Congenital Absence of NOS3 Potentiates Left Ventricular Dysfunction in a Murine Model of Diet Induced Obesity and Chronic Pressure Overload

by

Roien Ahmadie

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

Master of Science

Department of Physiology

Faculty of Medicine

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Of

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This thesis is dedicated to my family.

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ABBREVIATIONS

ATP: Adenosine triphosphate

BH4: Tetrahydrobiopterin

CAD: Coronary artery disease

DBP: Diastolic blood pressure

GCH: Guanylate cyclo-hydrolase

GTP: Guanylate triphosphate

Hi-FGF-2: High molecular weight fibroblast growth factor-2

HLD: High lipid diet

L-NMMA: N^G-monomethyl-L-arginin

LLD: Low lipid diet

LVH: Left ventricular hypertrophy

LV: Left ventricle

MI: Myocardial ischemia

NADPH: Nicotinamide adenine dinucleotide phosphate

NOS1: Nitric oxide synthase-1

NOS2: Nitric oxide synthase-2

NOS3: Nitric oxide synthase-3

$NOS3^{-/-}$: Nitric oxide synthase-3 knockout

NO: Nitric oxide

Pla2g1b: Group 1B phospholipase A_2

ROS: Reactive oxygen species

SBP: Systolic blood pressure

TAC: Transverse aortic constriction

TC: Total cholesterol

TG: Triglyceride

ABSTRACT

A high lipid diet (HLD) is causal to the induction of cardiomyocyte hypertrophy, fibrosis, insulin resistance and hyperlipidemia leading to adverse left ventricular (LV) remodeling and endothelial dysfunction, which may be exacerbated in conditions of hemodynamic stress such as chronic pressure overload. Although nitric oxide synthase 3 (NOS3) plays a major protective role in LV remodeling after transverse aortic constriction (TAC), the nature of interaction between a HLD and NOS3 in a chronic heart failure model of hypertension remains undefined.

Wild type C57Bl/6 (WT) and nitric oxide synthase-3 knockout mice ($NOS3^{-/-}$) were randomized into four groups: **a)** WT + low lipid diet (LLD); **b)** WT + HLD; **c)** $NOS3^{-/-}$ + LLD; and **d)** $NOS3^{-/-}$ + HLD for a total of three months. After one week of randomization to either diet, TAC was performed on all groups. Echocardiograms were obtained at baseline and monthly thereafter. Fasting lipid profiles were evaluated at baseline and at the end of the study. At month 3, the hearts were removed for histopathological and Western blot analyses.

Echocardiography revealed a decrease in left ventricular ejection fraction (LVEF) in WT and $NOS3^{-/-}$ mice fed a HLD compared to a LLD fed mice. However, $NOS3^{-/-}$ mice demonstrated a further reduction in LVEF compared to WT mice at 12 weeks post TAC (51±3% vs. 44±3% $P<0.05$). There was increased myocyte hypertophy and perivascular fibrosis in $NOS3^{-/-}$ mice fed HLD 12 weeks as compared to the other groups. Percentage change in triglyceride levels in $NOS3^{-/-}$ mice was increased at week 12 as compared to WT mice. High molecular weight

FGF-2 (hi-FGF-2), a marker of cardiac hypertrophy, was upregulated in $NOS3^{-/-}$ mice fed a HLD compared to WT mice.

In our model of chronic pressure overload state and diet induced obesity, $NOS3^{-/-}$ mice demonstrated greater coronary intimal thickening, LV hypertrophy, fibrosis and LV systolic dysfunction as compared to WT mice.

INTRODUCTION

In Canada, cardiovascular disease (CVD) is the leading cause of increased patient morbidity and mortality.[1] Cardiovascular disease is a major burden on the Canadian economy, with a total direct cost of CDN$ 6.8 billion and an indirect cost of CDN$ 11.6 billion in 1998.[2] Recent data indicates that 72, 743 deaths, 447,218 hospitalizations, and 3,885,588 patient days in hospital occur each year as a result of cardiovascular illness in Canada.[3]

Major risk factors for the development of CVD include smoking, high blood pressure, obesity, diabetes, elevated lipids, family history of premature CVD, and physical inactivity. Obesity is an increasing public health concern associated with increased patient morbidity and mortality.[4] As the leading modifiable risk factor for the development of premature CVD, obese individuals are at increased risk of developing hypertension, dyslipidemia, and impaired glucose tolerance.[5] The synergistic effects of a high lipid diet (HLD) and chronic pressure overload due to hypertension may lead to adverse left ventricular (LV) remodeling in obese individuals, with subsequent development of heart failure.[6]

Despite the increasing prevalence of heart failure, many of the mechanisms associated with its pathophysiologic development remain undetermined. One area holding promise is the role of nitric oxide (NO) in heart failure. Nitric oxide plays a cardioprotective role in left ventricular (LV) remodeling, through its endothelial dependent vasodilation and antiatherogenesis effects.[7] Nitric oxide induces angiogenesis, decreases interstitial fibrosis, and reduces cardiomyocyte

hypertrophy, all of which may have a beneficial role in LV remodeling. Nitric oxide is produced from the conversion of L-arginine to L-citrulline by three separate isoform enzymes, NOS1 (neuronal NOS), NOS2 (inducible NOS) and NOS3 (endothelial NOS).[8] As NOS3 is constitutively expressed both in cardiac tissue and the vascular endothelium,[9] this isoform may play an important cardiovascular role in the setting of diet induced obesity.

A growing body of evidence suggests that NOS3 plays a major protective role in LV remodeling after pressure overload induced by transverse aortic constriction (TAC).[8,10,11] Previous studies have demonstrated accentuated LV systolic dysfunction and cardiac fibrosis in systemic NOS3 deficient ($NOS3^{-/-}$) mice as compared to wild-type mice in a murine model of chronic pressure overload induced by TAC.[8,10,12] The nature of interaction between a HLD and chronic pressure overload in $NOS3^{-/-}$ mice however remains ill defined.

The aim of the study is to determine whether congenital absence of NOS3 potentiates cardiac dysfunction in a murine model of diet induced obesity and chronic pressure overload. We compared the LV remodeling response to TAC in C57Bl/6 wild type mice and $NOS3^{-/-}$ mice fed either a low lipid diet (LLD) or a HLD respectively. We report that $NOS3^{-/-}$ mice fed a HLD following TAC demonstrated greater LV hypertrophy, fibrosis, and systolic dysfunction as compared to wild-type mice.

LITERATURE REVIEW

1.1 Cardiovascular disease

Cardiovascular disease includes a number of conditions including coronary artery disease, stroke, high blood pressure and congestive heart failure.[13] Hypertension is a major modifiable risk factor of CVD. Reduction of blood pressure prevents vascular disease as well as stroke and heart failure. Discoveries in improving management of patients with chronic hypertension will improve quality of life (QOL) and reduce overall mortality and morbidity.[14]

1.2 Obesity and cardiovascular disease

Obesity is a growing epidemic around the world. According to the 2004 survey of the Canadian Community Healthy Survey (CCHS), 23% of Canadians above the age of 18 were obese, far greater than the 1978/1979 survey of 14%.[15] Obesity is associated with a number of complications including premature coronary artery disease (CAD), diabetes, hypertension, dyslipidemia and heart failure.[16-17,18] Obesity is classified using the body mass index (BMI) that is calculated by dividing the weight in kilograms with the height squared in meters. An individual with a BMI equal to or greater than 30 kg/m^2 is considered to be obese.[19]

Abdominal obesity has adverse effects on the hemodynamic characteristics of the heart.[20] First, it causes an incremental increase in total blood volume and cardiac output.[21] This is due to an increase in body adipose tissue that requires higher circulation at any given time due to changes in metabolic demand. Thus, as compared to lean individuals, obese subjects have a higher cardiac workload.

Furthermore, an increase in cardiac output in obese subjects is due to increased stroke volume.[21] This further shifts the cardiac hemodynamic profile to the left of the Frank-Starling curve due to an incremental increase in left ventricle filling pressures. An increased left ventricular (LV) filling pressure initially leads to concentric LV hypertrophy.[21] Over time, concentric LV hypertrophy can no longer compensate for the increased afterload, leading to LV dilatation and symptoms and signs of heart failure.[22]

Apart from having a direct effect on adverse LV remodeling, obesity is also strongly associated with hypertension.[18] Hypertension is one of the major risk factors for CAD and subsequent development of heart failure.[23] With every increase in body weight by 10 kg body weight, there is a 3 mmHg increase in systolic pressure and a 2 mmHg increase in diastolic pressure.[21] According to the National Health Institute, these incremental increases in systolic and diastolic pressures correspond to a 12% increase risk of CAD and 24% increased risk of stroke. Furthermore, data from Framingham Heart Study show a strong correlation between obesity and prevalence of hypertension, diabetes mellitus and CAD.[19,24,25]

The high prevalence of hypertension in obese individuals can be partially attributed to the development of insulin resistance and an increase in the concentration of free fatty acids.[26] Free fatty acids increase the production of NADPH oxidase which in turn increases reactive oxygen species (ROS) that reacts with the nitric oxide synthase-3 (NOS3) enzyme.[27-28] This decreases the bioavailability of nitric oxide (NO), a potent vasodilator that reduces hypertension.

Moreover, insulin acts as an agonist for NOS3 and increases the production of NO through a different pathway.[29]

1.3 High fat diet induces LV systolic dysfunction

In obese and hyperlipidemic individuals, the heart can change in size and shape, a process called ventricular remodeling.[21] These changes can occur due to a number of different stimuli including metabolic dysfunction. Under normal basal conditions, the heart uses free fatty acids (FFA) to produce 70-80% of their energy in the form of adenosine triphosphate (ATP).[30] However, in conditions of hyperlipidemia, the ratio between energy uptake and expenditure can change drastically. Fatty acids are stored inside the cell as triglyceride (TG) molecules ready to be released in the blood in response to metabolic demands. Under increased fat intake, an excess of TG molecules are stored inside the non-adipocyte cells leading to lipotoxicity.[31,32] Furthermore, increased uptake and storage of fat inside the cell may interfere with the normal metabolic function of cardiomyocytes leading to lipotoxicity and apoptosis. [32,33]

Mice fed a high fat diet can develop, cardiac hypertrophy, myocardial contractile dysfunction, and impaired intracellular Ca^{2+} handling.[34-37] Recent studies have demonstrated that mice fed a high fat diet, even for a short duration of 9 days after TAC and followed for 28 days, showed significant cardiac remodeling, insulin resistance, and impaired tolerance to glucose as compared to mice fed a standard diet.[34] This study demonstrated that even a short duration of high fat diet and its associated metabolic disorders significantly increases the impact of chronic LV

pressure overload, accelerates adverse LV remodeling and progression to heart failure.

It is evident from previous data that a high fat diet in murine models does increase the levels of cholesterol and TG along with an increase in fasting glucose concentration.[38, 33] Accumulation of TG in the heart does lead to an imbalance in uptake and expenditure of fatty acids in the cardiomyocytes.[39] An explanation of the deleterious effect of intramyocardial lipid accumulation is that an overload of TG and lipid may cause some changes in the expression of lipid oxidizing proteins. This will produce oxygen radicals or their intermediates that could be cytotoxic. Also, in the clinical arena, patients with congenital lipodystrophy, where they lack adipocytes to store fat, have increased accumulation of lipid in non-adipocyte tissues, such as cardiomyocytes, that can lead to apoptosis and premature cardiomyopathy. Therefore, an overload of lipid within cardiomyocytes, whether genetic or environmental, is deleterious.[33,35,40]

1.4 Nitric oxide and the cardiovascular system

Under conditions of hyperlipidemia, in cardiomyocytes, several lipid oxidizing enzymes can react with nitric oxide (NO) decreasing its bioavailability.[31, 41, 42] Also, in hypertension, hypercholesterolemia, atherosclerosis, diabetes, obesity and aging, the amount of bioavailable NO generated by the endothelial cells is decreased.[28] Nitric oxide is an important vasodilator that can reduce cardiomyocyte hypertrophy, adhesion of leukocytes on the endothelial surface, proliferation and migration of vascular smooth muscle cells and cardiac preload and afterload.[43] Furthermore, it also decreases extra-cellular matrix deposition and

suppresses platelet aggregation.[8,11,10,44-46] Although a number of studies have shown that NO protects against adverse LV remodeling during pressure overload alone the precise biological interaction between NO and diet induced obesity in a state of chronic pressure overload is not well understood.[8,10,47]

All of the major treatments for cardiovascular diseases target either the cardiomyocytes or the vascular tissue. Nitric oxide, a potent vasodilator of the vascular tissue is produced by three nitric oxide synthase (NOS) isoforms: NOS1 (neuronal NOS), NOS2 (inducible NOS) and NOS3 (endothelial NOS).[48-50] Nitric oxide synthase-3 is mainly produced in the cardiomyocytes and endothelial cells lining the vascular tissue. The NOS3 gene was cloned in 1993 and localized to chromosome 7q35-36.[51] Spanning 4.4 Kb of genomic DNA, this gene comprises of 26 exons that encode a 133-KDa protein containing 1,203 amino acids.[51-53] Nitric oxide synthase-3 (NOS3) constitutively produces nitric oxide that is a radical molecule comprising of only two atoms. It confers its vasodilatory effect via the cyclic-guanylate monophosphate (cGMP) pathway.[54] Soluble guanylate cyclase contains an iron containing protein called protoporphyrin IX that binds NO with great affinity.[54] Nitric oxide is a hydrophobic molecule and readily diffuses across the membrane and activates guanylate cyclase enzyme to produce cGMP. In turn cGMP activates cGMP dependent kinases in the target tissue that modulates intracellular calcium levels, which, in turn, controls many cellular activities in the target tissue (Figure 1).[55-57]

The role played by NOS3 in cardiac remodeling during chronic mechanical constraints such as chronic pressure overload is equally important as it directly

affects the basic contractile unit of the heart, the cardiomyocyte.[8,11,10,45,54] This is evident from experiments where either the NOS3 gene has been genetically deleted or over-expressed, in mice and subjected to aortic or abdominal constriction.[11,8,58] The initial compensatory response to increased afterload is concentric hypertrophy of LV, but if the causal stress persists chronically, the LV dilates leading to the development of congestive heart failure.[59] Changes in LV mass, size and geometry which define LV remodeling are major prognostic factors in patients with essential hypertension.[59,60] Although the mechanism behind NOS3 attenuation of cardiac hypertrophy is not very well known, mounting evidence points towards a reduction in the pro-hypertrophic stimuli and molecules, in particular fibroblast growth factor-2 (FGF-2).[61-64]

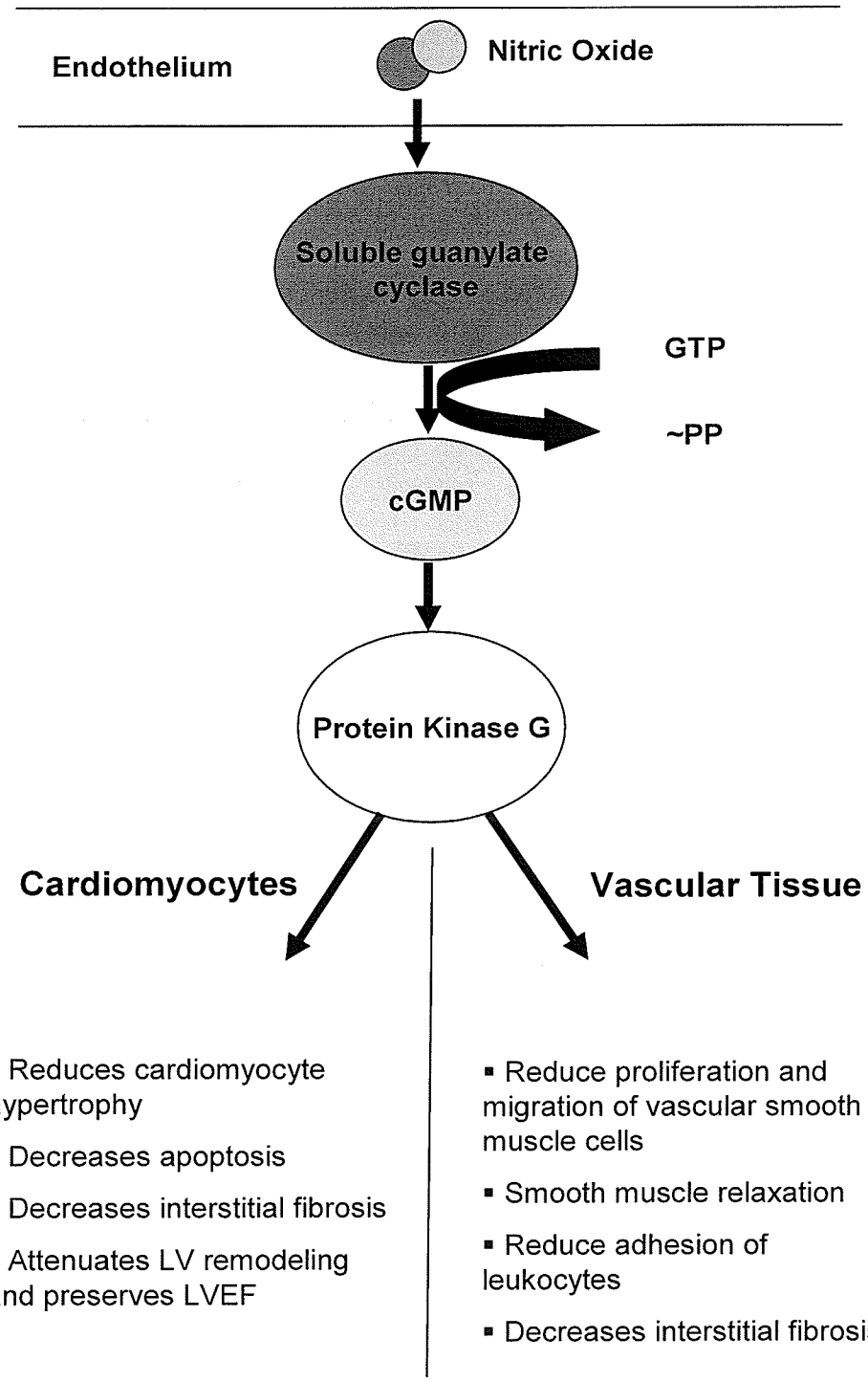


Figure 1: The effect of nitric oxide (NO) effect on cardiomyocytes and vascular tissue. In cardiomyocytes, NO attenuates hypertrophy, apoptosis, fibrosis while in vascular smooth muscle cells NO reduce proliferation, adhesion of leukocytes and interstitial fibrosis.

In recent years, viable and fertile mice knockouts of each NOS isoform have been generated through gene targeting procedures.[65-71] Their phenotypes reflect the importance of each isoform in physiologic and pathologic processes. These knockout mice serve as models of NOS deficiency and make it possible to study the importance of each one of them in healthy as well as diseased state. Of these isoforms, NOS3 stimulates angiogenesis, while reducing cardiac preload and afterload, cardiomyocyte hypertrophy, and extracellular matrix production by cardiac fibroblasts.

It has been shown that there is a link between decreased NO production and insulin resistance in murine models as well as in human subjects.[72,73] It has also been demonstrated that insulin mediates production of the NO radical.[33] Inhibition of NOS3 activity by N^G -monomethyl-L-arginin (L-NMMA) induces hypertension and insulin resistance in rats. Previous studies demonstrated that $NOS3^{-/-}$ mice have a higher concentration of fasting insulin as compared to wild type. To further examine whether insulin resistance was due to hypertension, they measured insulin resistance in a mouse model of renovascular hypertension. These mice were found to be equally hypertensive as $NOS3^{-/-}$, but they had normal insulin stimulated glucose uptake.[38,74,75] These findings indicate that in $NOS3^{-/-}$ mice, metabolic insulin resistance is not related to hypertension but rather to impaired NO synthesis.

Nitric oxide is an important signaling molecule in atherosclerosis as well. This is evident from the apoE-KO/$NOS3^{-/-}$ mice where the formation of atherosclerotic lesions were accelerated proving the beneficial role of NO in atherosclerosis. In

infracted and heart failure mice models *Jones et al* showed that mice over expressing NOS3 had reduced mortality and improved cardiac function as compared to wild types.[76]

1.5 Endothelial nitric oxide synthase and vascular diseases

Endothelial dysfunction has been referred to as several pathological conditions that affect the endothelium of vascular tissues, including altered anti-coagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling.[77]

The roughly 10^{14} endothelial cells of our vasculature protect us against atherosclerosis and thrombosis.[28] Endothelial Nitric Oxide synthase (NOS3) produces a vasoactive molecule, nitric oxide (NO). Nitric oxide released toward the vascular lumen is a potent inhibitor of platelet aggregation and adhesion. Nitric oxide also can inhibit leukocyte adhesion to the vessel wall, proliferation of smooth muscle cells and prevents intimal thickening.[41] As such it attenuates atherosclerosis, and fibrous plaque formation. Nitric oxide represents the most important anti-atherogenic defense principle in the vasculature. Risk factors such as hyperlipidemia, hypercholesterolemia, diabetes and others lead to excess production of superoxide O^{2-}.[41] A possible explanation for this could be the relative deficiency of the cofactor BH4 causing NOS3 uncoupling where the enzyme reduction of molecular oxygen is no longer coupled to L-arginine oxidation, resulting in production of superoxide rather than NO. In turn superoxide reacts further with NO to form peroxynitirite, and vasclular protection slowly vanishes.[69]

NOS isoenzyme is widely expressed in endothelial cells throughout the vascular bed and shear stress is a major regulator of endothelial NO formation.[78]

In a double knockout model of NOS3 and apo-E deficient mice, athersclerosis is increased suggesting a protective effect of NOS3 derived NO.[79] *Takaya et al* previously have shown that over expression of NOS3 in apo-E-KO mice fed a high fat diet increased atherosclerosis, superoxide generation and NOS3 uncoupling.[41] Furthermore, augmentation of BH4 production by over expressing GTP Cyclohydrolase I (GCH) enzyme led to reduced NOS3 uncoupling, reduced atherosclerosis and superoxide production as compared to controls and NOS3 over expressing mice.[41, 55]

1.5 Pressure overload, LV remodeling and heart failure

Sustained pressure overload stimulates pathological cardiac hypertrophy and dysfunction. At first compensatory hypertrophy occurs which will counteract a reduction in cardiac output. However, if the hemodynamic stress persists for a longer period of time the left ventricle (LV) dilates, leading to congestive heart failure.[22] Although this is beneficial at first, it may cause reduction in LV chamber distensibility and increase in elastic stiffness.[22] Left ventricular remodeling in response to pressure overload includes progressive cardiac myocyte contractile dysfunction, altered cardiac fibroblast function and extensive extracelluar matrix (ECM) deposition.[80]

In mice, chronic pressure overload is achieved by transverse aortic constriction (TAC). [11,10] This constriction imparts a pressure of 25-30 mmHg across the aorta,

which further increases the pressure within the LV chamber. As such aortic pressure, representing LV afterload, increases leading to greater LV filling pressure during diastole.[81] As early as 4 weeks post TAC, the LV chamber can no longer compensate for increased LV pressure and increased afterload, leading to LV failure.[10,46]

In previous experiments where TAC has been performed on mice, the LV has dilated as early as 4 weeks after the surgery, which was attenuated by administration of tetrahydrobiopten (BH-4), a cofactor of NOS3 enzyme that helps in preventing uncoupling of NOS3 dimerization.[77] Six weeks after abdominal aortic constriction, compared to wild type, $NOS3^{-/-}$ mice are shown to have increased anterior wall thickness, fibrosis and myocyte hypertrophy. Data from recent studies indicate that constriction of either the thoracic or abdominal aorta caused greater LV dysfunction in $NOS3^{-/-}$ mice as compared to wild type mice, indicating the importance of NO in attenuating the deleterious effects of chronic pressure overload.[11,10] It has also been shown that cardiomyocyte restricted restoration of nitric oxide synthase-3 attenuates left ventricular remodeling after chronic pressure overload, thus proving that NO protects against adverse LV remodeling. Furthermore, *Buys and colleagues* restored the NOS3 gene in cardiomyocytes of systemic $NOS3^{-/-}$ mice and found significantly less LV fibrosis and remodeling as compared to $NOS3^{-/-}$ mice, 4 weeks after TAC.[8] They also found that NOS3-transgenic mice, where the NOS3 gene is over expressed in cardiomyocytes, had preserved cardiac function as compared to $NOS3^{-/-}$ mice post TAC. Both hyperlipidemia and altered NO production have been implicated in the

pathogenesis of ventricular remodeling. In the current study we have explored the effects of a high-lipid diet (HLD) on cardiac response to pressure induced hemodynamic stress in $NOS3^{-/-}$ mice.

1.6 Nitric oxide and clinical studies

In recent years, clinical studies have evaluated the benefits and limitation of NO in CVD. Inhaled NO has been used in the clinical management of patients with pulmonary hypertension post coronary artery bypass grafting, left ventricular dysfunction in patients with cardiogenic shock, right ventricular dysfunction in patients with or without a left ventricular assist system (LVAS), and congenital heart disease with improved patient morbidity and mortality.[82-87] Additionally, nitrates are beneficial in the clinical entities of ischemic heart disease and end stage heart failure.[88]

However, little is known whether NO may be beneficial in treating LV ventricular dysfunction due to hypertension and diet induced obesity.

VI. Hypothesis

Chronic hypertension is a major cause of left ventricular dysfunction, cardiomyocyte hypertrophy, and apoptosis in mice the lacking NOS3 gene.[8] Separately, mice fed a high lipid diet (HLD) show increased LV hypertrophy, fibrosis and LV systolic dysfunction.[34] However, the combined effects of chronic pressure overload and diet induced obesity remains ill defined. Our experiment is designed to evaluate whether congenital absence of NOS3 ($NOS3^{-/-}$) will exacerbate adverse LV remodeling in a murine model of diet induced obesity and chronic pressure overload.

Objectives

- To determine whether congenital absence of NOS3 ($NOS3^{-/-}$) leads to greater LV systolic dysfunction in a state of chronic pressure overload and diet induced obesity. In vivo LV morphology and systolic function will be evaluated in wild type C57Bl/6 mice and $NOS3^{-/-}$ mice fed a low lipid diet (LLD) or a high lipid diet (HLD) respectively.

- To characterize the phenotypic difference between wild type C57Bl/6 mice and $NOS3^{-/-}$ mice fed either diet post transverse aortic constriction, we will evaluate lipid profiles, degree of LV hypertrophy and fibrosis, and the expression of hi-FGF-2.

Methods

2.1 Experimental Model

The experimental protocol was approved by the University of Manitoba protocol management and review committee in accordance to guidelines set forth by the Canadian Council on Animal Care. Only male C57BL/6 and NOS3$^{-/-}$ mice were studied. The mice were caged on their arrival in the R.O Burrell Animal Laboratory at St.Boniface Research Centre for a period of one week for acclimatization before the actual experiment was begun. The mice had free access to chow and water during this time period (Figure 1).

A total of 110 male mice (55 wild type C57Bl/6 and 55 NOS3$^{-/-}$, aged 6-8 weeks; weight 22-24g; Jackson Laboratories, Bar Harbor, ME) were fed either a low lipid diet (LLD: 10% kcal from fat – D12450B, Research Diets New Brunswick, NJ) or a high lipid diet (HLD: 60% kcal from fat – D12492, Research Diets New Brunswick, NJ) one week before TAC, The animals were divided into four groups **a) C57BL/6 + LLD (n = 32)**, **b) C57BL/6 + HLD (n = 33)**, **c) NOS3$^{-/-}$ + LLD (n = 33)**, **d) NOS3$^{-/-}$ + HLD (n = 32)**. One week after randomization into each diet, the animals underwent TAC as described below. Following TAC, the diets were continued for a total of 12 weeks (Figure 2).

A	Echocardiography measurement
B	Body weight measurement
C	Blood collection
D	Transverse aortic constriction
E	Randomized to receive either LLD or HLD
F	Euthanasia, storage of tissues and biochemical analysis

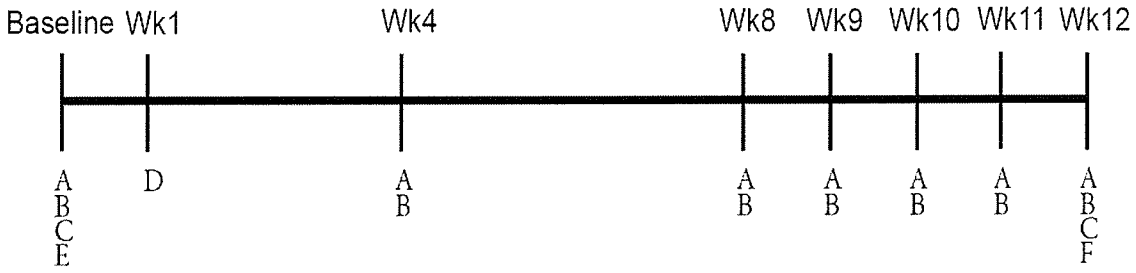


Figure 2: Timeline of different methods and procedures performed over a 12 week follow up of wild type and NOS3$^{-/-}$ mice.

2.2 Fasting Blood Collection

One week prior to transverse aortic constriction (TAC) and just prior to sacrifice, the animals were fasted for a 12 hour period, and blood was collected from the jugular vein using a 1cc heparinized syringe. The blood was collected in 1.5ml eppendorf test tubes and stored on ice before centrifugation at 5000g. The plasma was then transferred to 0.5 ml test tubes and stored at -80°C for biochemical analysis of triglyceride (TG), total cholesterol (TC) and glucose concentrations.

2.3 Transverse aortic constriction

Wild type and $NOS3^{-/-}$ mice were fed either a LLD or HLD for one week after which TAC was performed. The mice were first anesthetized with isoflurane 3% and the chest hair was shaved. Lubricating eye ointment was placed in each eye to prevent them from drying followed by intubation using a 20 gauge catheter and attached to a volume-controlled ventilator with a constant rate of 130 breaths per minute. A midline skin incision using surgical scissors was made from the mid neck region overlying salivary glands to the fourth rib. The transverse aortic arch was located between the right innominate and left common carotid artery and the overlying connective tissue was dissected. A 7-0 silk suture was passed underneath the aorta and both the aorta and a blunted 27 gauge needle were ligated. Once the tie was in place, the needle was removed followed by retractors. The chest and skin was closed with a simple interrupted pattern using 5-0 silk. The mouse was recovered, extubated and moved to a warm oxygenated incubator. They were then given their first buprenorphine after surgery and then put at room temperature overnight.

2.4 Murine echocardiography

All mice underwent baseline echocardiography with tissue Doppler imaging (TDI) and were serially followed at weeks 4, 8, and 12 week post-TAC.[46] Murine echocardiography was performed using a 13-Mhz probe (Vivid 7, GE Medical Systems, Milwaukee, WI) on awake mice. Hearts were imaged in the 2D parasternal short axis view and LV end-diastolic diameter ($LVID_{ED}$), LV end-systolic diameter ($LVID_{ES}$), posterior end diastolic wall thickness (PWT), and LV fractional shortening (FS) were acquired from M mode measurements. The LV end-systolic volumes (LVESV) and diastolic volumes (LVEDV) were measured from a parasternal long axis view using the prolate ellipsoid geometric model and the LV ejection fraction (EF) was calculated.

Tissue Doppler imaging (TDI) was acquired on a parasternal short axis view at the level of the papillary muscles, at a rate of 483 frames per second. For peak systolic endocardial velocity (V_{ENDO}), a region of interest (0.2 X 0.2mm) in the posterior wall was analyzed. Radial strain rate (SR) was measured over a distance of 0.6 mm (Echopac PC, GE medical, Milwaukee, WI). The temporal smoothing filters were turned off for all measurements. The values obtained in 5 consecutive cardiac cycles were averaged.

2.5 Histology

Following 12 weeks of either a LLD or HLD after TAC, the mice were euthanized using CO_2 inhalation. The heart was removed at sacrifice and fixed in 10% buffered formalin followed by paraffin embedding for histological evaluation. Sections were cut and stained with hematoxylin and eosin (H&E) for histological

examination and myocyte cross-sectional diameter measurement. Masson's trichrome was used to determine the degree of perivascular and interstitial fibrosis. Pathological observations regarding myocardial remodeling, fibrosis, vascular structure and cardiomyocyte size were made by a pathologist blinded to the study (In collaboration with Dr.Moghadasian's Pathology Lab, CCARM, SBRC, Winnipeg, MB, Canada).

2.6 Western Blot Analysis

The left ventricle was excised from wild type and $NOS3^{-/-}$ frozen hearts and grinded in liquid nitrogen. For the total cell lysates, the samples were mixed in a 1:5 ratio (v/v) with 5x SDS loading buffer (10% SDS, 50% glycerol, 0.5 M DTT, 300 mM Tris-HCl pH 6.8, 0.005% bromo phenol blue). The samples (50-100 µg of proteins) were boiled for 5 minutes before loading onto a 15% SDS-PAGE gel. Heparin-Sepharose-bound proteins were eluted by boiling directly in 3x SDS and then loaded onto the gel. Broad-range molecular weight standards and pre-stained molecular weight markers were also loaded in the gel.

Proteins were seperated electrophoretically transferred onto a 0.45 µM PVDF membrane at 200 V for 1 hour using a buffer containing 20% methanol, 192 mM glycine, and 25 mM Tris base. Membranes were stored in a Tris-Buffered Saline (10 mM Tris-HCl pH 7.6 or 8.0 and 150 mM NaCl) with 0.1% Tween-20 (TBS-T). Before probing, blots were checked for equal protein loading between lanes using Ponceau S stain. Also, the molecular weight standards were marked while the blots were stained with Ponceau S. The membranes were blocked in a TBS-T solution containing 10% dried non-fat (skim) milk powder for a period of 1 hour at

room temperature on an orbital shaker. The membranes were briefly rinsed with two changes of TBS-T. The blots were then incubated with primary antibody in 1% non-fat skim milk powder in TBS-T overnight at 4°C with constant agitation for one hour at room temperature. The antibodies used were diluted as follows: (a) monoclonal anti-FGF-2 antibody was used at 1:1000 dilution; (b) monoclonal anti-α-smooth muscle actin antibody was used between 1:500-1:1000 dilutions.

Membranes were briefly washed with two changes of 1% milk in TBS-T. The membranes were then washed again for 15 minutes once, followed by 3 washes for 5 minutes at room temperature on an orbital shaker. Secondary antibody, consisting of anti-mouse and anti-rabbit immunoglobulin conjugated to horseradish peroxidase (HRP), was then applied at 1:10000 dilutions in TBS-T containing 1% skim milk and incubated for 1 hour at room temperature on an orbital shaker. Following the administration of secondary antibody, the membranes were washed again following the same methods as described for the primary antibodies except the membranes were washed in TBS-T only. Protein bands on Western blots were visualized by enzyme chemiluminescence plus (ECL) according to the manufacturer's instructions, and developed on film. Exposures of film (Kodak) ranged from only a few seconds to over 20 minutes depending on the samples. The bands were normalized against α-smooth muscle actin signal and used to confirm even protein loading (In collaboration with Dr. Elissavet kardami's Muscle Cell Biochemistry Laboratory, SBRC, Winnipeg, MB, Canada).

2.7 Statistical Analysis

Repeated measures were analyzed by one way ANOVA and multiple comparisons were made among different groups using Tukey-HSD test. Probability values of ≤ 0.05 were considered significant.

Results

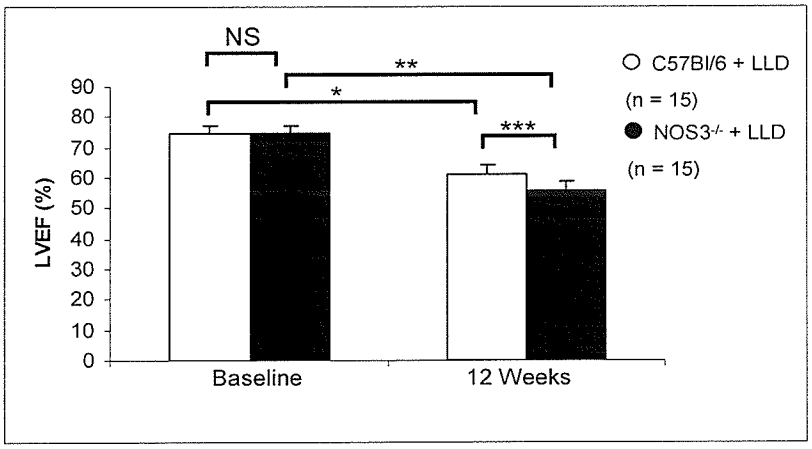
3.1 Murine echocardiography

At baseline, the LV dimensions and systolic function, as determined by both conventional parameters (fractional shortening (FS) and ejection fraction (EF)) and tissue Doppler imaging (TDI) indices (peak endocardial velocity (Vendo) and strain rate (SR)) were similar in WT and $NOS3^{-/-}$ mice (Table 1). Serial echocardiographic analysis of the $NOS3^{-/-}$ mice LV receiving either a LLD or HLD revealed progressive increase in LV cavity dimensions and a decrease in LVEF by week 12 post-TAC as compared to WT fed either diet (Table 1). At week 12 post-TAC, wild type mice fed a LLD vs. HLD demonstrated a LVEF of 61±3% and 51±3% respectively. Similarly, at 12 weeks post-TAC, $NOS3^{-/-}$ mice fed LLD vs HLD demonstrated a LVEF of 56±3% and 44±3% respectively (Figure 3). Additionally, $NOS3^{-/-}$ mice fed a HLD were found be have a lower LVEF, FS, Vendo and SR as compared to wild type mice fed a HLD at 12 weeks post TAC (Table 1). Representative M-mode echocardiograms of $NOS3^{-/-}$ mice fed a HLD at baseline and 12 weeks post-TAC are shown in Figure 4.

	Group	Baseline	4 weeks	8 weeks	12 weeks	P value
HR, bpm	WT+LLD	617±21	612±18	605±17	603±19	
	WT+HLD	612±19	602±25	625±15	611±19	
	$NOS3^{-/-}$+ LLD	621±12	618±24	611±19	632±11	
	$NOS3^{-/-}$+ HLD	631±11	613±19	617±16	618±11	NS
PWT, mm	WT+LLD	0.7±0.05	0.9±0.03*	1.0±0.03*	1.0±0.04*	
	WT+HLD	0.7±0.04	0.9±0.04*	1.0±0.05*	1.0±0.05*	
	$NOS3^{-/-}$+ LLD	0.7±0.03	0.9±0.02*	1.0±0.04*	1.0±0.02*	
	$NOS3^{-/-}$+ HLD	0.7±0.04	0.9±0.03*	1.0±0.02*	1.0±0.03*	NS
$LVID_{ED}$, mm	WT+LLD	3.0±0.3	3.2±0.2*	3.3±0.4*	3.3±0.3*	
	WT+HLD	3.0±0.2	3.2±0.2*	3.4±0.4*	3.5±0.2*	
	$NOS3^{-/-}$+ LLD	3.0±0.3	3.2±0.2*	3.4±0.4*	3.5±0.3*	
	$NOS3^{-/-}$+ HLD	3.0±0.2	3.3±0.2*	3.6±0.4*§	3.8±0.2*§	<0.05
EF, %	WT+LLD	75±2	66±3*	62±2*	61±3*	
	WT+HLD	76±3	67±2*	57±2*§	51±3*§	
	$NOS3^{-/-}$+ LLD	75±2	66±3*	58±2*§	56±3*§	
	$NOS3^{-/-}$+ HLD	76±3	67±2*	53±2*§	44±3*§	<0.05
Vendo, cm/s	WT+LLD	3.3±0.3	2.6±0.1*	2.4±0.3*	2.1±0.1*	
	WT+HLD	3.4±0.2	2.5±0.1*	2.1±0.2*§	1.6±0.3*§	
	$NOS3^{-/-}$+ LLD	3.3±0.3	2.5±0.1*	2.0±0.3*§	1.9±0.1*§	
	$NOS3^{-/-}$+ HLD	3.4±0.2	2.4±0.1*§	1.8±0.2*§	1.3±0.3*§	<0.05
SR, s^{-1}	WT+LLD	22.2±1	20.0±1*	19.2±1*	18.5±1*	
	WT+HLD	22.9±1	20.5±1*§	17.2±1*§	14.4±1*§	
	$NOS3^{-/-}$+ LLD	23.1±1	20.2±1*§	17.5±1*§	16.8±1*§	
	$NOS3^{-/-}$+ HLD	23.0±1	20.4±1*§	16.2±1*§	11.6±1*§	<0.05

Table 1: Echocardiographic parameters at baseline, 4, 8 and 12 weeks after TAC in wild-type C57Bl/6 and $NOS3^{-/-}$ mice fed a LLD or HLD respectively. Values are mean ± SEM. N= 15 for each group. HR, heart rate; WT, wild-type; LLD, low lipid diet; HLD, high lipid diet; PWT, posterior wall thickness; $LVID_{ED}$, left ventricular end-diastolic diameter; EF, ejection fraction; V_{endo}, peak endocardial systolic velocity; SR, strain rate; Absolute P values for the overall interaction of time and genotype are shown in the far right column. *$p<0.05$ vs. at baseline. §$p<0.05$ vs. WT+LLD at same time point.

A)



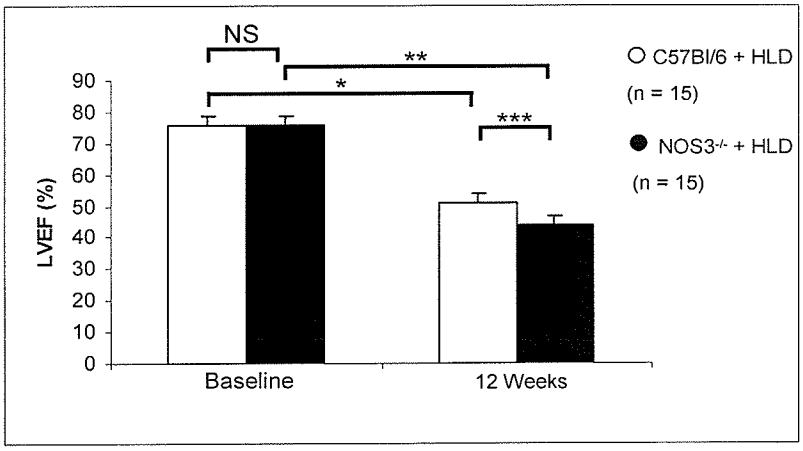
*P<0.05 as compared to C57Bl/6 + LLD at baseline

** P<0.05 as compared to NOS3$^{-/-}$ + LLD at baseline

***P<0.05 as compared to C57Bl/6 + LLD at 12 weeks

NS-Not significant

B)



*P<0.05 as compared to C57Bl/6 + HLD at baseline

** P<0.05 as compared to NOS3$^{-/-}$ + HLD at baseline

***P<0.05 as compared to C57Bl/6 + HLD at 12 weeks

NS-Not significant

FIGURE 3: A) Left ventricular ejection fraction (LVEF) in wild type and NOS3$^{-/-}$ mice fed LLD at baseline and 12 weeks post TAC. At baseline the LVEF was similar between both groups at 75±3%. At 12 weeks post TAC however, wild type and NOS3$^{-/-}$ mice fed LLD demonstrated a LVEFF of 61±3% and 56±3% resepectively ($p<0.05$) **B)** Left ventricular ejection fraction (LVEF) in wild type and NOS3$^{-/-}$ mice fed a HLD at baseline and 12 weeks post TAC. At baseline, the LVEF was similar between both groups at 75±3%. At week 12 post-TAC, wild type and NOS3$^{-/-}$ mice fed a HLD demonstrated a LVEF of 51±3% and 44±3% respectively ($p<0.05$) (Figure 2B).

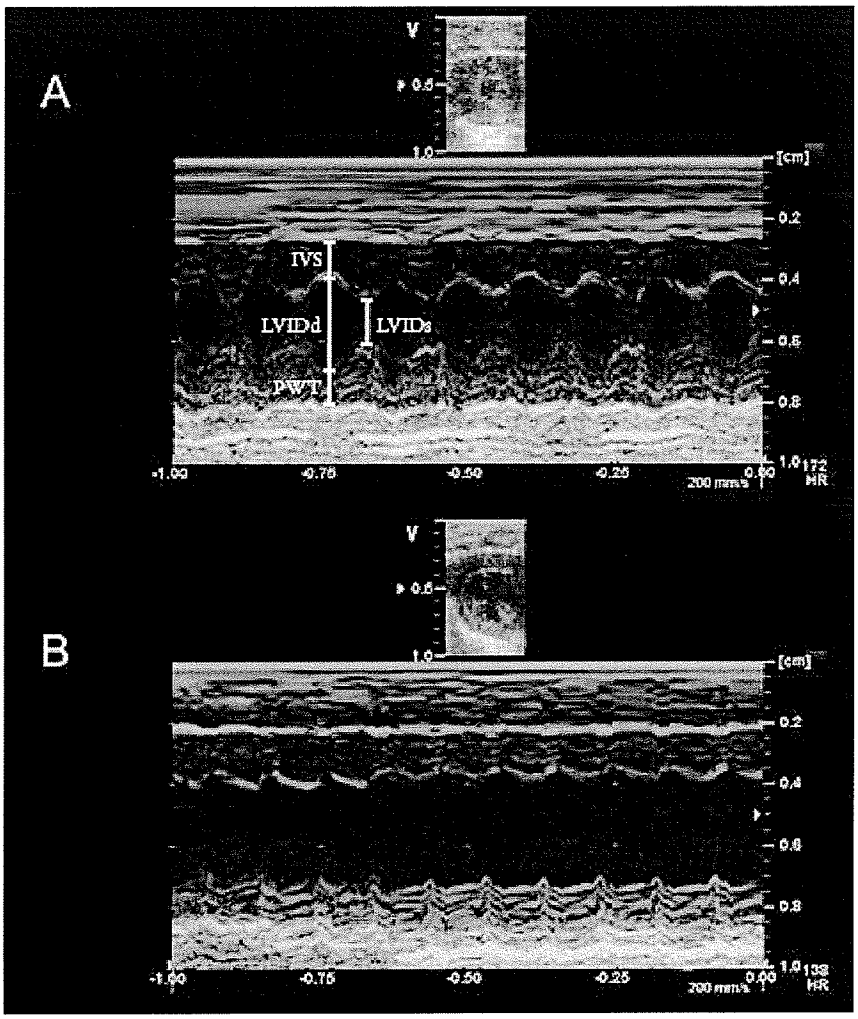


Figure 4: Representative M-mode echocardiograms of NOS3$^{-/-}$ mice fed a HLD at **A)** baseline and **B)** 12 weeks post-TAC. At baseline, the LV cavity dimensions are normal. At 12 weeks post TAC, the LV cavity is dilated with LV systolic dysfunction. IVS-Interventricular septum; LVIDd-Left ventricular internal diameter in diastole; PWT-Posterior wall thickness; LVIDs- Left ventricular internal diameter in systole.

3.2 Myocyte size and myocardial fibrosis

Cross-sections of myocardium from all four groups showed hypertrophy of cardiomyocytes at 12 weeks post TAC (Figure 5). Wild type and $NOS3^{-/-}$ mice fed a LLD, showed isolated cardiomyocyte cell hypertrophy whereas $NOS3^{-/-}$ fed a HLD showed aggregated patches of cardiomyocyte hypertrophy as compared to wild type mice fed a HLD.

In general, there was an increase in intimal thickening with smooth muscle proliferation into the intima of epicardial coronary arteries in $NOS3^{-/-}$ mice as compared to the wild type mice (Figure 6). This was further exacerbated in $NOS3^{-/-}$ fed a HLD as compared to the wild type mice fed a HLD. Additionally, irrespective of diet, there was increased perivascular fibrosis in mice lacking NOS3 gene in general as compared to wild type mice.

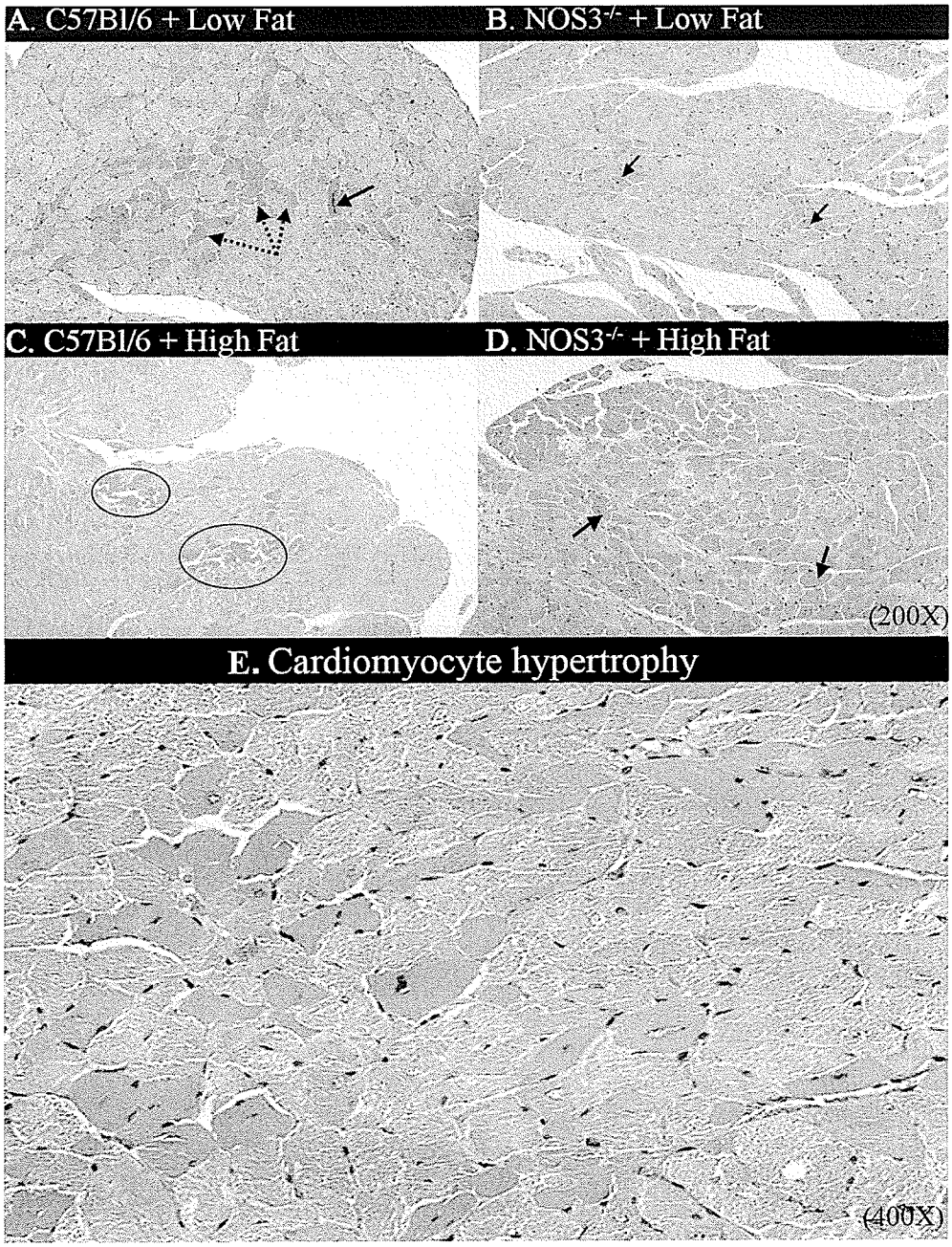


Figure 5: Heart sections from C57Bl/6 and $NOS3^{-/-}$ myocardium showing cardiomyocyte hypertrophy fed either a LLD or a HLD 12 week post-transverse aortic constriction (arrows). **A)** C57BL/6 + LLD (n = 6); **B)** $NOS3^{-/-}$ + LLD (n = 9); **C)** C57Bl/6 + HLD (n = 9); and **D)** $NOS3^{-/-}$ + HLD (n = 9) (200X Magnification). **E)** Isolated patches of cell hypertrophy were observed in C57Bl/6 and $NOS3^{-/-}$ mice. However, $NOS3^{-/-}$ mice fed a HLD showed increased number of isolated cardiomyocyte hypertrophy (400X Magnification).

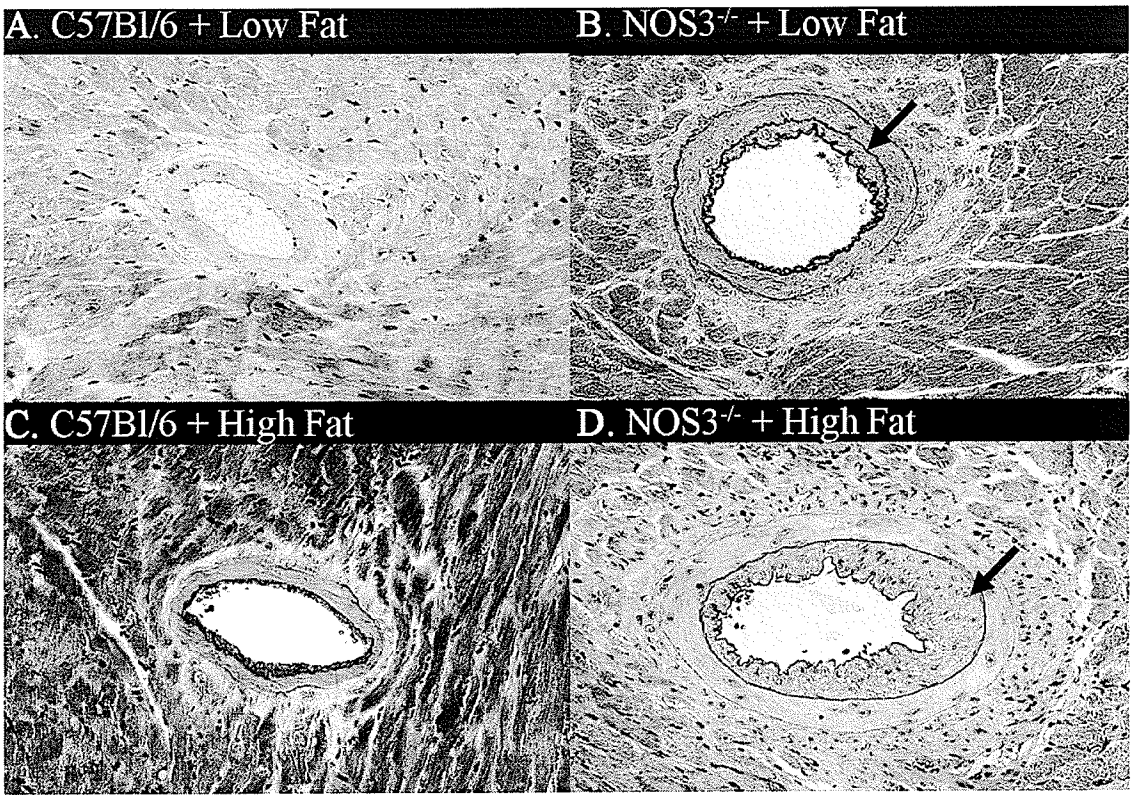


Figure 6: Sections of coronary artery from C57Bl/6 and $NOS3^{-/-}$ mice fed either a LLD or a HLD for 12 weeks. While in C57Bl/6 coronaries show normal endothelial lining, coronary arteries in $NOS3^{-/-}$ shows endothelial are thickening 12 week post-transverse aortic constriction. Note that even on LLD mice lacking endothelial nitric oxide synthase gene shows endothelial thickening 12 weeks post-TAC. **A)** C57Bl/6 + LLD (n = 6); **B)** $NOS3^{-/-}$ + LLD (n = 9); **C)** C57Bl/6 + HLD (n = 9); and **D)** $NOS3^{-/-}$ + HLD (n = 9). (Analyzed in collaboration with Dr. M. Moghadasian's pathology laboratory) (200X Magnification).

3.3 Plasma total cholesterol, triglyceride and glucose analysis

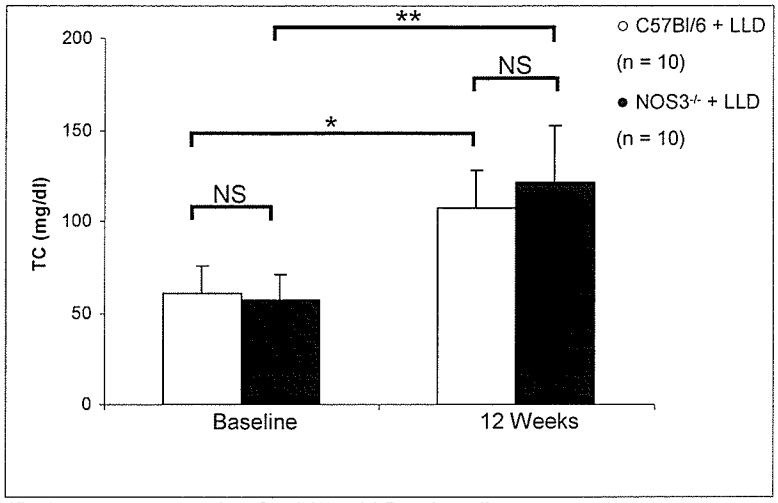
Fasting lipid profiles are shown in Figure 5 and 6. At baseline there was no significant difference in TC, TG and glucose levels amongst all four groups. In wild type mice and $NOS3^{-/-}$ mice fed a LLD TC increased by approximately 2 fold respectively (60 mg/dl to 107 mg/dl and from 57 mg/dl to 122 mg/dl respectively). Additionally, in the HLD stratum, TC levels increased by 2.5 fold in wild type mice (61 mg/dl to 155 mg/dl) while in $NOS3^{-/-}$ it increased by 3.8 fold (51 mg/dl to 194 mg/dl) (Figure 7).

Similar to TC levels, TG levels increased in wild type and $NOS3^{-/-}$ fed either a LLD or a HLD respectively (Figure 7). Wild type mice on LLD did not show any significant increase in TG levels from baseline to 12 weeks post TAC (43 mg/dl to 50 mg/dl). However, in $NOS3^{-/-}$ mice on LLD, TG increased by approximately 2 fold from baseline to 12 weeks post-TAC (52 mg/dl to 95 mg/dl). When given a HLD diet TG levels did increase in both wild type and $NOS3^{-/-}$ mice at 12 weeks post TAC in comparison to baseline. While TG did not reach any significant value in wild type mice, it did increased 4 fold in $NOS3^{-/-}$ mice (Figure 8).

Glucose values were also evaluated for all four groups at baseline and 12 weeks post-TAC. At baseline glucose values in wild type C57Bl/6 and $NOS3^{-/-}$ mice fed LLD diet were 113±35 and 146±37 mg/dl resepectively ($p>0.05$). At 12 weeks post TAC glucose values in these groups increased to 230±40 and 183±89 mg/dl respectively ($p>0.05$). Similarly, at baseline in wild type C57Bl/6 and $NOS3^{-/-}$ mice fed a HLD glucose values were 149±33 and 158±24 mg/dl respectively ($p>0.05$).

At 12 weeks post TAC glucose values in these groups increased to 316±65 and 267±63 mg/dl. Although in all four groups glucose values significantly increased by 2.5 fold compared to their baseline, there was no statistically significant difference amongst the groups.

A)

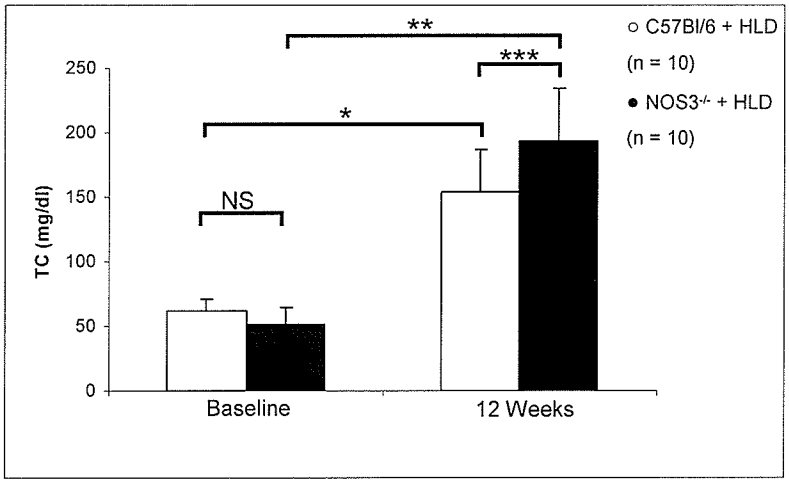


*P<0.05 as compared to C57Bl/6 + LLD at baseline

** P<0.05 as compared to NOS3$^{-/-}$ + LLD at baseline

NS - Not significant

B)



*P<0.05 as compared to C57Bl/6 + HLD at 12 Weeks

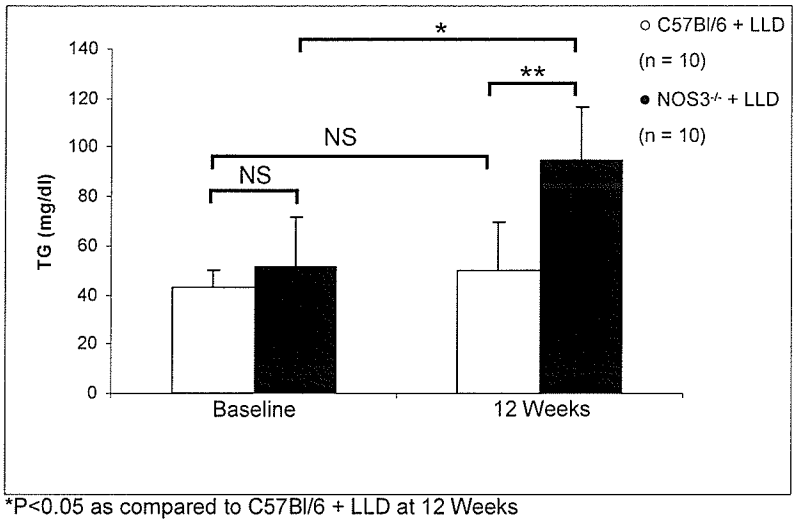
** P<0.05 as compared to NOS3$^{-/-}$ + HLD at baseline

***P<0.05 as compared to C57Bl/6 + HLD at baseline

NS - Not significant

Figure 7: A) Total fasting plasma cholesterol in wild type and NOS3$^{-/-}$ mice fed a LLD at baseline and 12 weeks post-TAC. **B)** Total fasting plasma cholesterol in wild type and NOS3$^{-/-}$ mice fed a HLD diet at baseline and 12 weeks post-TAC. In wild type and NOS3$^{-/-}$ mice fed a LLD, TC increased by approximately 2 fold respectively. In the HLD stratum, TC levels increased by 2.5 fold in wild type mice and 3.8 fold in NOS3$^{-/-}$ mice.

A)

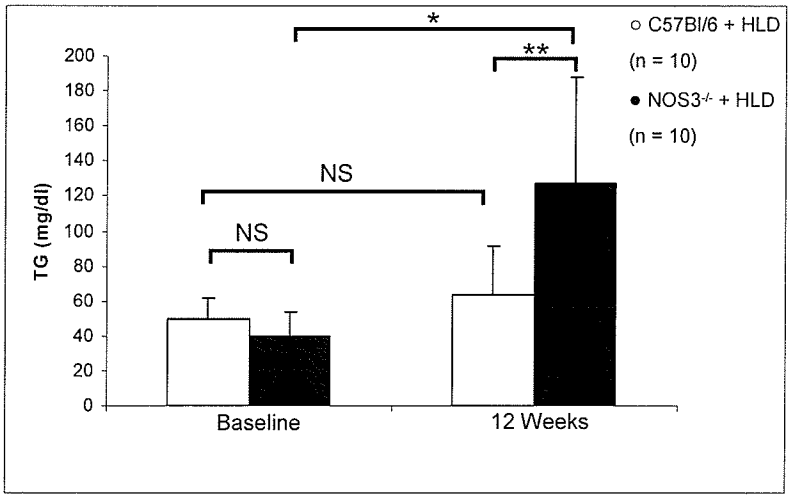


*P<0.05 as compared to C57Bl/6 + LLD at 12 Weeks

** P<0.05 as compared to $NOS3^{-/-}$ + LLD at baseline

NS - Not significant

B)



* P<0.05 as compared to C57Bl/6 + HLD at 12 Weeks

** P<0.05 as compared to $NOS3^{-/-}$ + HLD at baseline

NS - Not significant

Figure 8: A) Triglyceride (TG) levels in wild type and $NOS3^{-/-}$ mice fed a LLD at baseline and 12 weeks post-TAC. **B)** TG levels in wild type and $NOS3^{-/-}$ mice fed a HLD diet at baseline and 12 weeks post-TAC. In wild type mice on LLD, TG increased by approximately 0.4 fold at 12 weeks post-TAC vs. 2 fold increase seen in $NOS3^{-/-}$ mice. However on a HLD diet, TG levels increased by 0.3 fold in wild type mice at 12 weeks post-TAC vs. 4 fold in $NOS3^{-/-}$ mice at the same time period.

3.4 Pro-hypetrophic hi-FGF-2

At 12 weeks post TAC the high molecular weight isoform of FGF-2 (hi-FGF-2), but not low molecular weight isoform of FGF-2 (lo-FGF-2), was significantly upregulated in LV lysates of our murine model of diet-induced obesity and chronic pressure overload. Figure 9A is a representative western blot analysis of the left ventricular cardiac lysates probed for total FGF-2. Figure 9B is the corresponding quantitative data. In $NOS3^{-/-}$ mice subjected to TAC, hi-FGF-2 is increased compared to their wild type counterparts. Furthermore, our quantitative data analysis also showed that the level of pro-hypertrophic hi-FGF-2 accumulation is exacerbated when the mice were fed a HLD versus a LLD in the absence of NOS3.

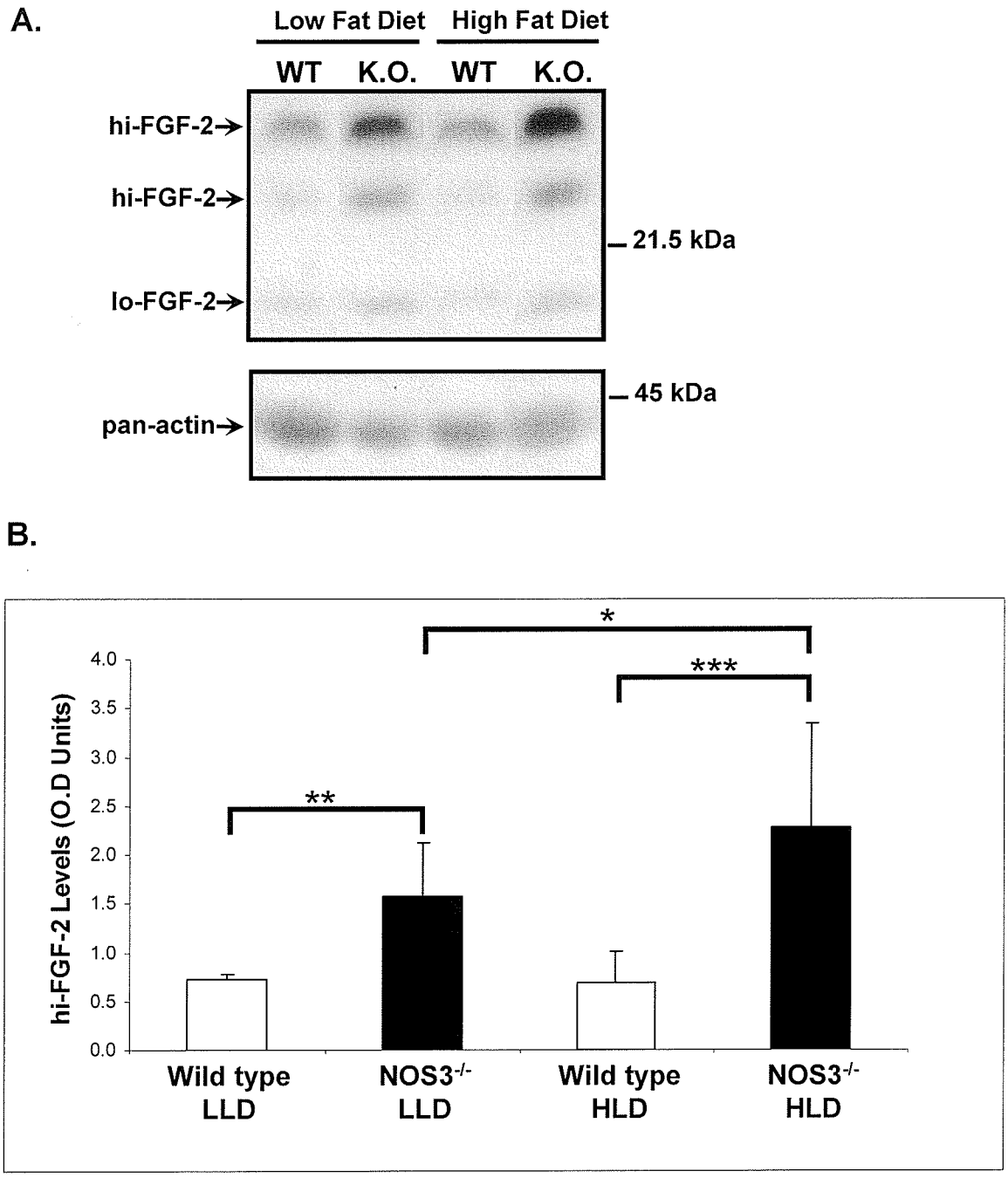


Figure 9 A) Representative western blot of total tissue extracts from wild type (low fat n=7, high fat n=6) and NOS3$^{-/-}$ mice (low fat n=12, high fat n=9) followed for 12 weeks post TAC. The blot was probed for total FGF-2 (lo-FGF-2, 18 kDa and hi-FGF-2, 22-24 kDa) and pan-actin (42 kDa) to show even protein loading between lanes. **(B)** Quantification of relative hi-FGF-2 expression in wild type vs. NOS3$^{-/-}$ mice on LLD (*P<0.05), NOS3$^{-/-}$ mice on LLD vs. NOS3$^{-/-}$ mice on HLD (**P<0.05), and wild type vs. NOS3$^{-/-}$ mice on HLD (***P<0.05). The densities of the bands were normalized to the loading control. No significant difference was observed with lo-FGF-2.

Discussion

The present study demonstrates that congenital absence of NOS3 ($NOS3^{-/-}$) significantly exacerbates left ventricular dysfunction in a state of diet induced obesity and chronic pressure overload. Before TAC, LV dimensions and contractile function did not differ between wild type and $NOS3^{-/-}$ mice fed either a LLD or HLD respectively. Three months after TAC, in vivo echocardiography demonstrated greater LV systolic dysfunction in $NOS3^{-/-}$ mice fed a HLD as compared to the other groups. Congenital absence of NOS3 demonstrated increased LV hypertrophy, pronounced interstitial fibrosis, hyperlipidemia and increased expression of hi-FGF-2 at 12 weeks post TAC. The combination of diet induced obesity and chronic hypertension produced greater LV dysfunction, LV hypertrophy and fibrosis in $NOS3^{-/-}$ mice as compared to wild type mice, stressing the importance of the NOS3 gene in heart failure.

At baseline, there were no significant difference between wild type and $NOS3^{-/-}$ mice in echocardiographic parameters of LV cavity dimensions and systolic function. At 12 weeks post TAC, LV dysfunction and remodeling was exacerbated in $NOS3^{-/-}$as compared to wild type mice. Adverse LV remodeling was more pronounced when $NOS3^{-/-}$ mice were administered a HLD post TAC as compared to wild type mice on a similar diet. The LV cavity dimensions were increased most substantially in $NOS3^{-/-}$ mice as compared to wild type mice on the same respective diet. Although both genotypes demonstrated systolic dysfunction as reflected by a decreased in both LV ejection fraction and TDI parameters, $NOS3^{-/-}$

mice fed a HLD demonstrated enhanced cardiomyopathic features of all four groups.

Our echocardiographic results of pronounced LV dysfunction in the $NOS3^{-/-}$ mice on a HLD post TAC are in agreement with those of previous studies.[10,12] *Ruetten et al* who independently reported that abdominal aortic banding of wild type and $NOS3^{-/-}$ mice for 6 weeks increased LV wall thickness in $NOS3^{-/-}$ to a greater extent than in wild type mice.[10] Furthermore, similar to our chronic pressure overload model, the morphological changes of LV were accompanied with systolic and diastolic dysfunction in $NOS3^{-/-}$ mice. Furthermore, *Ichinose et al* also reported an increase in LV cavity dimensions and decrease in fractional shortening (FS) in $NOS3^{-/-}$ as compared to wild type mice as early as one month post TAC.[11] Although FS was reduced in both groups, FS was reduced to a greater extent in $NOS3^{-/-}$ mice as compared to WT. Our study extends the observation that although $NOS3^{-/-}$ mice demonstrated adverse LV remodeling as compared to wild type three months post TAC, the degree of LV failure was exacerbated in the concomitant setting of diet induced obesity.

Although the absence of NOS3 exacerbates the degree of LV systolic dysfunction in the state of chronic pressure overload and diet induced obesity, overexpression of NOS3 attenuates the cardiomyopathic features to some degree. *Buys et al* demonstrated that NOS3 attenuates LV remodeling and dysfunction post TAC.[8] The selective restoration of the NOS3 gene, in cardiomyocytes of congenitally

NOS3 deficient mice ($NOS3^{-/-TG}$), attenuates LV hypertrophy and dysfunction post TAC. Interestingly, less LV hypertrophy and LV dysfunction was found in $NOS3^{-/-TG}$ mice than in $NOS3^{-/-}$ mice at one month post TAC. However, restoration of the NOS3 gene in cardiomyocytes of these $NOS3^{-/-}$ mice did not prevent the degree of fibrosis, suggesting that the presence of a systemic NOS3 gene, not cardiomyocyte specific, is needed to successfully attenuate LV fibrosis.[8]

In addition to the state of chronic pressure overload, NOS3 specific overexpression in cardiomyocytes attenuates the degree of LV systolic dysfunction in a chronic model of ischemia. *Jones* and colleagues demonstrated that over-expression of NOS3 within the systemic and pulmonary vascular endothelium of mice prevented cardiac and pulmonary dysfunction in murine model of heart failure due to myocardial infarction.[76] In future studies, we will explore whether overexpression of NOS3 will prevent adverse LV remodeling in a combined state of chronic pressure overload and diet induced obesity.

In our current model of diet induced obesity and chronic pressure overload the degree of cardiomyocyte hypertrophy on histological examination was found to be higher in $NOS3^{-/-}$ mice as compared to wild type mice. Cardiomyocyte hypertrophy was present in both genotypes fed either a LLD or a HLD. However, there were a greater number of isolated patches with cardiomyocyte hypertrophy in $NOS3^{-/-}$ fed a LLD as compared to wild type mice on a similar diet. Similarly, $NOS3^{-/-}$ mice fed a HLD showed greater cardiomyocyte hypertrophy as compared to wild type mice fed a similar diet. This is similar to the findings of *Ruetten et al* and *Ichinose et al*

who demonstrated greater myocyte hypertrophy in their respective NOS3$^{-/-}$ models of chronic hypertension.[10, 12] *Ichinose et al* observed myocyte hypertrophy as early as 4 weeks after TAC and even though the blood pressure was normalized in NOS3$^{-/-}$ mice by treatment with hydralazine to that of wild types, it did not prevent the myocyte hypertrophy and extensive fibrosis in these mice. Furthermore, in vitro exogenous NO attenuates cardiomyocyte hypertrophy induced by angiotensin II and decreases the expression of the angiotensin-II type 1 (AT1) receptors. In our study the presence of NOS3 gene in wild type mice was associated with decreased myocyte hypertrophy 12 weeks post TAC.

One potential mechanism for the difference in LV hypertrophy observed in NOS3$^{-/-}$ mice as compared to wild type mice may involve FGF-2. Fibroblast growth factor-2 is a multifunctional heparin-binding protein that regulates different physiologic processes including angiogenesis, cell proliferation, differentiation and migration.[64] It is expressed as 22-34 kDa high molecular weight (hi-FGF-2) and18 kDa low molecular weight (lo-FGF-2) isoforms. In the current model of diet induced obesity and chronic pressure overload, we demonstrated that pro-hypertrophic hi-FGF-2, but not lo-FGF-2, was up regulated and was associated with different levels of cardiac hypertophy. From our findings, we have determined that in NOS3$^{-/-}$ mice subjected to a state of chronic pressure overload and diet induced obesity, hi-FGF-2 was increased compared to their wild type counterparts on a similar diet. Furthermore, our quantitative data analysis also showed that the level of pro-hypertrophic hi-FGF-2 accumulation is exacerbated when the mice were fed a HLD versus a LLD in the absence of NOS3. Previous experiments have implicated

FGF-2 in abdominal banding and angiotensin-II induced cardiac hypertrophy.[62] However, in an acute rat model of myocardial infarction *Kardami et al* has recently showed that hi-FGF-2, but not lo-FGF-2, causes cardiac hypertrophy.[64] The mechanism of how an absence of NOS3 can lead to increased expression of hi-FGF-2 is not well known. Although, it is hypothesized that hi-FGF-2 does localize in the nucleus where it stimulates transcription of pro-hypertrophic genes.[61-64,89] Nitric oxide may play a role in either decreasing the gene expression or localization of hi-FGF-2 in the nucleus of cardiomyocytes.

In addition to the exacerbated LV hypertrophy, the degree of interstitial fibrosis was enhanced in $NOS3^{-/-}$ mice as compared to wild type mice fed a similar diet. The degree of interstitial fibrosis around the coronary arteries was more pronounced in $NOS3^{-/-}$ mice fed a HLD as compared to wild type mice fed similar diet and $NOS3^{-/-}$ mice fed LLD. These findings are in agreement with the previous study by *Ruetten et al*, who reported interstitial fibrosis and myocyte hypertrophy in $NOS3^{-/-}$ subjected to abdominal aortic constriction (AC) as early as 6 weeks post-AC. [10] However, unlike interstitial fibrosis observed by *Ruetten et al*, fibrosis in our model was concentrated around the coronary arteries. One potential mechanism that may contribute to the increased interstitial fibrosis observed in $NOS3^{-/-}$ mice fed a HLD in the chronic pressure overload state involve the renin-angiotensin-aldosterone system (RAAS).[90] There is considerable evidence that in the absence of NOS3 and chronic pressure overload, the RAAS is inappropriately activated. Chronic inhibition of NO by the NOS inhibitor L-NAME in rats activates the vascular and cardiac RAS, causing vascular thickening, perivascular and

myocardial fibrosis.[91] Secondly, it is postulated that activated angiotensin receptor type-1 (AT1) causes hypertrophy of adipose tissues which in turn release free fatty acids and pro-atherogenic adipocytokines, all of which inhibit signal transduction of insulin and cause insulin resistance. Insulin resistance in turn induces hypertension, hyperlipidemia, diabetes mellitus, and atherosclerosis. Although we did not explore the role of RAAS in our current model previous treatment of $NOS^{-/-}$ mice with angiotensin receptor blocker (ARB), olmesertan, has prevented fibrosis and reversed LV hypertrophy.[92]

Previously it is reported that NO attenuates smooth muscle proliferation and regulates vascular tone. In our study, mice lacking the NOS3 gene fed a HLD were found to have greater intimal thickening of coronary arteries as compared to those fed a LLD. Additionally, a HLD feeding in $NOS3^{-/-}$ mice was more associated with a significant decrease in the luminal diameter of coronary arteries as compared to the wild type mice fed either diet or $NOS3^{-/-}$ fed a LLD diet. This further proves the deleterious role of a HLD in LV remodeling and vascular disease in the setting of chronic pressure overload.

In our current model of chronic hypertension and diet induced obesity, metabolic abnormalities of dyslipidemia were significantly exacerbated in the $NOS3^{-/-}$ mice. At baseline we did not find any significant difference in triglyceride and total cholesterol levels between wild type and $NOS3^{-/-}$ mice fed either diet. However, plasma triglyceride and total cholesterol levels were significantly increased in wild

type and NOS3$^{-/-}$ mice at 12 weeks post TAC fed a LLD or a HLD compared to baseline. In LLD fed mice TC and TG both significantly increased at 12 weeks as compared to baseline. This is due to a difference in diet composition before blood collection at baseline and 12 weeks post TAC. While regular chow fed at baseline had negligible amounts of cholesterol, LLD and HLD had 0.95mg cholesterol per gram of lard. Secondly, wild type C57Bl/6 is more resistant to high fat diets.[38] Similarly, in mice fed HLD TC and TG significantly increased at 12 weeks compared to baseline. However, NOS3$^{-/-}$ mice fed a HLD had significantly higher levels of both TG and TC at week 12 as compared to their wild type counterparts.

Cardiomyocytes utilize mainly fatty acid (FA) derived from hydrolysis of TG circulating in the plasma. This process requires localization and activation of lipoprotein lipase (LPL) in the cardiomyocytes.[93] An increase in the levels of TG in NOS3$^{-/-}$ mice may involve a direct effect of NO on either localization or activation of LPL enzyme leading to an increase in the levels of TG in the plasma of these mice. However, the possibility of other mechanisms such as reduced binding to receptors and internalization of TG in NOS3$^{-/-}$could not be ruled out. Furthermore, in one study of type-2 diabetes (obesity related) patients, it was found that the level of plasma triglyceride increased as the levels of nitric oxide decreased.[94] One way to explain this finding is that an overload of TG and lipid may cause some changes in the expression of lipid oxidizing proteins that will produce oxygen radicals or their intermediates that could react with NO generating pathway and be cytotoxic.

The metabolic abnormalities of dyslipidemia in the $NOS3^{-/-}$ mice were also accompanied by impaired glucose intolerance in the current model. Although fasting blood glucose levels were similar in all four groups at baseline, there was evidence of glucose intolerance at 12 weeks post TAC. Our findings are in agreement with a previous study by *Raher et al* who reported glucose intolerance and an impaired insulin tolerance test in wild type C57Bl/6 mice fed a high fat diet (HFD).[34] Glucose intolerance precedes type-2 diabetes and is associated with cardiovascular complications and increased mortality. The mechanism of glucose intolerance is not very well known, however, group 1B phospholipase A_2 (Pla2g1b) has been suggested to play a role in obesity and glucose intolerance.[95] It is thought that Pla2g1b is associated with phospholipid digestion in the gastrointestinal tract, which is necessary step for absorption of lipid nutrients including triglycerides and cholesterol. A recent study by *Hui et al* demonstrated that inhibition of this enzyme by methyl indoxam did prevented diet induced obesity and glucose intolerance.[96] The roles of Pla2g1B and inhibition of methyl indoxam requires further study in our model.

4.2 Limitations of the current study

A number of limitations exist for the current study. First, a control groups that included sham operated animals that underwent a sternotomy, but not TAC, would be useful for isolating the effects of chronic pressure overload on the degree of LV systolic dysfunction, fibrosis, hypertophy and dyslipidemia in the current model. Diet rich in fat does lead to increase in low density lipoproteins (LDL) and a decrease in high density lipoproteins (HDL). In our study we did show an increase in triglyceride levels and total cholesterol at week 12 as compared to baseline. However, it does not tell us anything about the levels of LDL and HDL in the serum of wild type and $NOS3^{-/-}$ mice. To overcome this we could have used high pressure liquid chromatography (HPLC) to determine the levels of very low density lipoproteins (vLDL), LDL and HDL in wild type and $NOS3^{-/-}$ mice fed either a LLD or a HLD. This would have made it possible for us to be more specific in determining the beneficial role of NO in the setting of diet induced obesity and chronic pressure overload.

4.3 Conclusion and future directions

From this study we have determined that chronic consumption of a HLD and pressure overload is deleterious to LV pathophysiology in the congenital absence of NOS3.

Although we clearly demonstrated that a HLD diet has adverse effects on LV function and remodeling in a $NOS3^{-/-}$ mice as compared to wild type mice in the setting of chronic hypertension, we could evaluate the potential reversibility of this cardiomyopathy by reversing the diet from HLD to LLD. To do this, we can feed the mice with HLD for the first 6 weeks post-TAC and then followed by a reversal of the diet to a LLD for the next 6 weeks. This will allow us to determine if further progression of LV dysfunction and fibrosis could be attenuated by a change in diet. Additionally, the role of cardiomyocyte overexpression of NOS3 ($NOS3^{TG}$) as a protective mechanism in a murine model of diet induced obesity and chronic pressure overload requires further study.

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