslinks have been implicated in aging-related increases in vascular and ventricular stiffness.

Inhibitors of AGE formation, like aminoguanidine have been shown to prevent increased vascular and ventricular stiffness in diabetic rats. Recently a class of thiazolidine derivatives that catalytically break existing glucose crosslinks has been developed. One such molecule ALT-711 (3-phenylacyl-4, 5-dimethylthiazolium)) has been used with success in treatment of hypertension in diabetic rats as well as aged non-diabetic rhesus monkeys, \(^4\) and even preliminary human trials\(^5\). Improved arterial and ventricular functions were noted on treatment with ALT-711. This is a novel target for therapy, not a specific receptor or enzyme, but rather a chemical reaction.

Structural factors decreasing compliance (fibrosis) have been implicated in atrial fibrillation (AF). Further, both diabetes and increasing age are important predisposing factors for AF\(^6-8\). And finally we have shown that oxidative stress plays an important role in AF. Cross-linking results in increased stability of fibrosis by
decreasing collagen breakdown\textsuperscript{9}. Hence, a glucose crosslink breaker might result in reduction of fibrosis content.

Thus we tested the hypothesis that specifically targeting fibrosis stability with ALT-711 might have therapeutic value in AF. To do so, we used a dog model of mitral regurgitation (MR) induced AF, that has been extensively studied in our lab, in collaboration with Dr. C. Carnes.
METHODS

Canine MR Model and Study Design

Iatrogenic Mitral regurgitation was induced in 12 male beagles (20-40 lbs) by surgically dissecting the chordae tendinae under fluoroscopic guidance in the lab of Dr. R Hamlin. N=6 sham controls underwent sham surgical procedure. Before the production of mitral regurgitation, dogs received physical exams and submax exercise studies in which oxygen consumption and respiratory exchange quotients were measured to ascertain normal cardiovascular status.

At 6 months post MR induction, half the MR dogs (n=6) received treatment with ALT-711 (5 mg/kg/body weight daily) for 6 weeks. Electrophysiological and Echocardiographic parameters were studied in the dogs in the laboratories of Drs. R Hamlin and C. Carnes. Dogs then sacrificed, left atrial appendages and atrial free walls were isolated. Atrial tissues then formalin fixed for histological investigation and drop frozen in liquid nitrogen for further biochemical analysis in our laboratory.

Histology and Immunohistochemistry

Left atrial Free Wall (FW) and Atrial Appendages (AA) from MR, MR+ALT-711 and control dogs were paraffin embedded and blocked by standard procedures. General morphology and extent of fibrosis deposition were assessed using Masson’s trichrome stain (cytoplasm, red; collagen, blue; nuclei, black) with a kit based approach (Sigma Chemical).

Myocyte Hypertrophy measurements were conducted on H&E stained slides, a minimum of 5 images per tissue sample. Number of nuclei (blue)/view counted,
tissue area/view determined by image pro, and cellular area calculated as tissue area/nucleus. Intra-observer error <5%, Inter-observer error<14%

5µ sections were also assessed for evidence of 3-Nitotyrosine (3NT), β Tubulin, and AGE content using commercially available antibodies. Staining controls included antibodies preadsorbed with purified antigen; addition of antigen eliminated positive staining in each case, demonstrating antibody specificity, or equal concentration of host IgG. Diaminobenzidine (0.06% w/v) was used to provide visualization of immunoreactivity, with methyl green counterstaining.

**Digital Image Analysis**

Cross-sectional areas of each heart were visualized with an Olympus BX-40 microscope (400x magnification for 3NT, β Tubulin, and AGE, 800x magnification for myocyte hypertrophy; Olympus Inc., New York, NY) and captured using an Insight QE digital camera (Diagnostic Instruments, Sterling Heights, MI, 1290 × 960 resolution). Images were then analyzed for extent of diaminobenzadine signal in each tissue using research-based digital image analysis software (Image Pro Plus 4.0; Media Cybernetics, Silver Spring, MD), as previously described.

**Statistical Analysis**

All data presented represent 5-6 individual animals per group. Statistical comparisons were made by one-way ANOVA’s with Student-Newman-Keuls post hoc tests. P<0.05 denoted statistical significance.
RESULTS

Histochemical Studies: Cellular Hypertrophy

We observed an increase in myocyte significant increase (~47%) in myocyte area as determined by image analysis techniques in MR dogs at 6 month. We saw a similar, slightly larger increase in ALT-711 treated dogs.

Histochemical Studies: Intercellular Fibrosis

In the left atrial appendage there was a significant increase in fibrosis content as a percentage of total tissue at 6months. In Alt-711 treated MR dogs, however the fibrosis % did not increase.

We observed no significant increase in fibrosis in left atrial free wall (LFW) in the MR group, however we did see a significant decrease in fibrosis in the Alt-711 treated MR dogs as compared to control (~22% drop).

Histochemical Studies: β-tubulin

There was trend towards higher prevalence of microtubules as seen by total β tubulin content at 6 months in the LFW, and a significant increase in β tubulin content in the left atrial appendage, as shown in the figure. In both sites however, ALT-711 treatment did not cause any significant changes in total β tubulin content.
Histochemical Studies: AGE

There was no evidence of increased AGE production in the MR dogs as measured by IOD analysis. There was however a significant decrease in AGE content in the Alt-711 treated MR dogs, only in the left atrial free wall.
DISCUSSION

AF is the most common arrhythmia in the United States and effects about 1% of the population. Incidence of AF increases in both diabetes and aging populations\textsuperscript{6,8,10-13}. ALT-711 is a novel therapeutic agent, targeted against a chemical reaction, not a specific enzyme or receptor, and breaks down establish glucose crosslinking between structural protein, mainly collagen and elastin that occur in diabetes, aging, hypertension and oxidative conditions. Increased fibrosis (collagen) is often seen in AF has been hypothesized to be involved in development of substrate for maintenance of AF.

ALT-711 has been shown to be effective in cardiovascular diseases associated with aging and oxidative stress. Therefore we tested the effect of ALT-711 in a relevant model of Mitral regurgitance induced AF, that we have previously documented (see chapter 7). MR was surgically induced in 12 dogs and after 6 months of MR, \( n = 6 \) dogs received one dose of ALT-711. Age matched sham surgical controls were also incorporated into the study design and received identical handling. 6 weeks after treatment with ALT-711, the dogs were sacrificed. Cells were isolated for electrophysiological measurements in the lab of Dr. Cynthia Carnes. Tissue samples from left atrial appendage and left atrial free wall were also isolated and immediately formalin fixed for immunohistochemistry. Electrophysiology on isolated cells showed no significant changes in the alteon-711 treated group as compared to either control or treatment naïve MR dogs. Since only a single dose of drug was utilized, it is possible that the drug did not reach an effective concentration in the myocardium and thus was unable to produce an effect. We therefore investigated the formalin
fixed tissues immunohistochemically to determine changes in cell structure or myocardial protein expression.

Cellular hypertrophy is a well described parameter in MR and AF. We saw an expected increase in the myocyte size as determined by tissue area/nucleus in the MR dogs as compared to control. ALT-711 treatment did not affect the hypertrophy induced by MR.

We then investigated extent of fibrosis. We found evidence of increased fibrosis in the left atrial appendage in the naïve MR dogs, and this increase in fibrosis appeared to have been reversed by ALT-711 treatment. Further, even though the left atrial free wall did not show evidence of increased total fibrosis content, ALT-711 treatment, resulted in a decrease from basal fibrosis in control sham treatment dogs. Accumulation of glucose crosslink on collagen, can result in increase stability and decreased turnover of collagen, and therefore increases in the total fibrotic tissue. By breaking established crosslinks, ALT-711 might facilitate the breakdown of collagen and thus produce the seen reduction in fibrosis. Thus it might be construed as evidence that ALT-711 did reach the myocardium in an effective concentration.

A recent field that is gaining attention in cardiac disease milieu is the intracellular scaffolding of myocytes, the cytoskeleton which along with providing structural support and compartmentalization is also the embedding matrix for ion channels. Changes in the cytoskeleton can therefore affect conduction, as well as changing the contractility of the cell by affecting cellular stiffness. Microtubules are a major component of cardiac myocyte cytoskeleton, consisting of hollow protein cylinders of α and β tubulin heterodimers, aligned along the longitudinal axis of the cardiac
myocyte and disruption of the myotubular structure has been linked to various pathological conditions, like ischemia, and proliferation has been linked to decreased myocardial contractility due to hypertrophy. We therefore carried out investigations of β tubulin total content, and observed a trend towards increase in the left free wall with significant increase in the left atrial appendage, with no change from naïve MR dogs seen in the ALT-711 treated animals. Cytoskeleton modification (or intracellular stiffness) in addition to interstitial fibrosis (extracellular stiffness) might be of value.

Since ALT-711 might also have anti-oxidant properties, we investigated the effect of ALT-711 treatment on content of protein 3NT a stable biomarker of oxidative stress. Chronic MR lead to increase in total 3NT content in the left atrial appendage, while the left free wall did not show significant change from baseline. However, treatment with ALT-711, did not result in decreased protein 3NT, and might even have increased 3NT formation in the Left free wall.

It is interesting to note that we saw regional differences in staining for the markers investigated. Fibrosis, Protein 3NT and β tubulin were all significantly increased in the left atrial appendage at 6 months, while free wall did not show similar differences. This could be because of more turbulent flow, and consequently higher sheer stress on the atrial appendage as compared to the free wall.

Finally we looked at total content of AGE in the tissue as a whole. While the left atrial appendage did not show any changes in AGE content, we did see a significant reduction in AGE formation in the free wall, suggesting again that ALT-711 did
achieve effective concentrations in the atrium, at least regionally. However, it must be noted that in-spite of regional evidence of increased oxidant stress and fibrosis, there was no significant increase in AGE formation in the MR dogs. Thus it appears that AGE formation and hence AGE related crosslink breakers may not be of therapeutic value in this disease state.

In summary, electrophysiological experiments failed to show a significant effect of ALT-711 on MR induced changes in Ito and Iksus. MR induced site specific increases in myocyte hypertrophy, interstitial fibrosis, myotubular content, and oxidant stress. However, there does not seem to be a corresponding increase in AGE formation. While ALT-711 succeeded in reducing fibrosis formation and also resulted in lower AGE staining, there was no effect on hypertrophy and oxidant stress markers. In conclusion, ALT-711 does not seem to be of major therapeutic value in this setting. Furthermore, decreases in fibrosis failed to reduce vulnerability to arrhythmia, suggesting that increased fibrosis is not enough to explain the development of arrhythmias in chronic atrial dilatation models.
REFERENCE:


FIGURE LEGENDS:

Figure B.1: Mitral regurgitation induced hypertrophy is not reduced by ALT-711. Hypertrophy is represented by tissue area/nucleus ($\mu^2$). Data shown for left free wall, p<0.05.

Figure B.2: ALT-711 treatment results in reduced fibrosis. Mitral regurgitation induced fibrosis increase in the left atrial appendage is prevented/reversed ALT-711. Significant reduction in fibrosis content in ALT-711 treated free wall. All comparisons to control, p<0.05.

Figure B.3: Mitral regurgitation induces intracellular structural changes, unaltered by ALT-711 treatment. All comparisons to control, p<0.05.

Figure B.4: Increased protein 3NT, biomarker of oxidant stress in MR and ALT-711 treated dogs. All comparisons to control, p<0.05.

Figure B.5: No significant increases in AGE in MR dogs, ALT-711 treated dogs show reduced AGE formation. All comparisons to control, p<0.05.
Figure B.1: Mitral regurgitation induced hypertrophy is not reduced by ALT-711
Figure B.2: ALT-711 treatment results in reduced fibrosis
Figure B.3: Mitral regurgitation induces intracellular structural changes, unaltered by ALT-711 treatment
Figure B.4: Increased protein 3NT, biomarker of oxidant stress in MR and ALT-711 treated dogs
Figure B.5: No significant increases in AGE in MR dogs, ALT-711 treated dogs show reduced AGE formation
New AIDS cases, deaths hold steady: CDC report. *Clin Infect Dis* 33:i, 2001  


Kanmogne GD, Kennedy RC, Grammas P: Analysis of human lung endothelial cells for susceptibility to HIV type 1 infection, coreceptor expression, and cytotoxicity of gp120 protein. AIDS Res Hum Retroviruses 17:45-53, 2001


Cantaluppi V, Biancone L, Boccellino M, Doublier S, Benelli R, Carlone S, Albini A, Camussi G: HIV type 1 Tat protein is a survival factor for Kaposi's sarcoma and endothelial cells. AIDS Res Hum Retroviruses 17:965-976, 2001


Barillari G, Gendelman R, Gallo RC, Ensoli B: The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence. Proc Natl Acad Sci U S A 90:7941-7945, 1993


Dhawan S, Puri RK, Kumar A, Duplan H, Masson JM, Aggarwal BB: Human immunodeficiency virus-1-tat protein induces the cell surface expression of endothelial leukocyte adhesion molecule-1, vascular cell adhesion molecule-1, and


Cioni C, Annunziata P: Circulating gp120 alters the blood-brain barrier permeability in HIV-1 gp120 transgenic mice. *Neurosci Lett* 330:299-301, 2002

Ren Z, Yao Q, Chen C: HIV-1 envelope glycoprotein 120 increases intercellular adhesion molecule-1 expression by human endothelial cells. *Lab Invest* 82:245-255, 2002


Fantuzzi L, Canini I, Belardelli F, Gessani S: HIV-1 gp120 stimulates the production of beta-chemokines in human peripheral blood monocytes through a CD4-independent mechanism. *J Immunol* 166:5381-5387, 2001


Mallery SR, Bailer RT, Hohl CM, Ng-Bautista CL, Ness GM, Livingston BE, Hout BL, Stephens RE, Brierley GP: Cultured AIDS-related Kaposi's sarcoma (AIDS-KS)


Nolan D, Hammond E, James I, McKinnon E, Mallal S: Contribution of nucleoside-analogue reverse transcriptase inhibitor therapy to lipoatrophy from the population to the cellular level. *Antivir Ther* 8:617-626, 2003


Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, Mann D, Sidhu GD, Stahl RE, Zolla-Pazner S, Leibowitch J, Popovic M:


Roldan EO, Moskowitz L, Hensley GT: Pathology of the heart in acquired immunodeficiency syndrome. *Arch Pathol Lab Med* 111:943-946, 1987


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de Larrañaga GF, Petroni A, Deluchi G, Alonso BS, Benetucci JA: Viral load and disease progression as responsible for endothelial activation and/or injury in human immunodeficiency virus-1-infected patients. *Blood Coagul Fibrinolysis* 14:15-18, 2003


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