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THE ARCTIC UNIVERSITY OF NORWAY

Faculty of Health Sciences

Cardiac remodelling in obesity- and angiotensin II-mediated heart failure

Morphological, functional and metabolic alterations

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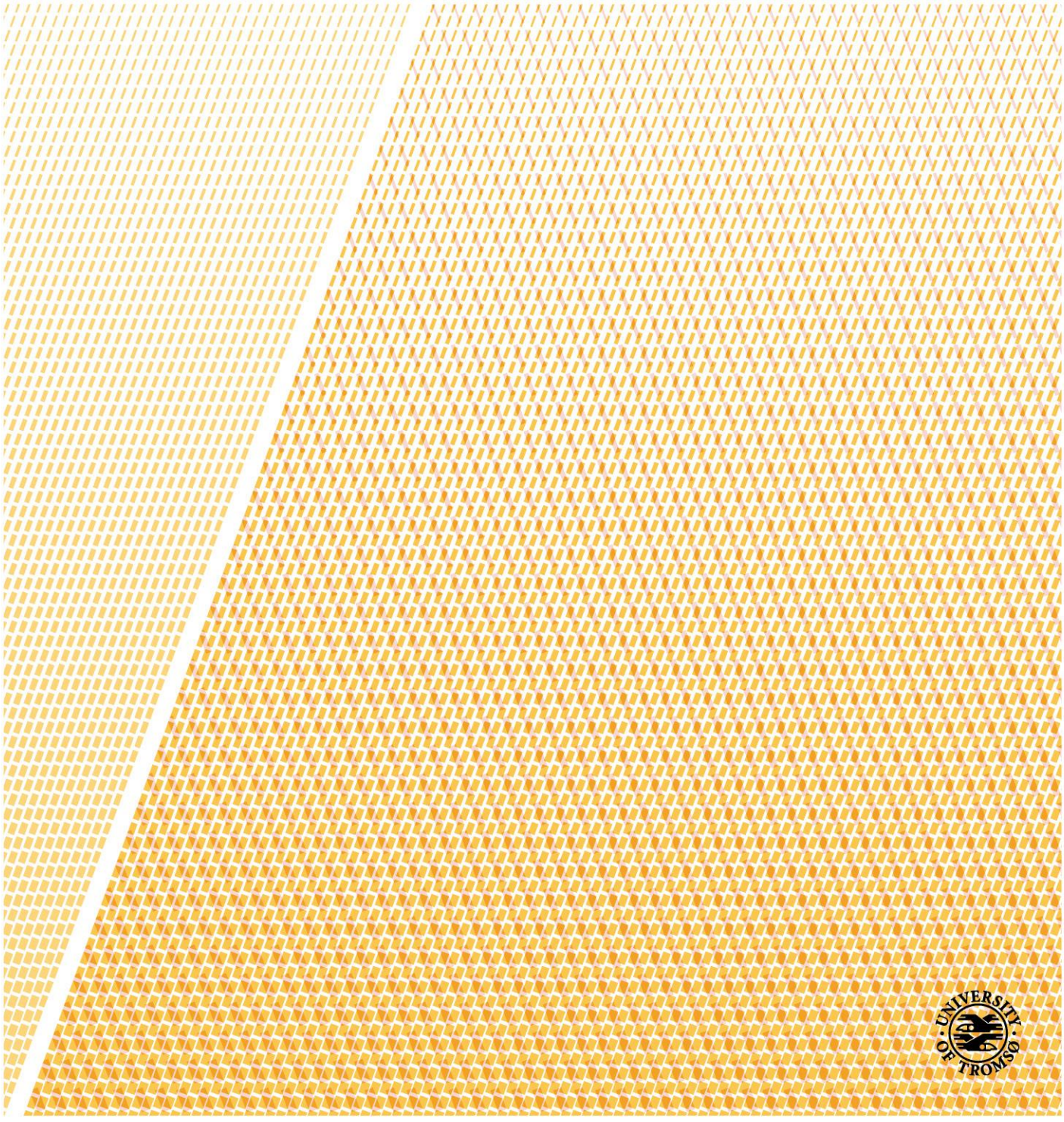
Tina Myhre Pedersen

A dissertation for the degree of Philosophiae Doctor – July 2019



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ACKNOWLEDGEMENTS

This project was started in 2014 and conducted at the Cardiovascular Research Group, Department of Medical Biology. It was funded by The Norwegian Health Association (Nasjonalforeningen for Folkehelsen), with additional funding from the University of Tromsø.

First, I would like to thank the person who has contributed the most: my main supervisor Ellen. Her knowledge, enthusiasm and attention to detail has been instrumental in my PhD journey, and she has sacrificed a great deal of her time for me and my project. Secondly, I'm very grateful to my two co-supervisors. Anne was my first landlord in Tromsø, and made the move to Tromsø a whole lot easier. Her academic curiosity and creativity has been of great help to me when I've been stuck in my ways. Neoma has been there when I've needed it the most - whether for experiments, talks or concerts - and she has endured frustration, excessive singing and bad jokes. No small feat.

Everyone in the Cardiovascular Research Group deserve a thanks, and especially my previous office mates Synne and Kirsten. I greatly appreciated all our conversations and shenanigans. I'm also very appreciative of Trine's technical help and her kind and smiling attitude. Thanks to Leif for teaching me how to do Doppler and for telling me that I'm good at what I do when I've needed to hear it.

I'm very grateful to all my friends in Tromsø who have made my existence a whole lot better. TOF janitsjaren and TAKk have been particularly important to me during my 5 years in Tromsø, and the fact that I've had somewhere to run to and distract myself from the stress of a PhD has been invaluable to me. I owe a big thanks to the French horn group - they are my family away from home.

Lastly, thanks to my friends and family whom I haven't been able to see a lot the last few years. Although I've been far away from you all, you've always felt close, and I've been comforted by many, MANY hours of phone calls. Thank you for all your encouragement. And thanks to Ragnhild, Thea, Anne Katrine, Léa, Line, Kamilla and Lene for joining me on my crazy stupid adventures. My closest family have my deepest gratitude; Mamma and Pappa have always given me advice when I'm close to freaking out. Ida, Chris, Alexander and Emily have provided many laughs and a welcome distraction from my stressful routine. Tiril is perhaps the person who knows and understands me the most - thanks for listening and laughing.

ABBREVIATIONS

AngII	angiotensin II
BM	basal metabolism
DCM	diabetic cardiomyopathy
DIO	diet-induced obese
E-C coupling	excitation-contraction coupling
FA	fatty acid
HF	heart failure
HFpEF	heart failure with preserved ejection fraction
HFrEF	heart failure with reduced ejection fraction
LV	left ventricle
MI	myocardial infarction
MVO2	myocardial oxygen consumption
NADPH	nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidase
PCr	phosphocreatine
PPAR	peroxisome proliferator-activated receptor
RAS	renin-angiotensin system
ROS	reactive oxygen species
TG	transgenic
P-V	pressure-volume
WT	wild type

LIST OF PAPERS

Paper 1 Pedersen TM, Boardman NT, Hafstad AD, Aasum E. Isolated perfused working hearts provide valuable additional information during phenotypic assessment of the diabetic mouse heart. *PLOS ONE.* 13: e0204843, 2018. doi: 10.1371/journal.pone.0204843

Paper 2 Boardman NT*, Pedersen TM*, Rossvoll L, Hafstad AD, Aasum E. Diet-induced obese mouse hearts show metabolic adaptation that enhances tolerance to acute high fatty acid exposure and reduces susceptibility to ischemic injury. *Submitted.* *Authors contributed equally

Paper 3 Pedersen TM, Hansen SS, Boardman NT, Aasum E, Hafstad AD. Overexpression of NOX2 exacerbates AngII-mediated cardiac remodelling and dysfunction in male, but not female mice. *Manuscript.*

INTRODUCTION

Heart failure (HF) is a major killer in both men and women worldwide. Obesity and diabetes mellitus are well known risk factors of HF, and cardiovascular diseases is the major cause of mortality and morbidity in these patients. Obese and diabetic patients also have greater incidence of angina and acute myocardial infarction (MI) and a higher mortality rate following MI. In addition to increased incidence of hypertension and concomitant macro- and microvascular disorders, diabetic patients develop a distinct pathology with alterations at the cardiomyocyte level.[1] The condition is termed diabetic cardiomyopathy (DCM) and is characterized by ventricular dysfunction in the absence of coronary heart disease or hypertension.[2]

Cardiac disease is commonly associated with altered myocardial substrate utilization and reduced cardiac efficiency, and metabolic remodelling and inefficiency are early hallmarks in HF independent of disease aetiology or various comorbidities.[3] As the heart consumes large amounts of energy, the supply and consumption of substrates is of major importance in order to sustain viable function. Thus, metabolic remodelling might have great impact on cardiac function, potentially leading to cardiac dysfunction. Whether metabolic alteration causes dysfunction or whether it is a consequence of dysfunction, is however not clear. As the heart shows great adaptability, there is also a question of whether such alterations are advantageous or disadvantageous. This thesis discusses cardiac morphological, functional and metabolic remodelling in two distinct disease aetiologies, namely obesity- and angiotensin II-induced cardiac disease.

HEART FAILURE AND CARDIAC DYSFUNCTION

The heart is essential in supplying all other tissues with oxygen and nutrients, by continuously pumping blood through the circulatory system. HF occurs when this ability is reduced, often characterized clinically by reduced ejection fraction, however, there are also patients with HF who have maintained ejection fraction. Thus, left ventricular HF is often dichotomized into HF with *preserved* ejection fraction (HFpEF) and HF with *reduced* ejection fraction (HFrEF). In HFpEF, the cardiac output (systolic function) is maintained, but the ventricular relaxation and subsequent filling (diastolic function) are impaired. As the causes and comorbid conditions of diastolic dysfunction are diverse, there is great pathophysiological heterogeneity, making diagnosis and treatment in these patients difficult. In addition, as systolic function is maintained, patients with diastolic failure often present with diffuse symptoms, further complicating diagnosis. In fact, it is suggested that as many as 50% of current HF cases are HFpEF,[4] substantiating the importance of establishing criteria of diagnosis and treatment for these patients. Accordingly, diastolic failure is emerging as a significant clinical problem for patients with diabetes and current clinical data suggest that 30-40% of patients with HFpEF, suffer from diabetes.[5; 6]

Obesity and diabetes

Obesity and diabetes are complex disorders associated with a range of chronic metabolic abnormalities affecting multiple signalling pathways (Fig 1), which also contribute to the development of DCM. Hallmarks of the diabetic heart include ventricular remodelling, fibrosis, oxidative stress, impaired calcium handling, mitochondrial dysfunction and lipotoxicity (Fig. 1). Additionally, alterations in substrate utilization and increased myocardial oxygen consumption, with subsequent impaired cardiac efficiency and energetic imbalance (Fig. 1), are also suggested to play a role in the development of dysfunction.[3; 7-9] Corroborating this, both clinical[10; 11] and preclinical studies[12-14] have shown that obesity and diabetes are associated with increased myocardial oxygen consumption and decreased cardiac efficiency.

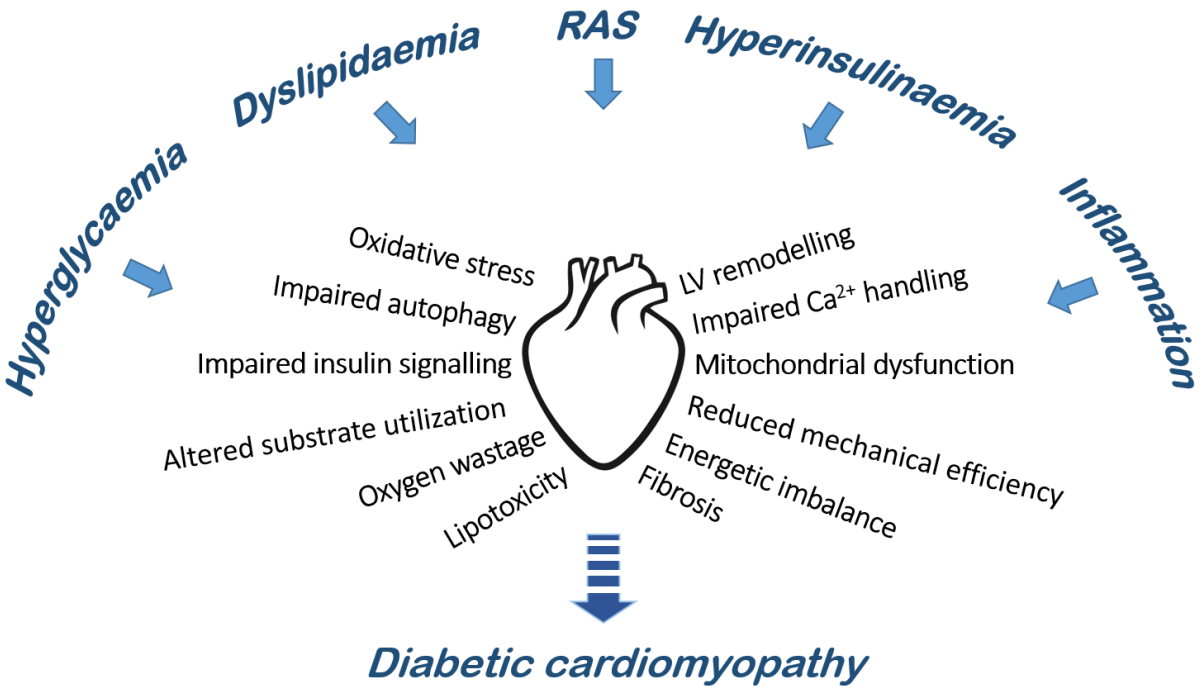

Figure 1: Hallmarks of diabetic cardiomyopathy. A range of systemic changes in obesity and diabetes such as hyperlipidaemia, hyperglycaemia, increased activation of the renin- angiotensin-system (RAS), hyperinsulinaemia and a chronic low-grade inflammation, are believed to lead to cellular changes in the cardiomyocyte with consequent development of diabetic cardiomyopathy. Modified from Hansen *et al.*[15]

Although the systemic metabolic changes in diabetes are likely to induce DCM, the heart might also initially be able to adapt to the chronically altered milieu. In diabetes, the heart predominantly relies on energy from fatty acids (FAs), and notably, there are studies showing that the presence of FAs can improve cardiac function in preclinical models of obesity and diabetes.[16-19] However, more studies are needed to elucidate a possible association between FAs and adaptation in diabetes.

The renin-angiotensin system (RAS)

The renin-angiotensin system (RAS) plays an important physiological role in haemodynamic controls. The overactivity of RAS in HF leads to excessive vasoconstriction, with eventual cardiac hypertrophy and fibrosis. The effector peptide angiotensin II (AngII) is produced both systemically and locally in the heart, and correspondingly, AngII has been shown to have direct effects on cardiomyocytes, inducing cardiomyocyte hypertrophy, fibrosis and apoptosis.[20] Although activation of RAS contributes to the development of obesity-mediated cardiac disease (Fig. 1), the direct effects of RAS on substrate utilization and mechanical

efficiency have not been fully elucidated. However, there are studies describing that the inhibition of RAS normalizes substrate metabolism in models of both cardiac hypertrophy[21] and severe heart failure,[22] and that metabolic preservation is protective in AngII-induced cardiac disease.[23] This supports the notion that AngII might play a role in cardiac metabolic remodelling.

Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are oxygen-derived small reactive molecules that react avidly with other molecules, such as cellular lipids, proteins and nucleic acids. This way, ROS act as signalling molecules in a variety of pathways, however when ROS production is high and/or the cellular antioxidant defences impaired, ROS can cause cell damage and death.[15; 24] The myocardial production of ROS occurs through a variety of mechanisms, the major being as a by-product of mitochondrial oxidative phosphorylation (Fig. 2). Another important source in the heart is nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX); the main cardiac isoforms are NOX2 and NOX4, with NOX2 activation showing the most deleterious effects.[15; 25; 26] Although their role in the pathogenesis of heart failure has yet to be fully elucidated, ROS have been implicated in many processes affecting cardiac function and metabolism,[15; 27] thus, the involvement of ROS has been described in several different aetiological pathogeneses, including AngII-, obesity- and diabetes-mediated cardiac disease.[26; 28-31]

CARDIAC METABOLISM

The storage of energy rich compounds in the heart is limited, and so the heart must renew its supply continuously. The heart is an omnivore and thus able to utilize a variety of substrates. However, FAs and glucose are preferred, with FA oxidation providing 60-90% of adenosine triphosphate (ATP) under normal conditions in adult mammals.[9; 24] Under different physiological and pathophysiological conditions, the relative energy contribution of FAs and glucose may change, as addressed below. The ability to switch between energy substrates according to workload, neurohormonal signalling and substrate and oxygen supply ("metabolic flexibility") is considered vital in sustaining cardiac function.[9; 32]

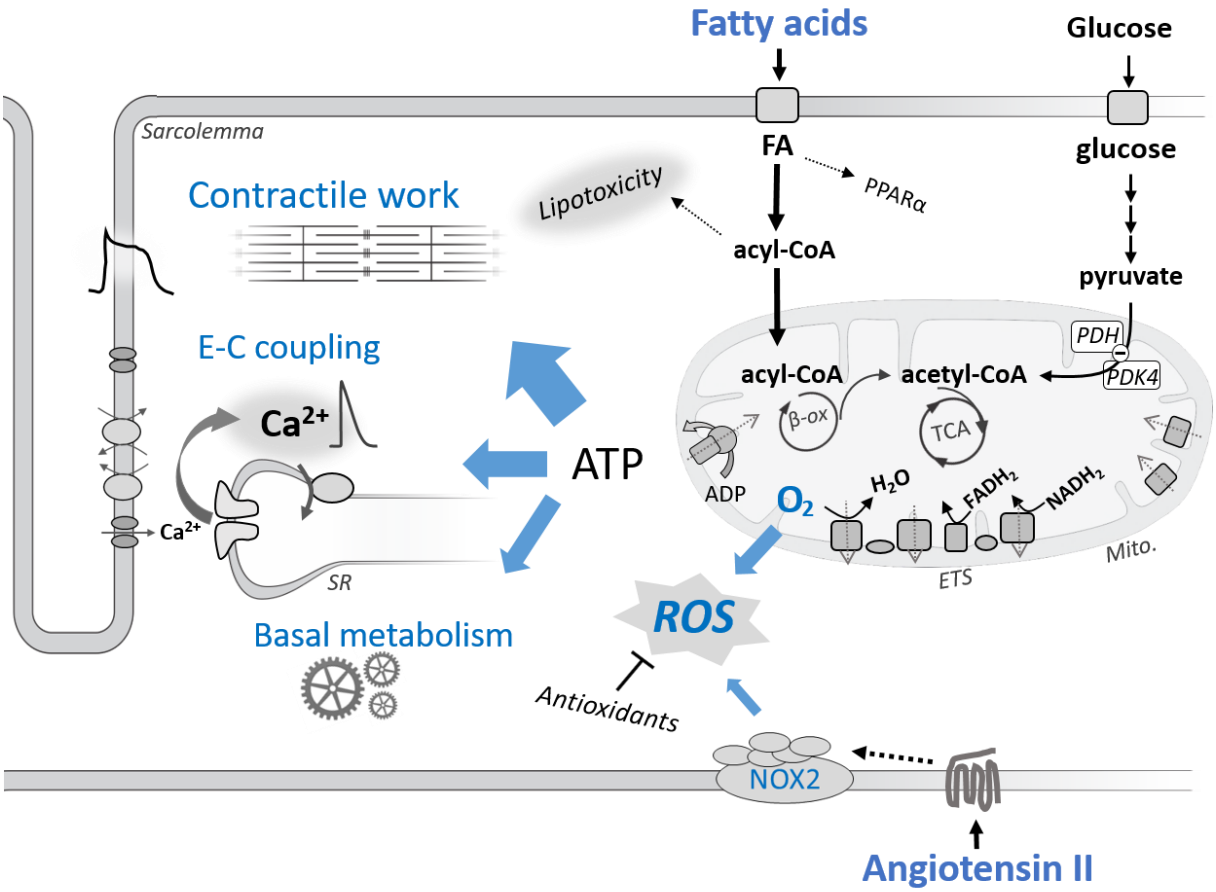

Figure 2: Schematic overview of metabolic pathways of main energy consuming processes described in this thesis. Fatty acids (FAs) are the preferred substrate in cardiomyocytes. FAs are taken up (by FA transport protein (FATP), FA binding protein (FABP) and FA translocase (FAT/CD36)), acetylated (to acyl-CoA) and transported into mitochondria (by the carnitine-palmitoyltransferase (CPT) system), where β-oxidation (β-ox) leads to generation of acetyl-CoA. An excess of acyl-CoA is stored as triacylglycerol (TAG) but may also result in the accumulation of toxic FA intermediates, leading to lipotoxicity. Glucose uptake (facilitated diffusion by the glucose transporters 1 and 4 (GLUT 1 and 4)) results in the generation of pyruvate during glycolysis. Pyruvate is taken up by the mitochondria and converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDH). PDH is inhibited by pyruvate dehydrogenase kinase 4 (PDK4). Reducing equivalents ($FADH_2$/NADH) are formed from acetyl-CoA in the tricarboxylic acid (TCA) cycle, and subsequently drive the proton motive force in the electron transport system (ETS), powering ATP synthesis.
Energy consuming processes in the cardiomyocyte include generation of contractile work (cell shortening), but also energy for non-mechanical processes, e.g. maintaining ion homeostasis following ion-flux associated with action potentials and Ca^{2+} transients. Cardiomyocyte contraction is dependent on the entry of Ca^{2+} via sarcolemmal Ca^{2+} channels that trigger release of Ca^{2+} from the sarcoplasmatic reticulum (SR) via the ryanodine receptor 2 (RyR2), and the subsequent activation of myofilaments. Relaxation occurs when Ca^{2+} is sequestrated from the cytosol to the SR by SR Ca^{2+} ATPase (SERCA2a), and extruded out of the cell. The energy used for the excitation-contraction (E-C) coupling in total represents approximately 2/3 of the non-mechanical energy consumption in the cardiomyocyte, while processes associated with basal metabolism account for the rest.

The oxygen molecule O_2 is the electron acceptor in the ETS, however, 1-2% of mitochondrial oxygen consumption results in formation of reactive oxygen species (ROS). ROS may also be generated from non-mitochondrial sources such as NADPH oxidase 2 (NOX2). Activation of NOX2 requires translocation of regulatory cytosolic subunits to the membrane-bound NOX2 flavocytochrome. Angiotensin II is known to cause production of NOX2-mediated ROS. Excess ROS production and/or impaired antioxidant capacity can lead to oxidative stress, which plays an important role in the pathogenesis of heart failure.

The endpoint of substrate utilization is the mitochondrial production of ATP coupled with O_2 consumption (oxidative phosphorylation), which is facilitated by the reducing equivalents NADH and $FADH_2$ (Fig. 2). In the healthy heart, the rate of oxidative phosphorylation is intricately linked to the hydrolysis of ATP to adenosine diphosphate (ADP), ensuring a stable concentration of ATP, even when cardiac work load increases rapidly, such as in physical exercise or acute catecholamine stress. Additionally, phosphocreatine (PCr) is an important component of the cardiac energy metabolism, acting as an energy buffer and ensuring a constant supply of ATP.[9; 33] When the energy demand outstrips the energy supply, the phosphocreatine level falls, keeping ATP at a normal level. Thus, although ATP levels may initially be maintained per se, the [PCr]/[ATP] ratio is a predictor of HF,[33; 34] and such energetic imbalance has been described both clinically and experimentally in various aetiologies, such as pressure-overload-induced cardiac hypertrophy, dilated cardiomyopathy and diabetes.[34-39]

Cardiac metabolism is complex and highly regulated, and the rate of different metabolic pathways is influenced by substrate availability and hormonal changes, but also translational and post-translational regulation of enzymes and transport proteins. The Randle cycle is of major importance in terms of regulation due to differences in substrate supply, as there is reciprocal interaction where FA intermediates can inhibit glucose oxidation, and vice versa.[40] The expression of metabolic target genes is dynamically regulated in response to physiological and pathophysiological conditions, and can thus contribute to both adaptive and maladaptive metabolic changes. The transport proteins and enzymes are regulated by several transcription factors, including peroxisome proliferator activated receptor (PPAR) α, where FA can serve as ligands (Fig. 2). PPARα target genes are numerous and include enzymes regulating both FA uptake and oxidation, such as FA translocase/CD36 that are involved in FA uptake, medium- and long-chain acyl CoA dehydrogenase (MCAD and LCAD) that facilitate FA β-oxidation, and carnitine palmitoyltransferase 1 (CPT1) that is involved in mitochondrial FA uptake.[3] Activation of PPARα also affects glucose oxidation as it increases

the expression of pyruvate dehydrogenase kinase 4 (PDK4), which inhibits the pyruvate dehydrogenase complex which in turn catalyses the decarboxylation of pyruvate and is a key rate-limiting step in glucose oxidation.[3]

Post-translational modifications of metabolic enzymes include a range of processes, including protein phosphorylation and acetylation. Acetyl-CoA (the majority of which is supplied by FA oxidation) serves as a co-factor in this acetylation, and a growing body of literature describes that most metabolic mitochondrial enzymes are acetylated.[41; 42] Recent studies suggest that reversible lysine acetylation of proteins can play an important role in the regulation of metabolism.[43; 44]

Cardiac metabolism in obesity and diabetes

Obesity and diabetes are accompanied by increased plasma levels of both free FAs and triacylglycerols, causing enhanced cardiac FA uptake and oxidation. Subsequently, PPARα-mediated transcriptional changes will further contribute to increase the FA uptake, oxidation capacity and inhibition of glucose oxidation.[3] Additionally, chronic hyperlipidaemia can also contribute to impaired insulin signalling, causing reduced glucose utilization, due to a diminished translocation of glucose transporter 4 (GLUT-4).[3] These changes increase the reliance on FA oxidation for ATP production in obese hearts. Obesity and elevated plasma FA levels are also associated with a cardiac-specific lipotoxicity, where the accumulation of toxic lipid intermediates (such as ceramide and diacylglycerols) can induce apoptosis and DNA damage.[45; 46] Moreover, mitochondrial protein acetylation has been shown to be increased in diet-induced obese mice, which is associated with decreased expression of the deacetylase enzyme sirtuin 3 (SIRT3).[43; 44] The significance of such post-translational modification in terms of metabolic adaptation and maladaptation has not been fully elucidated yet.

CARDIAC EFFICIENCY

The term efficiency is used widely, but is not uniformly defined. Since more than 95% of the cardiac ATP is produced by oxidative phosphorylation,[3] myocardial oxygen consumption (MVO_2) is a measure of total energy expenditure in the heart. The effectivity of the heart can therefore be assessed by examining the relationship between cardiac work and the measured MVO_2. Accordingly, cardiac efficiency is most commonly a measure of the ratio between MVO_2 and cardiac work, and this efficiency ratio was termed *mechanical* efficiency by Bing in the 1940's.[47] In the normal heart, it has been shown that the presence of high levels of FAs

causes an increase in myocardial oxygen consumption,[13; 48] with subsequent reduced cardiac efficiency. Interestingly, this FA-mediated oxygen waste is not due to altered mechanical function, but caused by aggravated O_2 wasting in non-contractile processes (Fig. 2).[13; 49]

As previously mentioned, both clinical[10; 11] and pre-clinical[13; 14; 50] studies have demonstrated that obese and diabetic hearts have increased oxygen consumption and reduced efficiency. Similarly to in normal hearts, the inefficiency in these hearts has been shown to be due to aggravated O_2 cost of non-contractile processes.[13; 49] Furthermore, the increased MVO_2 in obese and diabetic hearts is generally attributed to the elevated FA oxidation, since the O_2 cost of FA oxidation is higher than that of glucose, however the majority of the oxygen wasting is likely caused by other processes (as discussed later).[13; 49]

It is well known that an acute MI is accompanied by increased circulating levels of FAs.[51] The obligatory FA-mediated increase in O_2 consumption, poses a challenge in the hypoxic heart, and in accordance with this, exposure to high levels of FAs has been reported to exacerbate ischemic injury in normal hearts.[52-54] Although increased ischemic injury has been reported in diabetic hearts in clinical studies,[55] the reported ischemic tolerance varies in experimental studies.[56-58] Thus, it is unclear whether a MI-induced acute increase in FAs is detrimental in these hearts.

AIMS OF THE THESIS

This thesis comprises of three studies, aiming to:

1. Elucidate the functional remodelling in two different mouse models of obesity (representing prediabetes and diabetes), by comparing *in vivo* and *ex vivo* techniques
2. Investigate whether cardiac energetic alteration in obesity alters the heart's ability to tolerate exposure to an acute high FA load, and how these conditions influence ischemic injury
3. Examine the effects of a slow-pressor dose of angiotensin II on cardiac metabolism and efficiency, and to investigate how cardiac NOX2 overexpression alters these angiotensin II-mediated effects in both sexes

METHODOLOGICAL CONSIDERATIONS

Mouse models and treatments

The C57BL/6J strain is one of the most common inbred mouse strains in cardiovascular research and it is a common background strain for many genetically modified mouse models, including both the *db/db* strain and the transgenic (TG) NADPH oxidase 2 (NOX2) mice that were used in this thesis (described below). The strain is known to be particularly susceptible to obesity, due to a loss-of-function mutation in the Nicotinamide nucleotide transhydrogenase (Nnt) gene, causing decreased pancreatic insulin secretion and reduced glucose clearance.[59-61] Additionally, C57BL/6J shows reduced responsiveness to leptin and β-adrenergic signalling, hindering lipolysis.[60; 62; 63]

Diet-induced obesity was achieved by feeding mice a high-caloric diet for 20 weeks, starting at an age of 5 weeks. Diet-induced obese (DIO) C57BL/6J develop a prediabetic metabolic syndrome with hyperlipidaemia, impaired glucose tolerance and insulin resistance with or without hyperglycaemia.[14; 64] Thus, they mimic human disease progression and pathogenesis well, and fat distribution is similar to that in people when exposed to high-caloric diets.[59] Hearts from these animals generally have increased wall thickness and left ventricular (LV) mass *in vivo*.[65; 66] These hearts also display a shift towards higher reliance on fatty acids (FAs) as energy substrate and following long-term feeding they often display diastolic dysfunction, with or without mild systolic impairment,[14; 64] although the literature is inconsistent with regard to the severity of dysfunction.

Db/db mice (C57BL/KsJ-lepr^{db}/lepr^{db}) is a monogenic model where the leptin receptor is mutated and thus defective, creating a lack of satiety sensation and subsequent hyperphagia. The mice develop severe obesity and overt progressive type 2 diabetes mellitus, characterized by insulin resistance, with hyperinsulinaemia, hyperglycaemia and hyperlipidaemia.[67-69] They display cardiac hypertrophy, remodelling and ventricular dysfunction,[68-70] although the reported occurrence of the latter seems to vary in the literature. The metabolic shift in these hearts is characterized by a preference for FAs and a reduction in glucose oxidation, which is shown to precede cardiac dysfunction.[67; 68; 71]

csNOX2 TG mice are mice with a cardiac-specific overexpression of NOX2, made by cloning a human NOX2 cDNA downstream of the myosin light chain-2 promoter. The mice are backcrossed onto a C57BL/6J background and were supplied from colleagues at King's

College in London. They have a normal cardiac phenotype prior to intervention and basal myocardial NOX activity is unaltered, although their expression of the NOX2 enzyme is approximately five times that of wild type (WT) animals.[72] With increased stress however, i.e. AngII administration, myocardial NOX activity increases with 50% in TG compared to WT.[72]

Angiotensin II treatment in mice was done using mini-osmotic pumps. This facilitated the subcutaneous infusion of angiotensin II (AngII), allowing for continuous treatment without repeated injections or surgeries. Mini-osmotic pumps were inserted in the midscapular region, containing either a solution of AngII or saline. The chronic infusion of AngII is initiated with a prehypertensive period, characterized by trophic stimulation of vasculature muscle and potentiation of pressor and vasoconstrictor responses to AngII itself (*autopotentiation*).[73] The increase in pressure is dependent on the dose and treatment duration, thus different treatment regimens can be divided into categories depending on whether they induce hypertension; often categorized as no-pressor, sub-pressor, slow-pressor or pressor doses. In this thesis (paper 3), we used a low dose of AngII (400 ng/kg/min) which induced hypertension in WT males, and this was thus categorized as a slow-pressor dose.[74; 75]

Assessment of cardiac function

Echocardiography is valuable for the non-invasive evaluation of LV architecture and function, and the technological development of high-frequency transducers and improved image frame rates allows for ample phenotyping even in small animals. Hence, the echocardiographic assessment of cardiac morphology and function using conventional echocardiography is widely used in rodent models of heart disease. Despite the advancement of additional imaging techniques and modalities (i.e. echocardiographic speckle-tracking and 3D imaging, as well as magnetic resonance imaging), conventional echocardiographic examination (2D B-/M-mode and pulsed wave Doppler) is still more common. In our studies, hearts were imaged in the parasternal short-axis and apical four-chamber views to obtain M-mode and Doppler images respectively. Generally, parameters of wall thickness, volume and systolic function were assessed from M-mode, while diastolic function was evaluated from pulsed-wave and tissue Doppler, in addition to M-mode parameters. The animals were lightly anaesthetized during the procedure, with heart rate, body temperature and respiratory frequency being monitored continuously.

Ex vivo cardiac function can be evaluated in isolated perfused hearts using the working heart mode or the Langendorff mode. In the working mode, the heart is subjected to anterograde perfusion where hearts are ejecting perfusion buffer against a standardized afterload pressure (Fig. 3A). In the present thesis, LV pressure (P) and volume (V) changes were assessed using an intraventricular conductance catheter (i.e. P-V catheter) that was inserted into the LV lumen via the apex. P-V recordings allow for comprehensive evaluation of the *ex vivo* function, and the steady-state measurement of both load-dependent and -independent parameters.

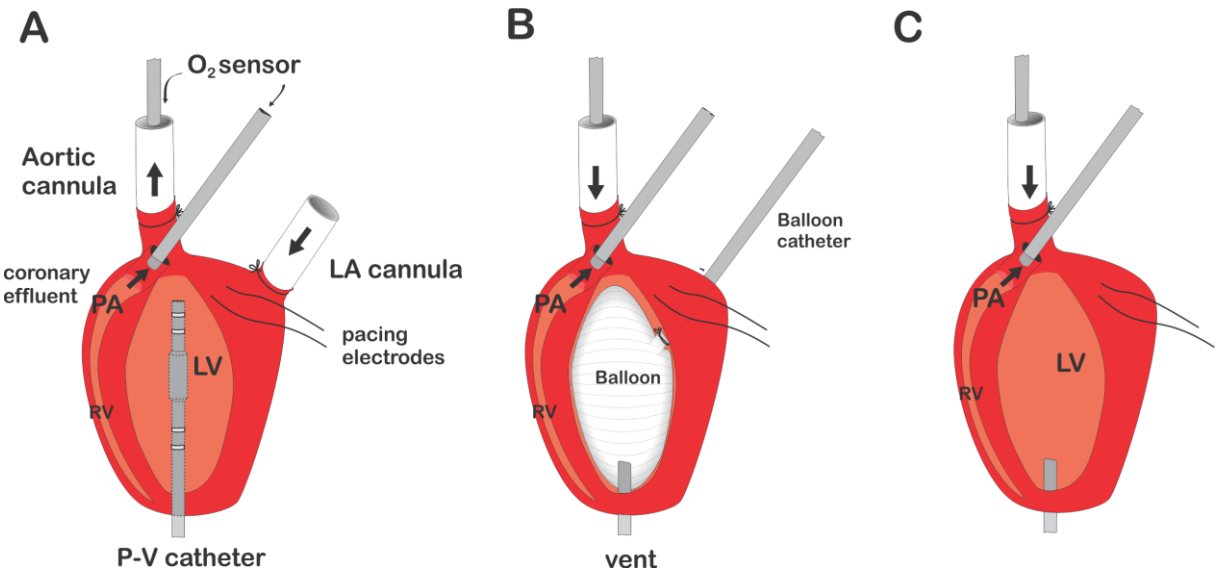

Figure 3. Illustration of the different modes of isolated perfused hearts. A) Working heart mode with pressure-volume catheter, B) Langendorff mode with intraventricular balloon, C) unloaded Langendorff mode.

The Langendorff mode is a good model for ischemia-reperfusion studies, and in this thesis, hearts were subjected to a global ischemia-reperfusion protocol in the Langendorff mode, in order to evaluate post-ischemic recovery and infarct sizes. LV pressure was monitored continuously using an intraventricular fluid-filled balloon connected to a pressure transducer (Fig. 3B). As changes in temperature during ischemia is an important variable in terms of development of ischemic damage, the temperature was monitored continuously and kept at 37 ± 0.5 °C. To prevent build-up of fluid in the ventricle, a cannula was inserted through the apex to allow drainage from the LV cavity. Due to the small size of the hearts, the size of the balloon was set after insertion into the LV, adjusting the LV end-diastolic pressure to be between 5 and 10 mmHg. As the end-diastolic pressure is predetermined, comparison of intrinsic diastolic pressures is not feasible using this protocol.

Assessment of myocardial oxygen consumption and efficiency

Myocardial oxygen consumption (MVO_2) was measured in isolated perfused hearts, using fibre-optic sensors measuring ΔPO_2 in the buffer, i.e. oxygen concentrations in the buffer entering (the aortic line) and leaving (the pulmonary trunk) the heart (Fig 3A). The cardiac mechanical efficiency was calculated as the ratio between cardiac work and the measured MVO_2 in working hearts. Cardiac efficiency can also be examined by analysing the linear relationship between MVO_2 and cardiac work (often assessed as P-V work) over a range of workloads.[13; 64; 76] The slope of this relationship defines the work-dependent MVO_2, and correspondingly, the extrapolated y-intercept (i.e. no cardiac work) defines the work-independent consumption.[76; 77] This work-independent MVO_2 can also be measured directly in Langendorff perfused hearts where cardiac work is minimized (unloaded state, $MVO_{2\ unloaded}$).[49] In the unloaded Langendorff mode (Fig 3C), we also measured MVO_2 following cardiac arrest, induced by the administration of KCl. The consumption of energy in the mechanically unloaded state includes energy consuming processes associated with basal metabolism (BM) and excitation-contraction (E-C) coupling.[78] Arresting the heart allows for measurement of oxygen consumption caused by BM ($MVO_{2\ BM}$), and from this, the O_2 cost of E-C coupling was calculated ($MVO_{2\ ECC} = MVO_{2\ unloaded} - MVO_{2\ BM}$).

Assessment of myocardial substrate utilization

Myocardial substrate metabolism was measured as described by Barr and Lopaschuk,[79] using radiolabelled isotopes ([U-^{14}C]-glucose and [9,10-^{3}H]-palmitate) added in trace amounts to the recirculating perfusion buffer. Glucose and FA oxidation rates were calculated based on the accumulation of $^{14}CO_2$ and $^{3}H_2O$ produced (respectively), from buffer samples collected at regularly timed intervals. ^{14}C-labelled bicarbonate was measured by injecting a sample of the perfusate into a sealed test tube containing sulphuric acid, where the subsequent released $^{14}CO_2$ was trapped on filter paper with hyamine hydroxide. $^{3}H_2O$ was separated from the buffer sample using vacuum sublimation, freezing the sample to allow sublimation of the water. The amount of total $^{14}CO_2$ and $^{3}H_2O$ at the different time points was determined using a liquid scintillation counter, where the slope of increased isotope concentration represents the rate of substrate oxidation.

Assessment of mitochondrial function and mitochondrial protein acetylation

Mitochondrial oxygen consumption was measured in isolated mitochondria using a high-resolution respirometer (O2-k, OROBOROS Instruments). Mitochondria were isolated using a conventional differential centrifugation method, based on sequential centrifugation, separating different components of the cell homogenate with increasing speed. Respiration was measured using pyruvate and malate or palmitoyl, carnitine and malate as energy substrates. Respiration rates were measured before (V_0) and after the addition of 100 μmol/L of ADP (V_{max}), and was normalized against the protein content in the samples. In order to further evaluate coupling efficiency and mitochondrial function, the ADP/O ratio and respiratory control ratio (RCR) were calculated. The ADP/O ratio is a rate-independent parameter of coupling efficiency and was calculated from the ratio between each phosphorylated ADP molecule to O_2 molecule consumed. The RCR was calculated as the ratio between V_{max} and V_0.

In order to evaluate the association between obesity, the presence of FAs and post-translational acetylation, total lysine acetylation of mitochondrial proteins was measured in LV tissue samples from hearts from normal and obese mice, perfused with low and high levels of FAs. Mitochondrial protein samples were separated by electrophoresis, and the membranes were then treated with acetylated lysine antibody. Densitometry of immunopositive bands was performed using western blot quantification software. The acetylation levels with increasing FA concentration were normalized to acetylation in LV samples perfused without FAs.

Assessment of gene expression

The measurement of expression of metabolic mediator genes and hypertrophy markers is an important tool in order to evaluate the cardiac phenotype. Expression of genes in the LV was measured using real-time quantitative PCR (qPCR). LV tissue from perfused hearts was immersed and stored in RNA later, prior to extraction of total mRNA. qPCR analysis was performed using a real-time thermal cycler and expression was normalized to a house-keeping gene whose expression was similar between groups.

Peroxisome proliferator-activated receptor α was examined as an indicator of regulation of both FA and glucose oxidation, in addition to other metabolic genes involved in FA (CD36) and glucose utilization (hexokinase, pyruvate dehydrogenase kinase 4 and lactate

dehydrogenase). We also measured the expression of SIRT3 and GCN5L1, which modulate mitochondrial acetylation.[41] Furthermore, the expression of hypertrophy markers (brain natriuretic peptide and atrial natriuretic factor) was also measured, in addition to confirming the transgenic overexpression of the NOX2.

SUMMARY OF RESULTS

Paper 1

Murine models of obesity and diabetes are well established, facilitating the study of the pathogenesis in cardiomyopathy. While the first reports on function in hearts from diabetic mice were based on *ex vivo* measurements using isolated heart perfusions,[12] echocardiography is the most common modality for cardiac functional assessment in experimental studies today. The aim in paper 1 was to examine the cardiac functional changes using conventional transthoracic echocardiography (*in vivo*) and isolated heart perfusion techniques (*ex vivo*), in hearts from two models of obesity, representing prediabetes (diet-induced obese (DIO) mice) and overt diabetes (*db/db* mice).

We found that there were marked discrepancies between left ventricular (LV) function measured *in vivo* and *ex vivo* (using pressure volume recording) in both animal models. While echocardiography demonstrated signs of diastolic dysfunction without systolic failure in *db/db* hearts, *ex vivo* assessment revealed both diastolic and systolic dysfunction. Similarly, only *ex vivo* LV pressure-volume analysis showed diastolic dysfunction in DIO hearts. The contrasting observations in *ex vivo* and *in vivo* measurements could be due to systemic adaptation that sustains *in vivo* function, or a lack of sensitivity using conventional transthoracic echocardiography. Thus, the study demonstrates that the isolated perfused working heart preparation provides unique additional information related to the development of cardiomyopathy, which might otherwise be masked by favourable systemic adaptation *in vivo* when only using conventional echocardiographic assessment.

Paper 2

Cardiac metabolic remodelling following obesity may alter the heart's ability to handle an increase in fatty acid (FA) levels. The aim of this paper was to examine the functional and metabolic effects of an acute exposure to elevated levels of FAs in hearts from normal and DIO mice. Whilst previous studies have mainly compared the effects of high FAs to conditions where FAs are absent, this study compared hearts perfused with high levels of FAs, to hearts perfused with low FA levels. Additionally, we examined LV functional recovery and infarct sizes in high FA conditions, as this mimics the acute increase in circulating FAs that is associated with myocardial infarction.

Even though the LV function remained unaltered in both groups, control hearts showed increased myocardial oxygen consumption (MVO_2), mechanical inefficiency and impaired mitochondrial respiration when perfused with high levels of FAs. Additionally, content of reactive oxygen species and mitochondrial lysine acetylation were increased with high FA exposure in controls. These effects were not seen in hearts from DIO mice, who, contrastingly, showed enhanced ischemic tolerance and post-ischemic recovery. Thus, this study highlights that obese hearts undergo adaptive changes that make them less vulnerable to disadvantageous effects of an acute FA-load.

Paper 3

Angiotensin II (AngII) is commonly used to study hypertension-induced overt heart failure, but the direct effect of AngII on cardiac energetics and substrate utilization is less known, especially following lower doses. In paper 3, the aim was therefore to study the morphological, functional and metabolic effects following two weeks of AngII-treatment, using a low (slow-pressor) dose of 400 ng/kg/min. We also elucidated the role of NADPH oxidase 2 (NOX2), by treating mice with cardiac-specific overexpression of NOX2 with the same dose.

Male mice treated with AngII developed hypertrophy and hypertension, while cardiac function and substrate utilization remained unaltered. Cardiac NOX2 overexpression exacerbated the effect, leading to a more rapid increase in blood pressure, an aggravated hypertrophic phenotype and the development of both systolic and diastolic failure. In these mice, heart failure was accompanied by a shift in substrate utilization, showing a preference for glucose oxidation when compared to their wild type littermates. Contrastingly, AngII treatment did not induce hypertrophy, cardiac failure or changes in substrate utilization in neither wild type nor transgenic female mice. Thus, this study illustrates that while a slow-pressor dose of AngII induces cardiac hypertrophy, it does not affect cardiac function or metabolism. NOX2 overexpression exacerbates the pathology in males, whereas females seem to be more resistant to the NOX2-mediated detrimental effects of AngII.

GENERAL DISCUSSION

Cardiovascular diseases, such as hypertension, coronary artery disease and cardiomyopathies, can lead to the development of diastolic and/or systolic dysfunction. Diastolic dysfunction, with reduced left ventricular (LV) relaxation and compliance, most commonly manifests prior to systolic dysfunction. While we currently have several strategies for treating heart failure with systolic dysfunction, increased diagnosis of diastolic dysfunction with preserved systolic function and a lack of effective therapies for these patients, demonstrates the need for new therapeutic approaches. Experimental studies have shown that the reversal to or maintenance of a normal cardiac substrate utilization is beneficial in the failing heart,[23; 37; 80; 81] but so far the outcome of such therapeutic approaches in clinical trials has not been consistent.[32; 82] Accordingly, the identification of potential metabolic modulators to treat heart failure is reliant on a better understanding of its pathogenesis. As various comorbidities influence the disease development, this adds to the complexity of studying cardiac pathogenesis, in terms of both experimental design and the discovery of new treatment strategies.

The functional phenotype in diabetic and obese models

Obesity and diabetes are risk factors of heart failure, and when failure is mediated by these factors, diastolic dysfunction precedes systolic dysfunction. Although this has been supported by functional assessment in hearts from animal models of diabetes and obesity,[30; 64; 65; 67; 83] the reported phenotype varies in the literature. This may be explained by differences in the severity of the model due to genetic background, age and the feeding regimen, but may also be related to the methods used for cardiac functional assessment. Echocardiography has become the most common modality for assessing cardiac function in mice due to technological advancement. Importantly, however, *in vivo* measurements of function do not necessarily depict the function of the heart per se, as it might be influenced by favourable haemodynamic and neurohormonal changes, improving the functional capacity. The *ex vivo* heart preparation, however, enables the examination of cardiac function under controlled conditions, both with regard to loading and neurohormonal conditions. In this thesis, we compared *ex vivo* and *in vivo* LV function in two distinct animal models, representing different severity of disease (paper 1). The *db/db* mice presented with overt obesity and diabetes, i.e. hyperlipidaemia, hyperglycaemia and insulin resistance, while diet-induced obese (DIO) animals had hyperlipidaemia and insulin resistance without hyperglycaemia, representing a prediabetic state. Despite a clear LV systolic and diastolic dysfunction in the

isolated perfused *db/db* hearts, conventional echocardiography did not reveal systolic dysfunction and only a mild diastolic dysfunction. Similarly, while the *ex vivo* perfused hearts from DIO displayed diastolic dysfunction, echocardiographic assessment did not reveal any dysfunction.

This paper therefore suggests that systemic adaptations may contribute to maintain cardiac function in models of obesity, masking the development of diabetic cardiomyopathy (DCM) in these hearts. This concept is supported in a study by Van den Bergh and colleagues [84], where *in vivo* pressure-volume recordings were evaluated in *db/db* mice. They found that load-independent parameters revealed cardiac dysfunction, whereas load-dependent parameters showed maintained function,[84] signifying that favourable haemodynamic loading conditions improved cardiac function. Our paper concludes that isolated perfused working hearts may be a valuable technique for functional assessment, as it can give additional information about the intrinsic function in the heart, which may be of major relevance for cardiac phenotyping in obesity and diabetes.

Metabolic and energetic phenotype in obesity

The obese and diabetic heart undergoes metabolic reprogramming to adapt to the altered systemic milieu. As a consequence of increased levels of circulatory fatty acids (FAs), clinical[10; 11] and experimental studies[12; 14; 50] report increased myocardial FA oxidation, with concomitant decreased glucose oxidation. Accompanying these changes is an increase in myocardial oxygen consumption (MVO_2), which may lead to a decrease in cardiac efficiency.[10; 11; 14; 64] Correspondingly, hearts from diet-induced obese (DIO) mice showed increased FA oxidation and decreased mechanical efficiency (paper 2). A metabolic shift from using glucose as the sole substrate to using only FAs, has been calculated to cause a theoretical 12% increase in O_2 consumption.[85] Notably however, this FA-induced increase in MVO_2 cannot account for 30-50% increase in MVO_2 reported in obese and diabetic models both in our present and previous studies.[14; 64; 86] In support of this, Boardman et al. showed that stimulating or impeding FA oxidation did not alter MVO_2, but rather that the *presence* of elevated levels of FAs caused increased oxygen consumption.[86] This suggests that other mechanisms contribute to the FA-induced increased MVO_2. A range of reported alterations that are associated with obesity and DCM might contribute to the increased oxygen waste in these hearts (Fig. 1), including increased oxidative stress, mitochondrial dysfunction, altered Ca^{2+} handling and disadvantageous FA cycling.

Both hyperglycaemia and diabetes are thought to directly impair the cardiac endogenous antioxidative capacity, and obesity and diabetes are associated with increased cardiac production of reactive oxygen species (ROS), both from mitochondrial and non-mitochondrial sources.[87] Accordingly, increased tissue content of ROS and oxidative stress has been reported in both obese and diabetic hearts,[14; 64; 88] and is corroborated by the findings in hearts from DIO mice in this thesis (paper 2). Although the direct link between increased ROS production and oxygen consumption has yet to be fully elucidated, ROS-mediated signalling and changes in the redox state, are likely to affect Ca^{2+} handling and mitochondrial function,[8; 87] giving reason to assume that they play a role in the development of cardiac mechanical inefficiency.

Mitochondrial dysfunction has been described in human diabetic hearts, showing impaired respiratory capacity and increased sensitivity to Ca^{2+}-induced opening of the mitochondrial permeability transition pore (mPTP).[89; 90] FA-mediated mitochondrial dysfunction has also been reported in experimental studies of obesity and diabetes,[14; 91] possibly triggered by the augmented generation of ROS.[29] Accordingly, isolated mitochondria from DIO hearts were found to have decreased coupled respiration (OxPhos) when compared to control hearts in our study (paper 2), with concomitant increased mitochondrial lysine acetylation. Increased acetylation has been associated with Complex I impairment,[92] which could explain the decrease in OxPhos activity.

Myocardial Ca^{2+} handling has been shown to be altered in cardiomyocytes from models of obesity and diabetes.[16; 93] Interestingly, the increased MVO_2 in *db/db* hearts has been shown to be associated with an increased O_2-cost for excitation-contraction (E-C) coupling.[86] In accordance with this, DIO hearts showed increased MVO_2 for E-C coupling when perfused under normal FA conditions (paper 2). Altered redox regulation is a possible mediator of these changes, resulting in increased Ca^{2+} leak from the sarcoplasmic reticulum through ryanodine receptors (RyR) and reduction in sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) 2a activity.[94; 95] Accordingly, aggravated leakage of Ca^{2+} through RYR and suppression of SERCA2a function has been reported in *db/db* mice,[93; 96] which ultimately increases Ca^{2+} recycling and thus the subsequent oxygen cost of E-C coupling.

Taken together, our previous and current studies show that the increased work-independent oxygen consumption in the obese and diabetic heart is likely associated with a variety of

processes, and cannot solely be explained by the higher O_2 requirement associated with the augmented FA oxidation.

Effects of an acute FA elevation

During acute physiological or pathophysiological stress, the circulating levels of free FAs are increased due to an increased lipolysis in white adipose tissue, initiated by an adrenergic activation of hormone-sensitive lipase.[3; 51] This catecholamine-induced increase in plasma FAs is considered unfavourable in normal hearts, as it is associated with increased ROS production,[16] altered mitochondrial function[16; 91] and increased MVO_2 with subsequent mechanical inefficiency.[48; 76] Whether an acute increase in FAs induces the same detrimental effects in obese and diabetic hearts, is less known. Hence, in paper 2, hearts from both normal and obese mice were exposed to low or high levels of palmitate. The rationale for comparing high FAs to low levels of FAs, as opposed to no FAs, was based on the fact that FAs are the preferred substrate in normal physiological settings and never completely absent.

Our study showed that hearts from DIO mice were able to handle an exposure to high FAs differently than normal hearts. While hearts from normal mice displayed a FA-mediated increase in MVO_2 and mechanical inefficiency, these changes were not seen in DIO hearts. Our results corroborate previous studies where control animals displayed an increase in MVO_2 in response to acute exposure to FAs, while *db/db* mice did not,[13; 86] indicating that diabetic hearts do not display the same FA-induced detriments as lean hearts. Notably, MVO_2 in the DIO hearts was unchanged despite the palmitate-induced increase in FA oxidation, supporting the fact that other mechanisms contribute to the FA-induced O_2 waste, rather than the increased O_2 cost of FA oxidation being the sole explanation (as discussed in the previous section).

We also saw a FA-mediated increase in cardiac ROS content and decrease in mitochondrial oxidative respiration in hearts from normal, but not DIO mice. Additionally, although mitochondrial proteins from DIO hearts had an endogenous higher degree of lysine acetylation, increasing the FA concentration in the perfusate only increased acetylation levels in normal hearts. Other studies have also shown that hearts from obese and lean animals have different ability to handle FAs, showing that the administration of palmitate is favourable in cardiomyocytes from obese and diabetic mice, while causing impaired Ca^{2+} handling, dissipation of mitochondrial membrane potential and increased ROS production in controls.[16-

[19] These observations, along with our own, indicate that FAs, while detrimental to lean control hearts, are less disadvantageous in obese hearts.

Metabolic adaptation and ischemia

Increased circulating FAs following the hyperadrenergic drive in acute myocardial infarction (MI) is considered disadvantageous in the normal heart, as the adverse effects of FAs (described in the previous section) pose an additional challenge in a hypoxic state. Accordingly, several experimental studies have shown that perfusing normal hearts with high levels of FAs decreases ischemic tolerance.[52-54] Similarly, clinical studies have also shown that patients with diabetes mellitus are particularly susceptible to various stresses, including ischemia and reperfusion.[55] Although the underlying mechanisms causing ischemic intolerance in these patients have not been fully elucidated, it is likely that the pathophysiological alterations associated with the development of DCM play a role. Importantly, however, the reported ischemic tolerance in preclinical diabetic models varies in the literature.[56-58]

In paper 2, the post-ischemic recovery and infarct size were determined following perfusion with both low and high levels of FAs in normal and DIO hearts. The ischemic susceptibility was not increased with high FAs in normal hearts in our study, however this may be related to mouse strain differences and, more importantly, the ischemia-reperfusion protocol and the resulting severity of the ischemic insult. Ischemic injury in DIO hearts on the other hand, was increased when perfused with low FAs, but, to our surprise, DIO hearts showed signs of improved ischemic tolerance when perfused with high FAs. Thus, FAs did not cause aggravation of ischemic injury in hearts from obese mice, but might conversely be advantageous.

The effects following elevated levels of FAs do not necessarily pose a risk to normal hearts under physiological conditions. FA-induced ROS production and mitochondrial acetylation may have signalling roles,[24; 41; 42] inducing adaptation to match the increased FA uptake, and a FA-mediated transient reduction in mechanical efficiency is not detrimental in a healthy heart. However, in pathophysiological conditions where oxygen supply is limited, such as MI, the effects of FAs are unfavourable, causing oxygen wasting in a hypoxic state. Contrastingly, the diabetic heart is continuously challenged by sustained hyperlipidaemia, causing metabolic remodelling, making these hearts uniquely adapted to high FA conditions. Accordingly, it has been suggested that the adaptive channelling of FAs in obese and diabetic hearts ensures a

sufficient supply of reducing equivalents to prevent ROS production and impaired energetics,[17] and that the loss of metabolic flexibility in these hearts render them energy starved when the FA concentration is low.[82] Our study shows that in MI, the increase in FAs does not represent an additive stress in hearts from obese mice, but contrastingly might be advantageous. Thus, although obese and diabetic hearts show increased susceptibility to ischemic damage compared to healthy controls, the acute elevation of FAs does not seem to be the cause of this, suggesting that other factors might contribute to the worsened outcome in obese patients.

Angiotensin II-induced cardiac failure

Angiotensin II (AngII) is a well-known modulator of NADPH oxidase 2 (NOX2)-mediated ROS production, having major impact on the pathogenesis of different types of heart failure.[25; 26; 31; 72] Although the renin-angiotensin system (RAS) has been studied extensively, its effect on the cardiac metabolic remodelling is not fully described. In this thesis, we studied the effects of a slow-pressor dose of AngII on cardiac function, MVO_2 and substrate utilization (paper 3). In order to also elucidate the role of NOX2 in AngII-induced cardiac pathology, we studied the effects in transgenic (TG) male and female mice with cardiac-specific overexpression of NOX2 (csNOX2).

Although the low dose of AngII induced hypertrophy in male mice, we did not find changes in cardiac function, substrate utilization, MVO_2 or mechanical efficiency. Contrastingly, AngII-induced systolic and diastolic dysfunction was observed in male csNOX2 TG mice. Hence, the study adds to the evidence of NOX2s role in AngII-induced hypertrophy and cardiac failure. Hearts from male TG mice also showed a progression towards eccentric hypertrophy and ventricular dilation. Interestingly, TG males also displayed increase in pressures earlier than wild types (WT). As NOX2-overxpression was cardiac-specific, it suggests that the NOX2-mediated increase in blood pressure stems from processes originating in the heart. However, compared to other studies reporting AngII-mediated increases in blood pressure,[75; 97-99] the elevation in both systolic and mean blood pressure was subtle in our study. This suggests that the observed cardiac phenotype was a result of direct effects in the heart, as opposed to pressure-induced.

Cardiac-specific transgenic mice overexpressing angiotensinogen have been reported to have an increase in glucose oxidation when also displaying cardiac failure.[100] Similarly, Choi and

colleagues showed that 4 weeks of treatment with an AngII dose 2 times higher than our dose, induced diastolic dysfunction, with reduction in FA oxidation and increased utilization of glucose.[23] Our study corroborates these findings, as AngII-treated male TG mice displayed cardiac failure with a concomitant metabolic switch towards glucose. Metabolic alterations were however not present in the absence of cardiac dysfunction in either our study or the study by Pellieux and colleagues.[100] This suggests that metabolic alterations are preceded by cardiac dysfunction in AngII-mediated pathology, contrasting metabolic remodelling in diabetes. Pellieux and co-workers have also described that an AngII-mediated decrease in FA oxidation was prevented by inhibition of ROS activity.[101] Thus, as increased ROS content triggered a metabolic switch in both this and our own study, this supports the notion that oxidative stress is a causative factor in AngII-mediated metabolic remodelling. Whether this AngII- and ROS-induced metabolic alteration is adaptive or maladaptive remains unclear,[102] and is potentially an important question to be answered regarding future therapy, particularly when considering the fact that the current treatment protocols largely rely on suppressing the RAS.

Angiotensin II in females

There are distinct differences between males and females in terms of clinical presentation and frequency of heart failure,[103] with females showing decreased development of fibrosis and a different hypertrophic phenotype.[104-106] Sex hormones greatly influence these differences, and oestrogen replacement therapy is suggested to be protective in terms of development of cardiac failure and hypertension in both males and ovariectomized females.[104; 107; 108] Accordingly, we found that females did not develop hypertrophy following AngII treatment, in neither WT nor TG hearts (paper 3). Similarly, the AngII-induced increase in blood pressure was more pronounced in males than in females, which has also been shown in previous studies.[97; 107] The discrepancies between the AngII-mediated effects have been suggested to be associated with ROS production and several studies have shown that NOX-mediated production of superoxide is correlated with increased blood pressure in males and ovariectomized females.[97; 109] Accordingly, the decreased susceptibility to cardiac disease has been attributed to an enhanced antioxidative ability in females,[105] signifying that protection from the AngII-mediated effects in the female mice in the present study may well be due to an oestrogen-mediated enhancement of the ability to modulate ROS production.

CONCLUDING REMARKS

Even though it is well established that metabolic alterations occur in cardiac disease, whether altered substrate metabolism is an advantageous adaptation or maladaptive, remains ambiguous. In fact, it is likely that the same metabolic phenotype is both beneficial and detrimental depending on the conditions, thus making it difficult to dichotomize alterations as purely adaptive or maladaptive. This can be exemplified by the finding in the present thesis, showing that obese hearts, even though they display remodelling, dysfunction and changed substrate utilization, also have increased ischemic tolerance when exposed to high levels of FAs. However, as disease progresses, initial adaptation might become maladaptive and cause progression of disease, i.e. mitochondrial dysfunction, increased ROS production and impaired Ca^{2+} handling. Furthermore, the failing heart is associated with a mismatch between energy supply and demand, as indicated by a reduced phosphocreatine-to-ATP (PCr/ATP) ratio. Energetic imbalance limits the contractile reserve, which may cause contractile dysfunction and the loss of inotropic reserve, which is a characteristic of the myocardium in HF.

Several studies have shown that interventions that prevent or reverse metabolic alterations impede the progression of HF, and that prevention of shifts in substrate utilization is beneficial. Notably however, as cardiac metabolic alterations can be both advantageous and disadvantageous depending on the disease progression, it can be derived that it is not necessarily specific alterations in substrate utilization *per se* that cause adverse effects, but rather the loss of metabolic flexibility. The issue of whether heart failure leads to "metabolic failure" or, conversely, whether changes in metabolism are the primary cause of heart failure, remains uncertain. Regardless of primacy however, changes in substrate utilization and function are probably so intimately linked, that the distinction or characterization becomes less pertinent. The scope of future research should rather be to further elucidate the mechanisms of cardiac metabolic regulation and how a normal phenotype can be maintained, thus preserving metabolic flexibility in the failing heart.

REFERENCES

1. **Rubler S, Dlugash J, Yuceoglu YZ, Kumral T, Branwood AW and Grishman A**. New type of cardiomyopathy associated with diabetic glomerulosclerosis. *The American Journal of Cardiology* 30: 595-602, 1972.

2. **Boyer JK, Thanigaraj S, Schechtman KB and Pérez JE**. Prevalence of ventricular diastolic dysfunction in asymptomatic, normotensive patients with diabetes mellitus. *The American Journal of Cardiology* 93: 870-875, 2004.

3. **Lopaschuk GD, Ussher JR, Folmes CDL, Jaswal JS and Stanley WC**. Myocardial fatty acid metabolism in health and disease. *Physiological Reviews* 90: 207-258, 2010.

4. **Steinberg BA, Zhao X, Heidenreich PA, Peterson ED, Bhatt DL, Cannon CP, Hernandez AF and Fonarow GC**. Trends in patients hospitalized with heart failure and preserved left ventricular ejection fraction. *Circulation* 126: 65-75, 2012.

5. **Klapholz M, Maurer M, Lowe AM, Messineo F, Meisner JS, Mitchell J, Kalman J, Phillips RA, Steingart R, Brown EJ, Berkowitz R, Moskowitz R, Soni A, Mancini D, Bijou R, Sehhat K, Varshneya N, Kukin M, Katz SD, Sleeper LA and Le Jemtel TH**. Hospitalization for heart failure in the presence of a normal left ventricular ejection fraction: Results of the New York heart failure registry. *Journal of the American College of Cardiology* 43: 1432-1438, 2004.

6. **Goyal P, Almarzooq ZI, Horn EM, Karas MG, Sobol I, Swaminathan RV, Feldman DN, Minutello RM, Singh HS, Bergman GW, Wong SC and Kim LK**. Characteristics of hospitalizations for heart failure with preserved ejection fraction. *The American Journal of Medicine* 129: 635, 2016.

7. **Bugger H and Abel ED**. Molecular mechanisms of diabetic cardiomyopathy. *Diabetologia* 57: 660-671, 2014.

8. **Hafstad AD, Boardman N and Aasum E**. How exercise may amend metabolic disturbances in diabetic cardiomyopathy. *Antioxidants & Redox Signaling* 22: 1587-1605, 2015.

9. **Bertero E and Maack C**. Metabolic remodelling in heart failure. *Nature Reviews Cardiology* 15: 457-470, 2018.

10. **Herrero P, Peterson LR, McGill JB, Matthew S, Lesniak D, Dence C and Gropler RJ**. Increased myocardial fatty acid metabolism in patients with type 1 diabetes mellitus. *Journal of the American College of Cardiology* 47: 598-604, 2006.

11. **Peterson LR, Herrero P, Schechtman KB, Racette SB, Waggoner AD, Kisrieva-Ware Z, Dence C, Klein S, Marsala J, Meyer T and Gropler RJ**. Effect of obesity and insulin resistance on myocardial substrate metabolism and efficiency in young women. *Circulation* 109: 2191-2196, 2004.

12. **Belke DD, Larsen TS, Gibbs EM and Severson DL**. Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (*db/db*) mice. *American Journal of Physiology-Endocrinology and Metabolism* 279: E1104-E1113, 2000.

13. **How OJ, Aasum E, Severson DL, Chan WYA, Essop MF and Larsen TS**. Increased myocardial oxygen consumption reduces cardiac efficiency in diabetic mice. *Diabetes* 55: 466-473, 2006.

14. **Hafstad AD, Lund J, Hadler-Olsen E, Höper AC, Larsen TS and Aasum E**. High- and moderate-intensity training normalizes ventricular function and mechanoenergetics in mice with diet-induced obesity. *Diabetes* 62: 2287-2294, 2013.

15. **Hansen SS, Aasum E and Hafstad AD**. The role of NADPH oxidases in diabetic cardiomyopathy. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1864: 1908-1913, 2018.

16. **Fauconnier J, Andersson DC, Zhang SJ, Lanner JT, Wibom R, Katz A, Bruton JD and Westerblad H**. Effects of palmitate on Ca^{2+} handling in adult control and *ob/ob* cardiomyocytes. *Diabetes* 56: 1136, 2007.

17. **Tocchetti CG, Caceres V, Stanley BA, Xie C, Shi S, Watson WH, O'Rourke B, Spadari-Bratfisch RC, Cortassa S, Akar FG, Paolocci N and Aon MA**. GSH or palmitate preserves mitochondrial energetic/redox balance, preventing mechanical dysfunction in metabolically challenged myocytes/hearts from type 2 diabetic mice. *Diabetes* 61: 3094, 2012.

18. **Bhatt NM, Aon MA, Tocchetti CG, Shen X, Dey S, Ramirez-Correa G, O'Rourke B, Gao WD and Cortassa S**. Restoring redox balance enhances contractility in heart trabeculae from type 2 diabetic rats exposed to high glucose. *American Journal of Physiology-Heart and Circulatory Physiology* 308: H291-H302, 2014.

19. **Smith W, Norton GR, Woodiwiss AJ, Lochner A and du Toit EF**. Dependence of cardiac systolic function on elevated fatty acid availability in obese, insulin-resistant rats. *Journal of Cardiac Failure* 22: 560-568, 2016.

20. **Zablocki D and Sadoshima J**. Angiotensin II and oxidative stress in the failing heart. *Antioxidants & Redox Signaling* 19: 1095-1109, 2012.

21. **Mervaala E, Biala A, Merasto S, Lempiäinen J, Mattila I, Martonen E, Eriksson O, Louhelainen M, Finckenberg P, Kaheinen P, Muller DN, Luft FC, Lapatto R and Oresic M**. Metabolomics in angiotensin II-induced cardiac hypertrophy. *Hypertension* 55: 508-515, 2010.

22. **Arsenault M, Zendaoui A, Roussel É, Drolet M, Dhahri W, Grenier A, Gascon S, Sarrhini O, Rousseau JA, Lecomte R and Couet J**. Angiotensin II-converting enzyme inhibition improves survival, ventricular remodeling, and myocardial

energetics in experimental aortic regurgitation. *Circulation: Heart Failure* 6: 1021-1028, 2013.

23. **Choi YS, de Mattos ABM, Shao D, Li T, Nabben M, Kim M, Wang W, Tian R and Kolwicz SC**. Preservation of myocardial fatty acid oxidation prevents diastolic dysfunction in mice subjected to angiotensin II infusion. *Journal of Molecular and Cellular Cardiology* 100: 64-71, 2016.

24. **Kanaan GN and Harper ME**. Cellular redox dysfunction in the development of cardiovascular diseases. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1861: 2822-2829, 2017.

25. **Byrne JA, Grieve DJ, Bendall JK, Li JM, Gove C, Lambeth JD, Cave AC and Shah AM**. Contrasting roles of NADPH oxidase isoforms in pressure-overload versus angiotensin II-induced cardiac hypertrophy. *Circ Res* 93: 802-805, 2003.

26. **Anilkumar N, Weber R, Zhang M, Brewer A and Shah AM**. NOX4 and NOX2 NADPH oxidases mediate distinct cellular redox signaling responses to agonist stimulation. *Arteriosclerosis, Thrombosis, and Vascular Biology* 28: 1347-1354, 2008.

27. **Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S and Shah AM**. NADPH oxidases in cardiovascular health and disease. *Antioxidants & Redox Signaling* 8: 691-728, 2006.

28. **Ritchie RH, Quinn JM, Cao AH, Drummond GR, Kaye DM, Favaloro JM, Proietto J and Delbridge LMD**. The antioxidant tempol inhibits cardiac hypertrophy in the insulin-resistant GLUT4-deficient mouse in vivo. *Journal of Molecular and Cellular Cardiology* 42: 1119-1128, 2007.

29. **Boudina S, Sena S, Theobald H, Sheng X, Wright JJ, Hu XX, Aziz S, Johnson JI, Bugger H, Zaha VG and Abel ED**. Mitochondrial energetics in the heart in obesity-related diabetes. *Diabetes* 56: 2457, 2007.

30. **Jeong EM, Chung J, Liu H, Go Y, Gladstein S, Farzaneh-Far A, Lewandowski ED and Dudley SC**. Role of mitochondrial oxidative stress in glucose tolerance, insulin resistance, and cardiac diastolic dysfunction. *Journal of the American Heart Association* 5: e003046, 2016.

31. **Bendall JK, Cave AC, Heymes C, Gall C and Shah AM**. Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. *Circulation* 105: 293-296, 2002.

32. **Karwi QG, Uddin GM, Ho KL and Lopaschuk GD**. Loss of metabolic flexibility in the failing heart. *Frontiers in Cardiovascular Medicine* 5: 68, 2018.

33. **Ingwall JS and Weiss RG**. Is the failing heart energy starved? On using chemical energy to support cardiac function. *Circ Res* 95: 135-145, 2004.

34. **Neubauer S, Horn M, Cramer M, Harre K, Newell JB, Peters W, Pabst T, Ertl G, Hahn D, Ingwall JS and Kochsiek K**. Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. *Circulation* 96: 2190-2196, 1997.

35. **Gupta A, Chacko VP and Weiss RG**. Abnormal energetics and ATP depletion in pressure-overload mouse hearts: *in vivo* high-energy phosphate concentration measures by noninvasive magnetic resonance. *American Journal of Physiology-Heart and Circulatory Physiology* 297: H59-H64, 2009.

36. **Conway MA, Allis J, Ouwerkerk R, Niioka T, Rajagopalan B and Radda GK**. Detection of low phosphocreatine to ATP ratio in failing hypertrophied human myocardium by ^{31}P magnetic resonance spectroscopy. *The Lancet* 338: 973-976, 1991.

37. **Abdurrachim D, Nabben M, Hoerr V, Kuhlmann MT, Bovenkamp P, Ciapaite J, Geraets IME, Coumans W, Luiken JJFP, Glatz JFC, Schäfers M, Nicolay K, Faber C, Hermann S and Prompers JJ**. Diabetic *db/db* mice do not develop heart failure upon pressure overload: a longitudinal *in vivo* PET, MRI, and MRS study on cardiac metabolic, structural, and functional adaptations. *Cardiovascular Research* 113: 1148-1160, 2017.

38. **Scheuermann-Freestone M, Madsen PL, Manners D, Blamire AM, Buckingham RE, Styles P, Radda GK, Neubauer S and Clarke K**. Abnormal cardiac and skeletal muscle energy metabolism in patients with type 2 diabetes. *Circulation* 107: 3040-3046, 2003.

39. **Diamant M, Lamb HJ, Groeneveld Y, Endert EL, Smit JWA, Bax JJ, Romijn JA, de Roos A and Radder JK**. Diastolic dysfunction is associated with altered myocardial metabolism in asymptomatic normotensive patients with well-controlled type 2 diabetes mellitus. *Journal of the American College of Cardiology* 42: 328-335, 2003.

40. **Randle PJ**. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes Metab Rev* 14: 263-283, 1998.

41. **Fukushima A and Lopaschuk GD**. Acetylation control of cardiac fatty acid b-oxidation and energy metabolism in obesity, diabetes, and heart failure. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1862: 2211-2220, 2016.

42. **Renguet E, Bultot L, Beauloye C, Horman S and Bertrand L**. The regulation of insulin-stimulated cardiac glucose transport via protein acetylation. *Frontiers in Cardiovascular Medicine* 5: 70, 2018.

43. **Alrob OA, Sankaralingam S, Ma C, Wagg CS, Fillmore N, Jaswal JS, Sack MN, Lehner R, Gupta MP, Michelakis ED, Padwal RS, Johnstone DE, Sharma AM and Lopaschuk GD**. Obesity-induced lysine acetylation increases cardiac fatty acid oxidation and impairs insulin signalling. *Cardiovascular Research* 103: 485-497, 2014.

44. **Thapa D, Zhang M, Manning JR, Guimarães DA, Stoner MW, O'Doherty RM, Shiva S and Scott I**. Acetylation of mitochondrial proteins by GCN5L1 promotes enhanced fatty acid oxidation in the heart. *American Journal of Physiology-Heart and Circulatory Physiology* 313: H265-H274, 2017.

45. **Wende AR and Abel ED**. Lipotoxicity in the heart. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1801: 311-319, 2010.

46. **Schulze PC, Drosatos K and Goldberg IJ**. Lipid use and misuse by the heart. *Circ Res* 118: 1736-1751, 2016.

47. **Bing RJ, Hammond MM, Handelsman JC, Powers SR, Spencer FC, Eckenhoff JE, Goodale WT, Hafkenschiel JH and Kety SS**. The measurement of coronary blood flow, oxygen consumption, and efficiency of the left ventricle in man. *American Heart Journal* 38: 1-24, 1949.

48. **Mjøs OD**. Effect of free fatty acids on myocardial function and oxygen consumption in intact dogs. *J Clin Invest* 50: 1386-1389, 1971.

49. **Boardman NT, Hafstad AD, Larsen TS, Severson DL and Aasum E**. Increased O_2 cost of basal metabolism and excitation-contraction coupling in hearts from type 2 diabetic mice. *American Journal of Physiology-Heart and Circulatory Physiology* 296: H1373-H1379, 2009.

50. **Boudina S, Han YH, Pei S, Tidwell TJ, Henrie B, Tuinei J, Olsen C, Sena S and Abel ED**. UCP3 regulates cardiac efficiency and mitochondrial coupling in high fat-fed mice but not in leptin-deficient mice. *Diabetes* 61: 3260, 2012.

51. **Oliver MF, Kurien VA and Greenwood TW**. Relation between serum-free-fatty-acids and arrhythmias and death after acute myocardial infarction. *The Lancet* 291: 710-715, 1968.

52. **Gambert S, Vergely C, Filomenko R, Moreau D, Bettaieb A, Opie LH and Rochette L**. Adverse effects of free fatty acid associated with increased oxidative stress in postischemic isolated rat hearts. *Molecular and Cellular Biochemistry* 283: 147-152, 2006.

53. **du Toit EF, Smith W, Muller C, Strijdom H, Stouthammer B, Woodiwiss AJ, Norton GR and Lochner A**. Myocardial susceptibility to ischemic-reperfusion injury in a prediabetic model of dietary-induced obesity. *American Journal of Physiology-Heart and Circulatory Physiology* 294: H2336-H2343, 2008.

54. **Dalgas C, Povlsen JA, Løfgren B, Erichsen SB and Bøtker HE**. Effects of fatty acids on cardioprotection by pre-ischaemic inhibition of the malate-aspartate shuttle. *Clin Exp Pharmacol Physiol* 39: 878-885, 2012.

55. **Stone PH, Muller JE, Hartwell T, York BJ, Rutherford JD, Parker CB, Turi ZG, Strauss HW, Willerson JT, Robertson T, Braunwald E and Jaffe AS**. The effect of diabetes mellitus on prognosis and serial left ventricular function after acute myocardial infarction: Contribution of both coronary disease and diastolic left ventricular dysfunction to the adverse prognosis. *Journal of the American College of Cardiology* 14: 49-57, 1989.

56. **Miki T, Itoh T, Sunaga D and Miura T**. Effects of diabetes on myocardial infarct size and cardioprotection by preconditioning and postconditioning. *Cardiovascular Diabetology* 11: 67, 2012.

57. **Whittington H, Ganesha Babu G, Mocanu M, Yellon M and Hausenloy J**. The diabetic heart: Too sweet for its own good? *Cardiology Research and Practice* 2012: 845698, 2012.

58. **Apaijai N, Chattipakorn SC and Chattipakorn N**. Roles of obese-insulin resistance and anti-diabetic drugs on the heart with ischemia-reperfusion injury. *Cardiovascular Drugs and Therapy* 28: 549-562, 2014.

59. **Surwit RS, Feinglos MN, Rodin J, Sutherland A, Petro AE, Opara EC, Kuhn CM and Rebuffe-Scrive M**. Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and AJ mice. *Metabolism* 44: 645-651, 1995.

60. **Collins S, Martin TL, Surwit RS and Robidoux J**. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. *Physiology & Behavior* 81: 243-248, 2004.

61. **Nicholson A, Reifsnyder PC, Malcolm R, Lucas CA, MacGregor GR, Zhang W and Leiter EH**. Diet induced obesity in two C57BL/6 substrains with intact or mutant Nicotinamide Nucleotide Transhydrogenase (Nnt) gene. *Obesity (Silver Spring)* 18: 1902-1905, 2010.

62. **Collins S, Daniel KW, Petro AE and Surwit RS**. Strain-specific response to b_3-adrenergic receptor agonist treatment of diet-induced obesity in mice. *endo* 138: 405-413, 1997.

63. **Prpic V, Watson PM, Frampton IC, Sabol MA, Jezek GE and Gettys TW**. Differential mechanisms and development of leptin resistance in A/J versus C57BL/6J mice during diet-induced obesity. *endo* 144: 1155-1163, 2003.

64. **Lund J, Hafstad AD, Boardman NT, Rossvoll L, Rolim NP, Ahmed MS, Florholmen G, Attramadal H, Wisløff U, Larsen TS and Aasum E**. Exercise training promotes cardioprotection through oxygen-sparing action in high fat-fed

mice. *American Journal of Physiology-Heart and Circulatory Physiology* 308: H823-H829, 2015.

65. **Abdurrachim D, Ciapaite J, Wessels B, Nabben M, Luiken JJFP, Nicolay K and Prompers JJ**. Cardiac diastolic dysfunction in high-fat diet fed mice is associated with lipotoxicity without impairment of cardiac energetics in vivo. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1841: 1525-1537, 2014.

66. **Zeng H, Vaka VR, He X, Booz GW and Chen JX**. High-fat diet induces cardiac remodelling and dysfunction: assessment of the role played by SIRT3 loss. *J Cell Mol Med* 19: 1847-1856, 2015.

67. **Aasum E, Hafstad AD, Severson DL and Larsen TS**. Age-dependent changes in metabolism, contractile function, and ischemic sensitivity in hearts from *db/db* mice. *Diabetes* 52: 434-441, 2003.

68. **Buchanan J, Mazumder PK, Hu P, Chakrabarti G, Roberts MW, Yun UY, Cooksey RC, Litwin SE and Abel ED**. Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity. *endo* 146: 5341-5349, 2005.

69. **Yue P, Arai T, Terashima M, Sheikh AY, Cao F, Charo D, Hoyt G, Robbins RC, Ashley EA, Wu J, Yang PC and Tsao PS**. Magnetic resonance imaging of progressive cardiomyopathic changes in the *db/db* mouse. *American Journal of Physiology-Heart and Circulatory Physiology* 292: H2106-H2118, 2007.

70. **Dludla PV, Essop MF, Gabuza KB, Muller CJF, Louw J and Johnson R**. Age-dependent development of left ventricular wall thickness in type 2 diabetic (*db/db*) mice is associated with elevated low-density lipoprotein and triglyceride serum levels. *Heart and Vessels* 32: 1025-1031, 2017.

71. **Aasum E, Cooper M, Severson DL and Larsen TS**. Effect of BM 17.0744, a PPARα ligand, on the metabolism of perfused hearts from control and diabetic mice. *Can J Physiol Pharmacol* 83: 183-190, 2005.

72. **Zhang M, Prosser BL, Bamboye MA, Gondim ANS, Santos CX, Martin D, Ghigo A, Perino A, Brewer AC, Ward CW, Hirsch E, Lederer WJ and Shah AM**. Contractile function during angiotensin-II activation: Increased NOX2 activity modulates cardiac calcium handling via phospholamban phosphorylation. *Journal of the American College of Cardiology* 66: 261-272, 2015.

73. **Simon G, Abraham G and Cserep G**. Pressor and subpressor angiotensin II administration: Two experimental models of hypertension. *American Journal of Hypertension* 8: 645-650, 1995.

74. **Kawada N, Imai E, Karber A, Welch WJ and Wilcox CS**. A mouse model of angiotensin II slow pressor response: role of oxidative stress. *J Am Soc Nephrol* 13: 2860, 2002.

75. **Brand S, Amann K and Schupp N**. Angiotensin II-induced hypertension dose-dependently leads to oxidative stress and DNA damage in mouse kidneys and hearts. *Journal of Hypertension* 31: 2013.

76. **How OJ, Aasum E, Kunnathu S, Severson DL, Myhre ESP and Larsen TS**. Influence of substrate supply on cardiac efficiency, as measured by pressure-volume analysis in *ex vivo* mouse hearts. *Am J Physiol Heart Circ Physiol* 288: H2979, 2005.

77. **Suga H**. Total mechanical energy of a ventricle model and cardiac oxygen consumption. *American Journal of Physiology-Heart and Circulatory Physiology* 236: H498-H505, 1979.

78. **Suga H**. Ventricular energetics. *Physiological Reviews* 70: 247-277, 1990.

79. **Barr RL and Lopaschuk GD**. Methodology for measuring *in vitro*/*ex vivo* cardiac energy metabolism. *Journal of Pharmacological and Toxicological Methods* 43: 141-152, 2000.

80. **Luptak I, Balschi JA, Xing Y, Leone TC, Kelly DP and Tian R**. Decreased contractile and metabolic reserve in peroxisome proliferator-activated receptor-α-null hearts can be rescued by increasing glucose transport and utilization. *Circulation* 112: 2339-2346, 2005.

81. **How OJ, Larsen TS, Hafstad AD, Khalid A, Myhre ESP, Murray AJ, Boardman NT, Cole M, Clarke K, Severson DL and Aasum E**. Rosiglitazone treatment improves cardiac efficiency in hearts from diabetic mice. *Archives of Physiology and Biochemistry* 113: 211-220, 2007.

82. **Bertero E, Sequeira V and Maack C**. Let's face the fats: palmitate restores cellular redox state in the diabetic heart. *J Physiol* 0: 2018.

83. **Semeniuk LM, Kryski AJ and Severson DL**. Echocardiographic assessment of cardiac function in diabetic *db/db* and transgenic *db/db*-hGLUT4 mice. *American Journal of Physiology-Heart and Circulatory Physiology* 283: H976-H982, 2002.

84. **Van den Bergh A, Flameng W and Herijgers P**. Type II diabetic mice exhibit contractile dysfunction but maintain cardiac output by favourable loading conditions. *European Journal of Heart Failure* 8: 777-783, 2006.

85. **Opie LH and Lopaschuk GD**. Fuels: Aerobic and anaerobic metabolism. In: *Heart physiology: from cell to circulation*, edited by Opie LH. Philadelphia: Lippincott Williams & Wilkins, 2004.

86. **Boardman NT, Larsen TS, Severson DL, Essop MF and Aasum E**. Chronic and acute exposure of mouse hearts to fatty acids increases oxygen cost of excitation-contraction coupling. *American Journal of Physiology-Heart and Circulatory Physiology* 300: H1631-H1636, 2011.

87. **Huynh K, Bernardo BC, McMullen JR and Ritchie RH**. Diabetic cardiomyopathy: Mechanisms and new treatment strategies targeting antioxidant signaling pathways. *Pharmacology & Therapeutics* 142: 375-415, 2014.

88. **Kajstura J, Fiordaliso F, Andreoli AM, Li B, Chimenti S, Medow MS, Limana F, Nadal-Ginard B, Leri A and Anversa P**. IGF-1 overexpression inhibits the development of diabetic cardiomyopathy and angiotensin II-mediated oxidative stress. *Diabetes* 50: 1414, 2001.

89. **Anderson EJ, Kypson AP, Rodriguez E, Anderson CA, Lehr EJ and Neufer PD**. Substrate-specific derangements in mitochondrial metabolism and redox balance in the atrium of the type 2 diabetic human heart. *Journal of the American College of Cardiology* 54: 1891-1898, 2009.

90. **Anderson EJ, Rodriguez E, Anderson CA, Thayne K, Chitwood WR and Kypson AP**. Increased propensity for cell death in diabetic human heart is mediated by mitochondrial-dependent pathways. *American Journal of Physiology-Heart and Circulatory Physiology* 300: H118-H124, 2010.

91. **Boudina S, Sena S, O'Neill BT, Tathireddy P, Young ME and Abel ED**. Reduced mitochondrial oxidative capacity and increased mitochondrial uncoupling impair myocardial energetics in obesity. *Circulation* 112: 2686-2695, 2005.

92. **Vazquez EJ, Berthiaume JM, Kamath V, Achike O, Buchanan E, Montano MM, Chandler MP, Miyagi M and Rosca MG**. Mitochondrial complex I defect and increased fatty acid oxidation enhance protein lysine acetylation in the diabetic heart. *Cardiovascular Research* 107: 453-465, 2015.

93. **Belke DD, Swanson EA and Dillmann WH**. Decreased sarcoplasmic reticulum activity and contractility in diabetic *db/db* mouse heart. *Diabetes* 53: 3201, 2004.

94. **Kuster GM, Lancel S, Zhang J, Communal C, Trucillo MP, Lim CC, Pfister O, Weinberg EO, Cohen RA, Liao R, Siwik DA and Colucci WS**. Redox-mediated reciprocal regulation of SERCA and Na^+-Ca^{2+} exchanger contributes to sarcoplasmic reticulum Ca^{2+} depletion in cardiac myocytes. *Free Radical Biology and Medicine* 48: 1182-1187, 2010.

95. **Santos CXC, Hafstad AD, Beretta M, Zhang M, Molenaar C, Kopec J, Fotinou D, Murray TV, Cobb AM, Martin D, Zeh Silva M, Anilkumar N, Schröder K, Shanahan CM, Brewer AC, Brandes RP, Blanc E, Parsons M, Belousov V, Cammack R, Hider RC, Steiner RA and Shah AM**. Targeted redox inhibition of

protein phosphatase 1 by Nox4 regulates eIF2a-mediated stress signaling. *EMBO J* 2016/01/07: 319-334, 2016.

96. **Stølen TO, Høydal MA, Kemi OJ, Catalucci D, Ceci M, Aasum E, Larsen T, Rolim N, Condorelli G, Smith GL and Wisløff U**. Interval training normalizes cardiomyocyte function, diastolic Ca^{2+} control, and SR Ca^{2+} release synchronicity in a mouse model of diabetic cardiomyopathy. *Circ Res* 105: 527-536, 2009.

97. **Ebrahimian T, He Y, Schiffrin EL and Touyz RM**. Differential regulation of thioredoxin and NAD(P)H oxidase by angiotensin II in male and female mice. *Journal of Hypertension* 25: 1263-1271, 2007.

98. **Glenn DJ, Cardema MC, Ni W, Zhang Y, Yeghiazarians Y, Grapov D, Fiehn O and Gardner DG**. Cardiac steatosis potentiates angiotensin II effects in the heart. *American Journal of Physiology-Heart and Circulatory Physiology* 308: H339-H350, 2014.

99. **Wang Q, Wang H, Wang J, Venugopal J, Kleiman K, Guo C, Sun Y and Eitzman DT**. Angiotensin II-induced hypertension is reduced by deficiency of P-selectin glycoprotein ligand-1. *Scientific Reports* 8: 3223, 2018.

100. **Pellieux C, Aasum E, Larsen TS, Montessuit C, Papageorgiou I, Pedrazzini T and Lerch R**. Overexpression of angiotensinogen in the myocardium induces downregulation of the fatty acid oxidation pathway. *Journal of Molecular and Cellular Cardiology* 41: 459-466, 2006.

101. **Pellieux C, Montessuit C, Papageorgiou I and Lerch R**. Angiotensin II downregulates the fatty acid oxidation pathway in adult rat cardiomyocytes via release of tumour necrosis factor-α. *Cardiovascular Research* 82: 341-350, 2009.

102. **Lopaschuk GD**. Cardiac energy metabolism alterations in angiotensin II induced hypertrophy. *Journal of Molecular and Cellular Cardiology* 41: 418-420, 2006.

103. **Levinsson A, Dubé MP, Tardif JC and de Denus S**. Sex, drugs, and heart failure: a sex-sensitive review of the evidence base behind current heart failure clinical guidelines. *ESC Heart Fail* 5: 745-754, 2018.

104. **Wang M, Tsai BM, Reiger KM, Brown JW and Meldrum DR**. 17-β-Estradiol decreases p38 MAPK-mediated myocardial inflammation and dysfunction following acute ischemia. *Journal of Molecular and Cellular Cardiology* 40: 205-212, 2006.

105. **Lagranha CJ, Deschamps A, Aponte A, Steenbergen C and Murphy E**. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. *Circ Res* 106: 1681-1691, 2010.

106. **Fliegner D, Schubert C, Penkalla A, Witt H, Kararigas G, Dworatzek E, Staub E, Martus P, Noppinger PR, Kintscher U, Gustafsson JA and Regitz-Zagrosek V**. Female sex and estrogen receptor-β attenuate cardiac remodeling and apoptosis in pressure overload. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 298: R1597-R1606, 2010.

107. **Xue B, Pamidimukkala J and Hay M**. Sex differences in the development of angiotensin II-induced hypertension in conscious mice. *American Journal of Physiology-Heart and Circulatory Physiology* 288: H2177-H2184, 2005.

108. **Iorga A, Li J, Sharma S, Umar S, Bopassa JC, Nadadur RD, Centala A, Ren S, Saito T, Toro L, Wang Y, Stefani E and Eghbali M**. Rescue of pressure overload-induced heart failure by estrogen therapy. *Journal of the American Heart Association* 5: e002482, 2016.

109. **Dantas AP, Franco MdoC, Silva-Antonialli MM, Tostes RCA, Fortes ZB, Nigro D and Carvalho MH**. Gender differences in superoxide generation in microvessels of hypertensive rats: role of NAD(P)H-oxidase. *Cardiovascular Research* 61: 22-29, 2004.

PAPER I

PLOS ONE

RESEARCH ARTICLE

Isolated perfused working hearts provide valuable additional information during phenotypic assessment of the diabetic mouse heart

Tina M. Pedersen, Neoma T. Boardman, Anne D. Hafstad, Ellen Aasum*

Cardiovascular Research Group, Department of Medical Biology, Faculty of Health Sciences, UiT-The Arctic University of Norway, Tromsø, Norway

* ellen.aasum@uit.no

OPEN ACCESS

Citation: Pedersen TM, Boardman NT, Hafstad AD, Aasum E (2018) Isolated perfused working hearts provide valuable additional information during phenotypic assessment of the diabetic mouse heart. PLoS ONE 13(10): e0204843. https://doi.org/10.1371/journal.pone.0204843

Editor: Jun Yu, Temple University School of Medicine, UNITED STATES

Received: January 30, 2018

Accepted: September 14, 2018

Published: October 1, 2018

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by grants from The Norwegian Council on Cardiovascular Disease (TMP, ADH, NTB). This publication also arises from research funded by UiT - The Arctic University of Norway.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Although murine models for studying the development of cardiac dysfunction in diabetes mellitus are well established, their reported cardiac phenotypes vary. These reported divergences may, in addition to the severity of different models, also be linked to the methods used for cardiac functional assessment. In the present study, we examined the functional changes using conventional transthoracic echocardiography (*in vivo*) and isolated heart perfusion techniques (*ex vivo*), in hearts from two mouse models; one with an overt type 2 diabetes (the *db/db* mouse) and one with a prediabetic state, where obesity was induced by a high-fat diet (HFD). Analysis of left ventricular function in the isolated working hearts from HFD-fed mice, suggested that these hearts develop diastolic dysfunction with preserved systolic function. Accordingly, *in vivo* examination demonstrated maintained systolic function, but we did not find parameters of diastolic function to be altered. In *db/db* mice, *ex vivo* working hearts showed both diastolic and systolic dysfunction. Although *in vivo* functional assessment revealed signs of diastolic dysfunction, the hearts did not display reduced systolic function. The contrasting results between *ex vivo* and *in vivo* function could be due to systemic changes that may sustain *in vivo* function, or a lack of sensitivity using conventional transthoracic echocardiography. Thus, this study demonstrates that the isolated perfused working heart preparation provides unique additional information related to the development of cardiomyopathy, which might otherwise go unnoticed when only using conventional echocardiographic assessment.

Introduction

The transition to a more sedentary lifestyle and overnutrition, has led to increased incidence of obesity, hyperglycaemia, insulin resistance, dyslipidaemia, and metabolic syndrome—all known risk factors of cardiovascular disease. Consequently, cardiovascular disease is the primary cause of morbidity and mortality in diabetes patients. Diabetes also predisposes to a distinct cardiomyopathy defined as ventricular dysfunction in the absence of coronary heart

disease or hypertension [1–3], which leads to the development of diastolic dysfunction prior to systolic dysfunction and finally heart failure.

A range of murine models is used to elucidate underlying mechanisms in the development of obesity and diabetes related cardiac dysfunction. Mice homozygous for the obese (Lep^{ob}) and the diabetes ($Lepr^{db}$) mutations are among the earliest characterized models of obesity-related insulin resistance and diabetes. In these monogenic models, leptin deficiency (*ob/ob* mice) or leptin receptor deficiency (*db/db* mice) lead to lack of satiety sensation, which consequently causes hyperphagia and hypoactivity. Studies using diabetic *db/db* mice have reported both development of ventricular remodelling and cardiac dysfunction [4–8], although there are studies reporting normal cardiac structure and function [9–11].

Diet-induced obese (DIO) mouse models are currently in extensive use in diabetic cardiomyopathy research as they recapitulate aspects of the metabolic syndrome associated with human obesity, and because they are comparable to commonly used genetically modified models. The reported cardiac phenotype from DIO models range from no change in cardiac function [12–15] to more severe remodelling and dysfunction [16–19]. Although, these differences may be linked to the variations between feeding protocols (nutritional composition of the diet and feeding initiation and duration) and the mouse strain used [20], there is also reason to believe that the techniques and modalities used for cardiac phenotyping influence the observed functional outcome. Today, assessment of cardiac function in mice primarily involves different *in vivo* techniques. The development of high-frequency transducers has made echocardiography a common modality for cardiac functional assessment of mouse hearts, and the parameters are mainly derived from M-mode and Doppler imaging. Although additional parameters from multi-planar images and speckle tracking echocardiography, as well as other imaging modalities (e.g. magnetic resonance imaging) have become more easily accessible, conventional echocardiography (M-mode and Doppler imaging) is still the most widespread *in vivo* modality [21].

The first reports of cardiac function in mice, published more than 25 years ago, describe cardiac function using isolated perfused hearts using the working mode [22;23] and the Langendorff mode [24]. Despite technical challenges due to the small size of the mouse heart, *ex vivo* assessment has proven valuable, not only in studying pharmacological effects, but also in the investigation of disease states and the impact of gene modification in the intact heart. It is important to emphasize that the *ex vivo* heart preparation has the advantage of facilitating elucidation of the ventricular performance under defined loading conditions and in the absence of neurohormonal influences, and may therefore provide unique additional information related to the development of dysfunction in the myocardium *per se*.

In the present study, we examined the functional changes in hearts from two mouse models of obesity/diabetes; the *db/db* mouse, a model of severe obesity and diabetes, and a DIO model induced by a high-fat diet, representing a model of obesity and prediabetes. Here, we compared *in vivo* cardiac function to that obtained *ex vivo*, using the most used imaging modality in small animal research (i.e. M-mode echocardiography and Doppler imaging) and isolated perfused heart techniques, respectively.

Materials and methods

Animals and diet

Mice were purchased from Charles River (Germany). Diet-induced obesity was obtained by feeding 5-6-week-old male C57BL/6J mice a high-fat diet (HFD) with 60% kcal from fat (lard), 20% kcal from carbohydrates and 20% kcal from protein (58Y1, TestDiet, UK; http://www.testdiet.com/Diets/High-Fat-DIO/index.html) for 20 weeks. Male diabetic *db/db* mice

(C57BL/KsJ-lepr^{db}/lepr^{db}) arrived at an age of 8 weeks and were fed a regular chow diet until 12 weeks of age. We used age-matched mice fed a regular chow diet as controls. All mice were housed in a room with a constant temperature of 23˚C and 55% humidity, with a 12:12-h reversed light:dark-cycle. They were given ad libitum access to water and their respective diets and were treated in accordance with the guidelines on accommodation and care of animals given by the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes. The experiments were approved by the local representation of the National Animal Research Authority in Norway, and carried out in our laboratory at the University of Tromsø –The Arctic University of Norway.

Blood sampling

Blood was collected from the saphenous vein (14 days prior to sacrificing the animals) after 4 hours of fasting or after a fasting/re-feeding protocol, where the mice were fasted for 3 hours, and then re-fed for 2 hours. Fasting blood glucose levels were measured using a glucometer system (FreeStyle Lite, Abbott, Australia), and insulin levels were measured in plasma from the same blood sample, using a mouse insulin ELISA kit (DRG Instruments GmbH, Germany). Plasma free fatty acids levels were analysed using commercial kits from Wako Chemicals (Neuss, Germany), in plasma collected following fasting/re-feeding.

Echocardiography

Echocardiography was done using the VisualSonics 2100 Vevo Imaging System (Toronto, Canada) with a 550D probe (frequency of 35–40 MHz) during light isoflurane (1.5–2%) anaesthesia. The body temperature was monitored using a rectal thermometer, and maintained at approximately 37˚C using a heated platform and a heat lamp.

A single operator imaged all mice. Left ventricular (LV) mass, wall thickness, end-systolic and end-diastolic diameter (LVID;s and LVID;d) and end-systolic and end-diastolic volumes (ESV and EDV) were determined from parasternal short-axis M-mode images of the midventricle at the level of papillary muscles. The LV mass was calculated from the measured cardiac borders in diastole, including the interventricular septum (IVS;d), LVID;d and the posterior wall (LVPW;d), using this formula: LV mass = $(1.053^{*}((\text{LVID;d}+\text{LVPW;d}+\text{IVS;d})^{3}-\text{LVID;d}^{3}))^{*}0.8$. Functional parameters like stroke volume (SV), ejection fraction (EF) and fractional shortening (FS) were obtained from further calculations of the M-mode data. Ejection fraction was calculated as EF = $(\text{SV/EDV})^{*}100$ and fractional shortening was calculated from FS = $(\text{LVID;d-LVID;s})/\text{LVID;d}^{*}100$. The ratio between LV Volume and LV mass was used as a measure of hypertrophy. The early (E) and late/atrial (A) peak ventricular filling velocities, E/A ratio and deceleration time of early filling (DT) were obtained from transmitral flow, in the apical 4-chamber view. Early diastolic (E') and late diastolic (A') mitral annular myocardial velocity of the left ventricle septal wall was recorded from the 4-chamber view with pulsed-wave tissue Doppler. The E/E' was calculated as an index of LV filling pressure.

Isolated heart perfusions

To evaluate the changes in heart function in the hearts independent of neurohormonal or loading conditions, we also characterised heart function *ex vivo*. After an intraperitoneal injection of pentobarbital, hearts were excised and perfused using a modified Krebs-Henseleit with added glucose (5mM) and palmitate (0.5 mM) bound to BSA (3%) [25]. Cardiac temperature was maintained at 37˚C and data were obtained and analysed using LabChart 7Pro software (ADInstruments, Bella Vista, Australia).

One group of hearts was perfused in the working heart mode with an 8 mmHg preload and 50 mmHg afterload [25]. Left ventricular pressure changes were assessed using a 1.0-Fr conductance catheter (Millar Instruments, Houston, TX), inserted into the ventricle through the apex [26]. In another group, the hearts were perfused in the Langendorff mode [27], where left ventricular pressure was assessed using an intraventricular fluid-filled balloon. The volume of the balloon was adjusted so that the end-diastolic pressure was between 5–10 mmHg. To prevent build-up of fluid in the ventricle, a cannula (25 G) was inserted through the apex and into the lumen to allow drainage of fluid [27].

Statistical analysis

The results are presented as means ± SE in tables and column bar graphs. In order to compare differences between two groups, unpaired Student's t-tests were performed.

Results

Diet-induced obese mice

5-6-week-old male mice were randomly divided into two groups with similar body weight (20.7 ± 0.2 g and 20.5 ± 0.2 g). Twenty weeks of feeding with the high-fat diet (HFD) resulted in obesity as indicated by higher body weight (Fig 1), and a significant increase in perirenal fat deposits (0.3 ± 0.1 vs 1.0 ± 0.1 g in controls (n = 12) and HFD (n = 13), respectively). Similar tibia lengths in the two groups of mice supports that the observation of increased weight gain in HFD mice is attributable to fat deposits and not the animal size per se (Fig 1). In this study, the HFD mice had elevated fasted blood glucose (p = 0.05, Fig 1), and HOMA-IR values (representing insulin resistance) were increased due to a marked increase in fasting insulin levels (Fig 1). In addition, HFD mice displayed a near 2.4-fold increase in free fatty acids (Fig 1).

***In vivo* cardiac function.** Left ventricular (LV) remodelling and function *in vivo*, were assessed using transthoracic echocardiography in anesthetized mice. HFD mice did not show altered wall thickness, intraventricular diameter or LV mass (Table 1). Fractional shortening (FS) was increased in the HFD mice, due to a trend towards a decreased LV end-systolic volume (ESV, p = 0.09, Table 1). There was also a significant increase in stroke volume and ejection fraction (EF) in HFD mice, but due to a somewhat lower heart rate, cardiac output was not altered when compared to the age-matched controls (Table 1). Interestingly, the volume-mass ratio was significantly lower in HFD mice, indicating concentric hypertrophic remodelling (Table 1). Pulsed-wave tissue Doppler showed that the peak velocity of early mitral filling (E wave—depicting the velocity of blood flow in early ventricular diastolic filling), was not significantly altered. However, due to a lowered late mitral filling velocity (A wave—depicting late ventricular filling), the E/A was unexpectedly augmented in HFD hearts (Table 1). Notably though, due to the high heart rates in mice, the E and A waves are challenging to consistently measure [28], and the E/A ratio is not a reliable parameter on its own. E/E' was unchanged, while there was a trend towards an elevated mitral valve deceleration time (p = 0.09) in the HFD mice (Table 1), indicating a mild impairment of diastolic function.

***Ex vivo* cardiac function.** Isolated perfused working hearts from HFD mice showed a slight but not significant decrease in intrinsic heart rate (p = 0.06, Fig 2). HFD hearts also displayed elevated LV end-diastolic pressure (LVEDP) and impaired relaxation as indicated by a decreased dP/dt_{min} (p = 0.06) and increased Tau (the relaxation time constant) (Fig 2). On the other hand, HFD hearts displayed no change in dP/dt_{max} or cardiac power (the product between cardiac output and LV developed pressure, Fig 2). Developed pressure (53.0 ± 1.6 vs 52.7 ± 0.9 mmHg in controls (n = 6) and HFD (n = 8), respectively) and cardiac output

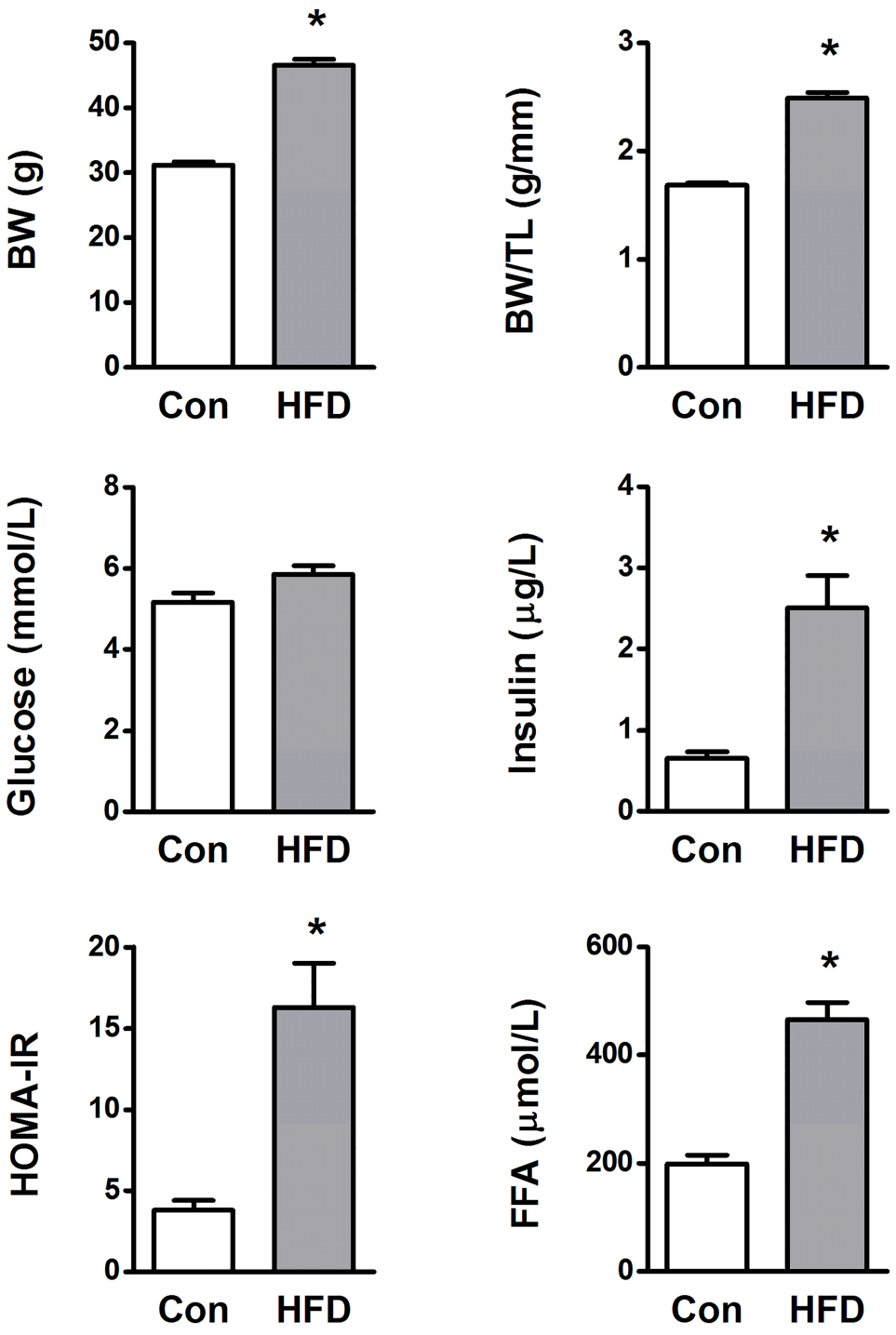

Fig 1. Body weight (BW), BW to tibia length (TL) ratio, blood glucose (fasted), plasma insulin (fasted), plasma free fatty acids (FFA, fasted/re-fed) and insulin resistance (HOMA-IR) from high-fat diet mice (HFD) and age-matched controls (Con). BW and tibia lengths were obtained from 25–30 mice per group, while blood samples were obtained from 13 controls and 23 HFD mice. HOMA-IR; Homeostatic Model Assessment—Insulin Resistance. * $p < 0.05$ vs Con.

https://doi.org/10.1371/journal.pone.0204843.g001

Table 1. Left ventricular function assessed by transthoracic echocardiography of high-fat diet-fed mice (HFD) and age-matched controls (Con).

	Con (n = 9)	HFD (n = 12)
Heart rate (BPM)	452 ± 10	439 ± 9
LVPW;d (mm)	0.79 ± 0.02	0.80 ± 0.01
LVID;d (mm)	4.1 ± 0.1	4.1 ± 0.1
LV mass (mg)	98 ± 3	107 ± 4
LVEDV (μL)	76 ± 3	74 ± 3
LVESV (μL)	36 ± 3	29 ± 2
SV (μL)	41 ± 1	45 ± 2 *
EF (%)	54 ± 2	61 ± 2 *
FS (%)	28 ± 1	32 ± 1 *
Volume/LV mass (μL/mg)	0.78 ± 0.02	0.70 ± 0.02 *
E/A	1.4 ± 0.1	1.7 ± 0.1 *
E/E'	32 ± 2	30 ± 1
DT (ms)	25 ± 1	27 ± 1

Data are means ± SE. LVPW;d and LVID;d, left ventricular (LV) posterior wall thickness and internal diameter in diastole, respectively; EDV and ESV, end-diastolic and end-systolic volumes; SV, stroke volume; EF, ejection fraction; FS, fractional shortening; E/A; ratio of velocities of early to late ventricular filling, E/E'; ratio of velocity of early ventricular filling to early diastolic mitral annular velocity, DT; deceleration time.

*$p < 0.05$ vs Con.

https://doi.org/10.1371/journal.pone.0204843.t001

(11.3 ± 0.6 vs 10.8 ± 1.1 mL/min in controls (n = 6) and HFD (n = 8), respectively) were not different from controls either.

The Langendorff perfusion mode has become the most commonly used perfusion mode for evaluating *ex vivo* function in mouse hearts, as it less technically challenging when compared to the working mode. Due to the small size of these hearts, the LV balloon has to be adjusted after it has been inserted into the LV, and this is generally done so that the LVEDP is between 5–10 mmHg. In this study, the mean values of the control and HFD hearts were 6.9 ± 0.7 and 8.6 ± 0.9 mmHg, respectively. In this perfusion mode, we did not find an increase in dP/dt_{min}, which would have indicated impaired relaxation (Table 2). Additionally, none of the parameters of systolic function (maximal systolic pressure, LV developed pressure, dP/dt_{max} or the rate-pressure-product) were altered in HFD hearts (Table 2).

db/db mice

12-week-old *db/db* mice had twice the body weight of the age-matched lean mice (Fig 3). The *db/db* animals also displayed a substantial elevation in HOMA-IR level due to a considerable increase in both fasting insulin and glucose levels. We also found plasma free fatty acids levels to be significantly increased in the *db/db* mice (Fig 3).

***In vivo* cardiac function.** Echocardiographic examination revealed elevated LV masses in *db/db* mice (Table 3), which is in contrast to previous data obtained by weighing these hearts [25;29]. This discrepancy may be related to potential differences in myocardial density, which is not included in the LV mass calculation. There was no change in heart rate between the two groups, but the *db/db* hearts showed increased LV diastolic diameter and end-diastolic volume (EDV, Table 3). As systolic diameter and ESV was unchanged, stroke volume and cardiac output were significantly increased (Table 3). These changes were also associated with a significant increase in EF and FS (Table 3). Doppler measurements showed that the E wave was decreased in *db/db* hearts (p = 0.003), but since the A wave was also decreased (p = 0.04), the

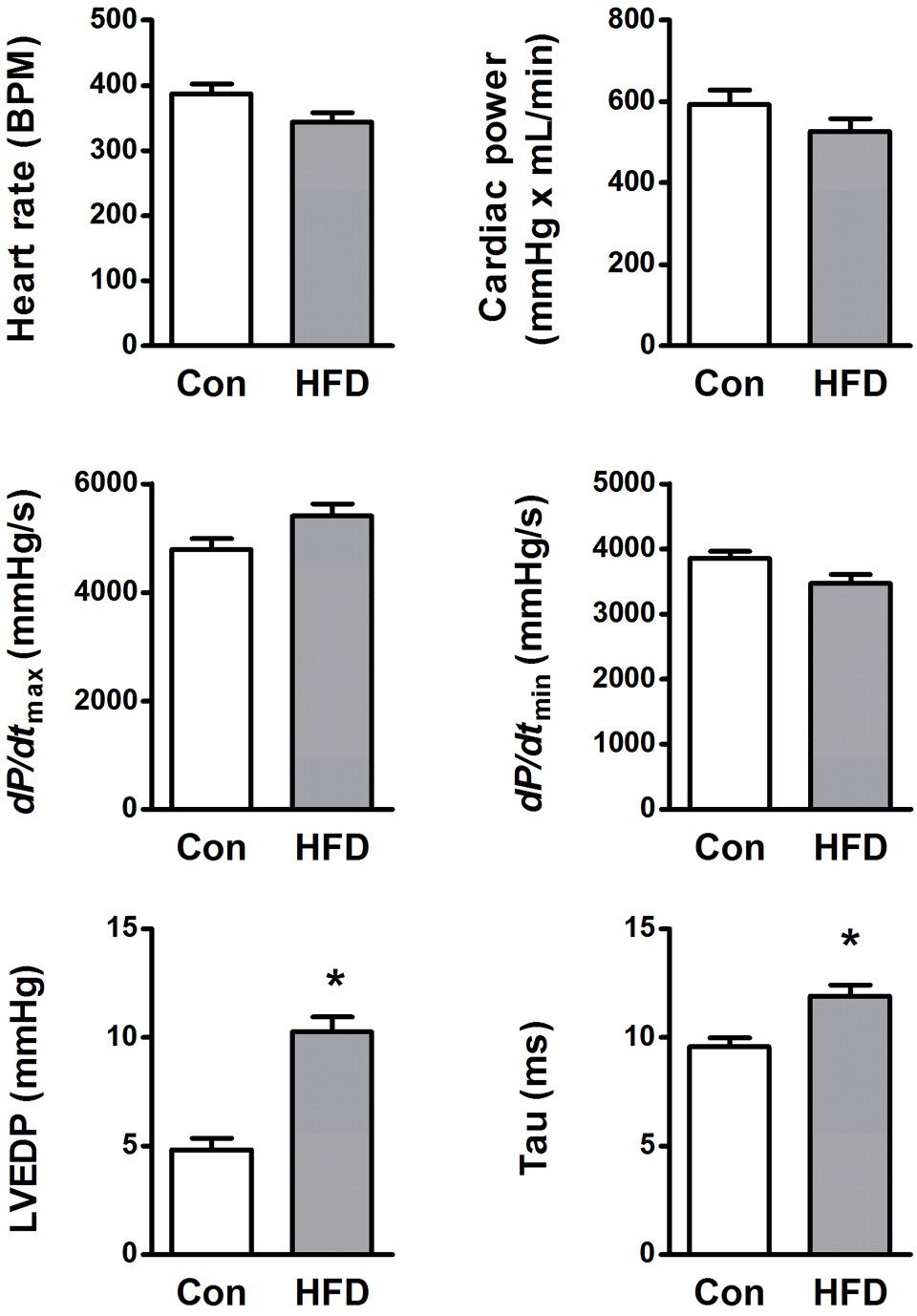

Fig 2. Left ventricular function assessed in isolated perfused working hearts from high-fat diet fed mice (HFD, n = 6) and age-matched controls (Con, n = 8). dP/dt_{max} and dP/dt_{min}; maximum positive and negative first-time derivative of left ventricular (LV) pressure respectively, left ventricular end-diastolic pressure (LVEDP), Tau; LV relaxation time constant calculated by the Weiss method. * $p < 0.05$ vs Con.

https://doi.org/10.1371/journal.pone.0204843.g002

Table 2. Left ventricular function assessed in isolated Langendorff perfused hearts from high-fat diet-fed mice (HFD) and age-matched controls (Con).

	Con (n = 12)	HFD (n = 12)
Heart rate (BPM)	318 ± 11	307 ± 14
Coronary flow (mL/min)	3.2 ± 0.2	3.4 ± 0.4
Developed pressure (mmHg)	139 ± 8	139 ± 14
dP/dt_{max} (mmHg/sec)	5282 ± 367	5623 ± 552
dP/dt_{min} (mmHg/sec)	-3610 ± 119	-3654 ± 292
RPP (mmHg x BPM)	44579 ± 3403	42238 ± 4366

Data are means ± SE. dP/dt_{max} and dP/dt_{min}; maximum positive and negative first-time derivative of left ventricular (LV) pressure respectively. RPP; rate-pressure-product (the product of developed pressure and heart rate).

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E/A remained unchanged (Table 3). Regardless of the unchanged ratio, the lowered filling velocities might portray increased resistance in the ventricle, and as the *db/db* hearts also showed a marked increase in the E/E' and mitral deceleration time (Table 3), this proves that these hearts have decreased ventricular compliance and increased myocardial stiffness.

***Ex vivo* cardiac function.** In the isolated working mode, the *db/db* hearts showed a diastolic dysfunction as indicated by increased LVEDP, lowered dP/dt_{min} and increased Tau (Fig 4). In addition, a combination of reduced stroke volume (23.8 ± 1.3 vs 14.6 ± 2.7 μL in controls (n = 10) and *db/db* (n = 7), respectively) and lowered heart rate (Fig 4), led to a marked decrease in cardiac output (9.8 ± 0.4 vs 5.1 ± 0.8 mL/min in controls (n = 10) and *db/db* (n = 7), respectively). These hearts also showed decreased cardiac power (Fig 4) and finally, despite the lower HR, a significantly lower LV developed pressure (58.2 ± 1.4 vs 50.7 ± 2.4 mmHg in controls (n = 10) and *db/db* (n = 7), respectively) and dP/dt_{max} (Fig 4), which further supports a decreased systolic function in these hearts. It is, however, important to appreciate that the impaired cardiac function in these hearts could also be related to the HR.

In Langendorff perfused hearts, the volume of the balloon was adjusted so that the LVEDP mean values were 6.2 ± 0.6 and 6.7 ± 0.2 mmHg in the control and *db/db* hearts, respectively. We did not find dP/dt_{min} to be altered in the *db/db* hearts, and neither were parameters of systolic function (LV developed pressure, rate-pressure-product and dP/dt_{max}) (Table 4).

Discussion

The present study demonstrates differences between the *ex vivo* and *in vivo* cardiac functional phenotype within the same murine models of diabetes. While isolated perfused working hearts from diabetic mice showed clear signs of dysfunction, conventional transthoracic echocardiography did not reveal this to the same extent. Although lack of *in vivo* cardiac dysfunction may be due to a low sensitivity of the M-Mode and Doppler assessment, it may also relate to systemic factors (such as altered neurohormonal status and/or changes in pre- and afterload) which might mask subtle functional changes *in vivo*. This study therefore shows how *ex vivo* examination can add valuable information when describing the progression of cardiomyopathy.

The cardiac phenotype in diet-induced obese mice

In accordance with previous studies, mice fed a high-fat diet (HFD) for 20 weeks were in a pre-diabetic state with obesity, hyperlipidaemia and insulin resistance, but without hyperglycaemia. In most studies, long-term high-fat feeding will increase left ventricular (LV) mass and induce LV concentric hypertrophy [17;30–34]. The reported incidence of systolic and diastolic

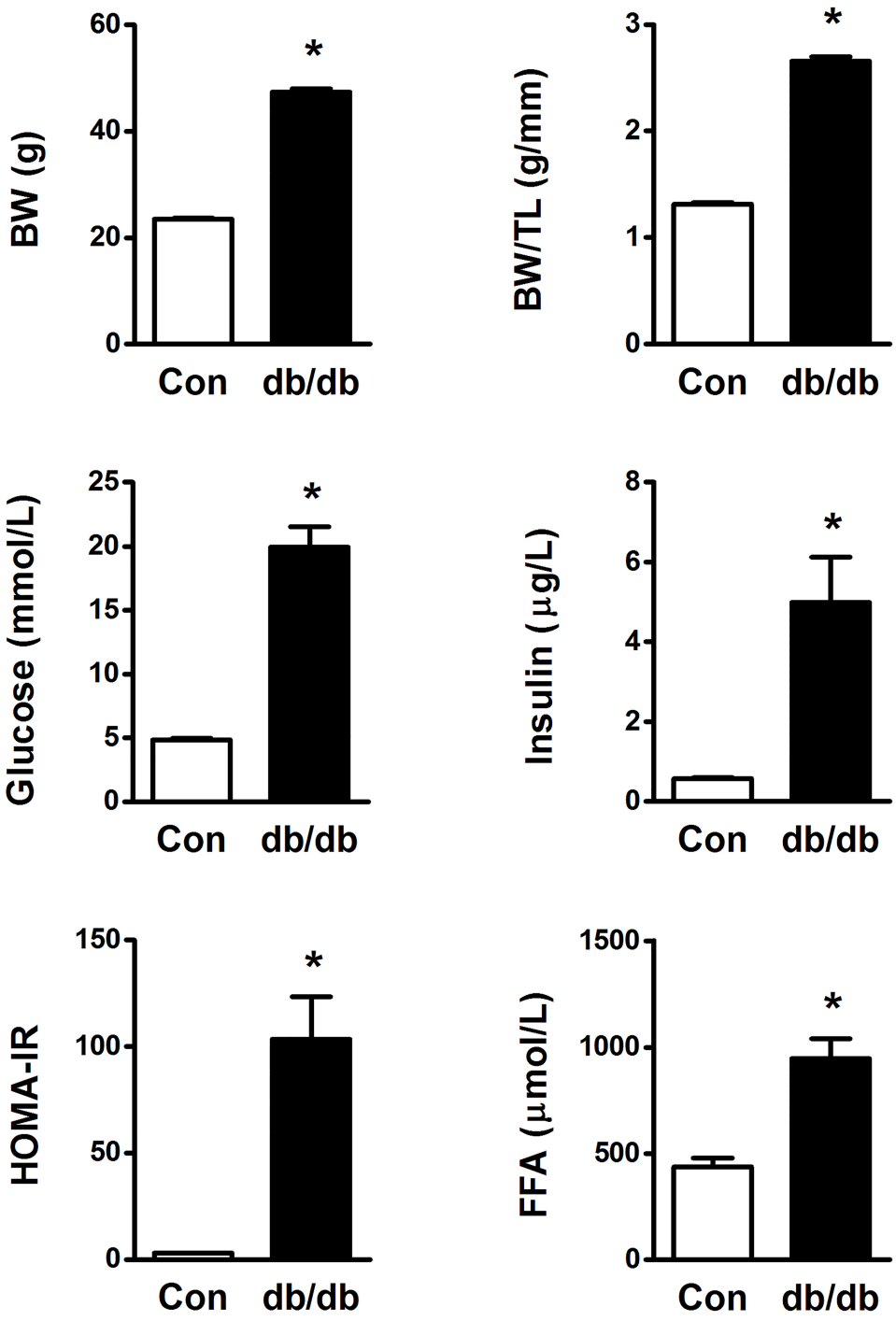

Fig 3. Body weight (BW), BW to tibia length (TL) ratio, blood glucose (fasted), plasma insulin (fasted), plasma free fatty acids (FFA, fasted/re-fed) and insulin resistance from *db/db* mice and age-matched controls (Con). BW and tibia lengths were obtained from 25–28 mice per group, while blood samples were obtained from 11 controls and 6 *db/db* mice. HOMA-IR; Homeostatic Model Assessment—Insulin Resistance. * $p < 0.05$ vs Con.

https://doi.org/10.1371/journal.pone.0204843.g003

Table 3. Left ventricular function assessed by transthoracic echocardiography of *db/db* mice and age-matched controls (Con).

	Con (n = 8)	*db/db* (n = 10)
Heart rate (BPM)	454 ± 10	464 ± 10
LVPW;d (mm)	0.76 ± 0.03	0.81 ± 0.02
LVID;d (mm)	3.83 ± 0.05	4.00 ± 0.04 *
LV mass (mg)	86 ± 4	100 ± 3 *
LVEDV (μL)	63 ± 2	70 ± 2 *
LVESV (μL)	23 ± 2	21 ± 1
SV (μL)	41 ± 2	49 ± 2 *
EF (%)	64 ± 2	70 ± 1 *
FS (%)	35 ± 2	39 ± 1 *
Volume/LV mass (μL/mg)	0.74 ± 0.02	0.71 ± 0.03
E/A	1.6 ± 0.1	1.6 ± 0.1
E/E'	30 ± 1	41 ± 1 *
DT (ms)	26 ± 1	31 ± 2 *

Data are means ± SE. LVPW;d and LVID;d, left ventricular (LV) posterior wall thickness and internal diameter in diastole, respectively; EDV and ESV, end-diastolic and end-systolic volumes; SV, stroke volume; EF, ejection fraction; FS, fractional shortening; E/A; ratio of velocities of early to late ventricular filling, E/E'; ratio of velocity of early ventricular filling to early diastolic mitral annular velocity, DT; deceleration time.

*$p < 0.05$ vs Con.

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dysfunction is, however, inconsistent. Systolic dysfunction has been reported following feeding regimes that varied from 4–6 [33;35] to 15–16 weeks [19;32;36], while the present study and other studies report no evidence of systolic dysfunction after 20–24 weeks [15;27;31;37;38]. In general, diastolic dysfunction is considered to advance prior to the development of systolic dysfunction, and although some studies [17;27;34] support this by showing impaired diastolic dysfunction without systolic dysfunction, the duration of HFD feeding required to evoke diastolic impairments remains unclear. Additionally, as the functional modalities/techniques have different sensitivities, lack of reported diastolic dysfunction by one modality/technique, does not necessarily exclude its occurrence. In accordance with this, a recent study by Schnelle and colleagues [28] shows how combining several ultrasound-based techniques of diastolic function (including additional views and speckle tracking echocardiography) will not only improve the detection of diastolic dysfunction in murine models, but also reveal the underlying pathophysiology for this dysfunction.

The present study showed that while the echocardiographic parameters (obtained by M-mode and Doppler) did not demonstrate diastolic dysfunction, *ex vivo* assessment (using perfused working hearts) confirmed previous reports of diastolic dysfunction [16;27]. This shows how *ex vivo* assessment of function using the working heart mode represents a sensitive and additional method for evaluation of diastolic dysfunction.

The cardiac phenotype in *db/db* mice

The *db/db* mouse represents a type 2 diabetic mouse model, as indicated by obesity, insulin resistance, hyperglycaemia and hyperlipidaemia. These hearts show an age-dependent increase in LV mass and wall thickness [5;11;39;40], but the literature is not consistent with regard to the development of cardiac dysfunction. Semeniuk and colleagues [4] were the first to describe echocardiographic examination of the *db/db* mice in 2002, where they reported reduced fractional shortening (FS) and E/A in 12 but not 5-week-old mice. Consistent with this, studies

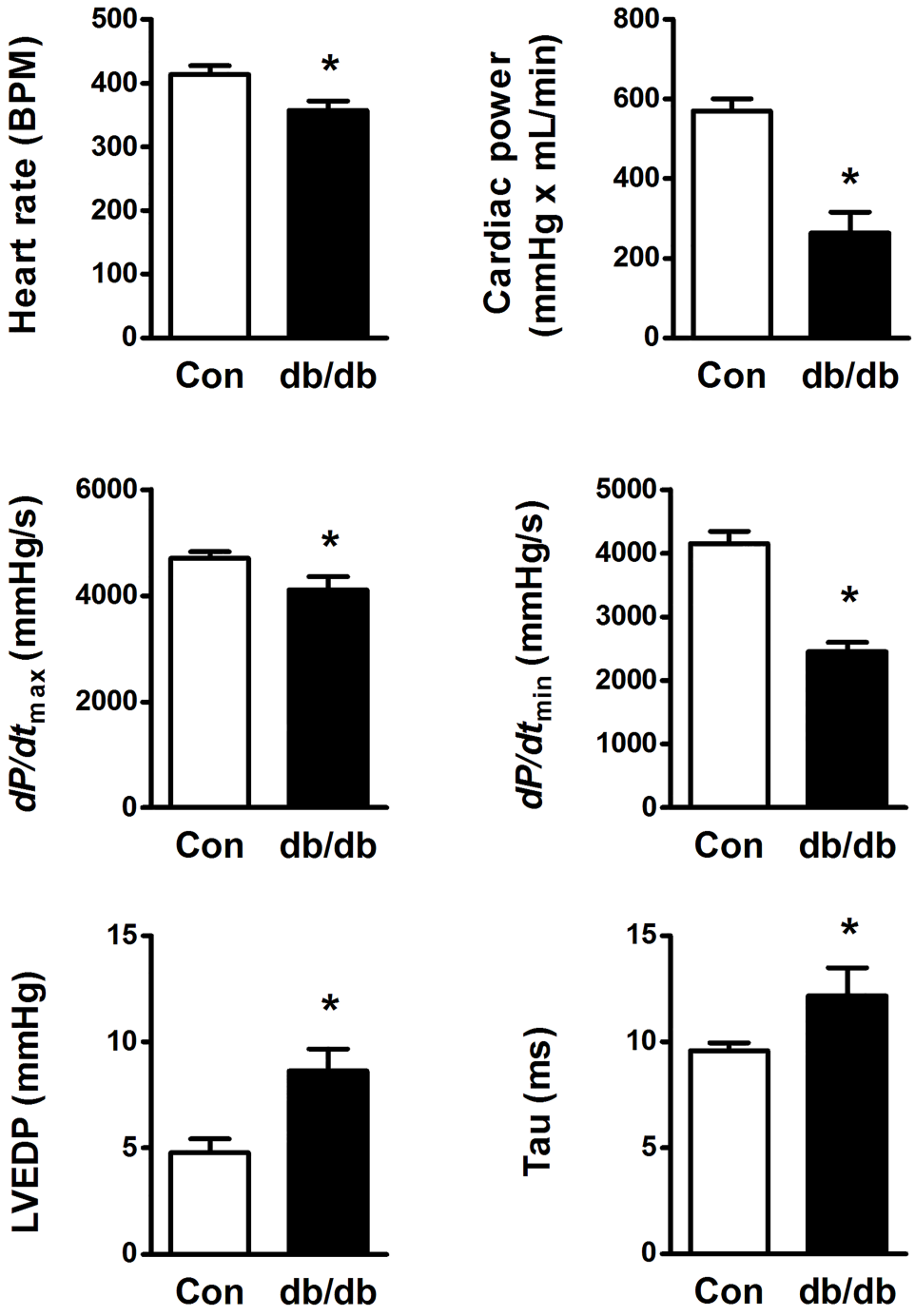

Fig 4. Left ventricular function assessed in isolated perfused working hearts from *db/db* mice (n = 10) and age-matched controls (Con, n = 7). dP/dt_{max} and dP/dt_{min}; maximum positive and negative first-time derivative of left ventricular (LV) pressure respectively, left ventricular end-diastolic pressure (LVEDP), Tau; LV relaxation time constant calculated by the Weiss method. * $p < 0.05$ vs Con.

https://doi.org/10.1371/journal.pone.0204843.g004

Table 4. Left ventricular function assessed in isolated Langendorff perfused hearts from *db/db* mice and age-matched controls (Con).

	Con (n = 8)	*db/db* (n = 10)
Heart rate (BPM)	292 ± 26	269 ± 22
Coronary flow (mL/min)	2.7 ± 0.2	2.3 ± 0.2
Developed pressure (mmHg)	133 ± 13	123 ± 8
dP/dt_{max} (mmHg/sec)	4793 ± 672	4844 ± 446
dP/dt_{min} (mmHg/sec)	-3362 ± 335	-3271 ± 234
RPP (mmHg x BPM)	36778 ± 4954	30683 ± 2690

Data are means ± SE. dP/dt_{max} and dP/dt_{min}; maximum positive and negative first-time derivative of left ventricular (LV) pressure respectively. RPP; rate-pressure-product (the product of developed pressure and heart rate).

https://doi.org/10.1371/journal.pone.0204843.t004

from our laboratory later reported that *ex vivo* working hearts from 12, but not 5-6-week-old mice, showed severe dysfunction [25;41–45]. We were therefore surprised to find that echocardiographic examination in the present study only revealed a mild diastolic impairment with no (or even improved) systolic dysfunction. The literature on echocardiographic examination of *db/db* mice (14–18 weeks of age), have reported both reduced [8], unaltered [9;11], and increased systolic function [10]. Although this inconsistency could be due to differences in the severity of the diabetic phenotype caused by using different background strains in these mice [46], it may also reflect the lack of sensitivity of conventional echocardiography.

Accordingly, Yue and colleagues [5] reported normal function in young (5-week-old) mice, but reduced FS and increased end-diastolic volume at an age of 9–13 weeks when using MRI. Interestingly, despite decreased FS, they found a slight (although not significant) increase in cardiac output at all ages. Again, the literature is inconsistent, as other studies (using the same modality) have not reported the same changes [6;47]. It should be noted that Stuckey *et al.* reported impairments in contractility and diastolic function in 12-week-old mice when analysing data from high-temporal resolution MRI, despite finding no functional changes when using standard MRI [6]. Interestingly, maintained systolic function in 12-week-old mice was supported both by a recent study by Li et al. [48] using both STE and conventional echocardiography. In this study only STE revealed a dysfunction when these mice were 16 weeks old [48], which suggests that conventional echocardiography lacks the sensitivity to capture subtle variations in left LV performance.

The very first functional assessment of the *db/db* hearts was not *in vivo* but performed using the isolated perfused working heart. In this study, Belke and colleagues measured intraventricular pressure (using a fluid-filled catheter) and demonstrated a higher end-diastolic pressure, as well as reduced cardiac output and cardiac power in hearts from 10-14-week-old mice [49]. Impaired *ex vivo* systolic function was later confirmed in 12-week-old mice [25;41;50]. In accordance with previous studies [29;44], the present study confirmed both diastolic and systolic dysfunction, using intraventricular pressure-volume recordings, which allow more accurate assessment of a range of load-dependent and -independent functional parameters.

It should be noted that when these hearts were perfused in the Langendorff mode, there were no detectable changes in parameters of LV function at neither 12 (present study) nor 24 weeks [47]. Thus, although the Langendorff mode is a suitable *ex vivo* mode for vascular reactivity studies and acute studies of changes in contractility following drug or ischemic damage, the present study reinforces that this mode is less suitable for detailed functional assessment, particularly in models of diastolic dysfunction.

The cardiac phenotype obtained *in vivo* vs *ex vivo*

The functional capacity of the *in vivo* heart is determined by the endogenous working capacity of the myocardium, coronary perfusion and changes in systemic factors known to influence the myocardial function, such as loading conditions and neurohormonal status. Thus, the functional phenotype of the diabetic heart may be altered due to pathological alterations in the myocardium itself and/or systemic changes representing compensatory or pathophysiological alterations.

In the present study, as well as in previous reports, there seems to be a discrepancy between the *in vivo* and *ex vivo* function in *db/db* hearts, when comparing parameters obtained by conventional echocardiography and the isolated perfused working hearts. As the mechanical function of the hearts *ex vivo* is examined under identical loading and neurohormonal conditions, it could be argued that favourable load-dependent and/or load-independent changes may have contributed to improve *in vivo* function. Haemodynamic measurements using LV *in vivo* catheterization in *db/db* hearts have supported this notion, first by showing impaired *load-independent* parameters of diastolic dysfunction (increased slope of the end-diastolic pressure-volume relationship) [5;51–53] and contractility (diminished preload recruitable stroke-work index and end-systolic elastance) [5;51]. On the other hand, *load-dependent* parameters of systolic function (dP/dt_{max} and cardiac output) were reported to be elevated [50;51], strongly supporting changes in the pre- and/or after-load. The increased LVEDP [50–52] and decreased arterial elastance in these mice [51;53] (both *load-dependent* parameters), have been argued to signify increased preload and decreased afterload, respectively [51], further supporting the notion of compensated haemodynamic conditions in *db/db* hearts.

Neurohormonal changes (as a compensatory mechanism) may also affect cardiac function *in vivo*. Discrepancy between the *in vivo* and *ex vivo* heart function has also been reported after a genetic impairment of SERCA2 function in the heart. While isolated cardiomyocytes from these hearts showed impaired cell shortening and reduced Ca^{2+}-transient amplitude [54], and isolated perfused working hearts displayed severe heart failure [55], echocardiographic examination showed a near normal *in vivo* function [54]. In support of this, Land et al. [56] demonstrated, using computational whole-organ simulation, the important role of the compensatory systemic changes to maintain *in vivo* function. They showed that the model would only be able to match the reported *in vivo* function if they included the effect of β-adrenergic stimulus i.e. the enhanced Ca^{2+}-transient amplitudes, and increased venous return with its subsequent Frank-Starling effect [56]. The fact that cardiomyocytes from the *db/db* hearts also show impaired SERCA2 function and an accompanying decrease in Ca^{2+}-transient amplitude and cell shortening [57;58], further supports that compensatory systemic changes can contribute to sustain *in vivo* cardiac function in these mice.

As the β-adrenergic drive also influences heart rate (HR), a lower HR in *db/db* hearts *ex vivo* [25;44;50] but not *in vivo* [39;48;51], supports that the neurohormonal status is altered in *db/db* mice. Although we cannot fully exclude that the reduced *ex vivo* HR in *db/db* hearts could have contributed to the reduced function, decreased contractility and impaired relaxation have also been reported in studies where HR was not significantly different in isolated perfused *db/db* hearts [29;45].

Limitations

The present study, cannot fully exclude the presence of *in vivo* systolic or diastolic dysfunction. Here we used the M-mode and Doppler images, as these are the most widely used images, and are regarded as suitable approaches for assessment of heart disease where the structural remodelling develop in a uniform manner [21]. It should be noted, however, that a recently

published guideline for measuring cardiac physiology in mice [21] recommends that detection of subtle systolic changes requires the use of other imaging modalities or techniques, to give a comprehensive and full description of cardiac function.

Oxygen delivery/supply can limit the function of an *ex vivo* heart, as demonstrated by altered function in rabbit hearts perfused with a buffer that had increased oxygen carrying capacity [59]. To what extent this also relates to smaller hearts (such as the mouse heart) is not known, and whether a resulting decrease in myoglobin oxygen saturation will have an impact on oxidative phosphorylation, remains unresolved [59]. In addition, it is not clear whether the function of the diabetic heart is more sensitive to mild hypoxia, and thus we do not know to what extent this may have influenced the systolic dysfunction observed *ex vivo*, but not *in vivo*, in diabetic hearts.

Conclusion

Although continued advances in *in vivo* imaging will provide access to new and more sensitive modalities for cardiac phenotyping, this study demonstrates that the isolated heart preparation remains a valuable tool for assessment of the myocardial function *per se*, and by that may bridge *in vitro* assays and *in vivo* approaches.

Acknowledgments

The expert technical assistance provided by Trine Lund is gratefully acknowledged. Additionally, the authors would like to thank professor Ajay Shah's group at the BHF Centre of Research Excellence, Cardiovascular Division at King's College London for their advice with regard to echocardiographic assessments.

Author Contributions

Conceptualization: Tina M. Pedersen, Ellen Aasum.

Formal analysis: Tina M. Pedersen, Neoma T. Boardman, Anne D. Hafstad.

Funding acquisition: Ellen Aasum.

Investigation: Tina M. Pedersen, Neoma T. Boardman, Anne D. Hafstad.

Project administration: Ellen Aasum.

Resources: Ellen Aasum.

Supervision: Ellen Aasum.

Validation: Tina M. Pedersen.

Visualization: Tina M. Pedersen.

Writing – original draft: Tina M. Pedersen.

Writing – review & editing: Tina M. Pedersen, Neoma T. Boardman, Anne D. Hafstad, Ellen Aasum.

References

1. Regan TJ, Lyons MM, Ahmed SS, Levinson GE, Oldewurtel HA, Ahmad MR et al. Evidence for Cardiomyopathy in Familial Diabetes Mellitus. J Clin Invest 1977; 60(4):885–899.
2. Fein FS. Diabetic cardiomyopathy. Diabetes Care 1990; 13(11):1169–79. PMID: 2261838
3. Shehadeh A, Regan TJ. Cardiac consequences of diabetes mellitus. Clin Cardiol 1995; 18(6):301–305. PMID: 7664503

4. Semeniuk LM, Kryski AJ, Severson DL. Echocardiographic assessment of cardiac function in diabetic *db/db* and transgenic *db/db*-hGLUT4 mice. Am J Physiol Heart Circ Physiol 2002; 283(3):H976–H982. https://doi.org/10.1152/ajpheart.00088.2002 PMID: 12181126
5. Yue P, Arai T, Terashima M, Sheikh AY, Cao F, Charo D et al. Magnetic resonance imaging of progressive cardiomyopathic changes in the *db/db* mouse. American Journal of Physiology-Heart and Circulatory Physiology 2007; 292(5):H2106–H2118. https://doi.org/10.1152/ajpheart.00856.2006 PMID: 17122193
6. Stuckey DJ, Carr CA, Tyler DJ, Aasum E, Clarke K. Novel MRI method to detect altered left ventricular ejection and filling patterns in rodent models of disease. Magn Reson Med 2008; 60(3):582–587. https://doi.org/10.1002/mrm.21677 PMID: 18727095
7. Plante E, Menaouar A, Danalache BA, Broderick TL, Jankowski M, Gutkowska J. Treatment with brain natriuretic peptide prevents the development of cardiac dysfunction in obese diabetic *db/db* mice. Diabetologia 2014; 57(6):1257–1267. https://doi.org/10.1007/s00125-014-3201-4 PMID: 24595856
8. Pei XM, Yung BY, Yip SP, Chan LW, Wong CS, Ying M et al. Protective effects of desacyl ghrelin on diabetic cardiomyopathy. Acta Diabetologica 2015; 52(2):293–306. https://doi.org/10.1007/s00592-014-0637-4 PMID: 25192951
9. Daniels A, Van Bilsen M, Janssen BJA, Brouns AE, Cleutjens JPM, Roemen THM et al. Impaired cardiac functional reserve in type 2 diabetic *db/db* mice is associated with metabolic, but not structural, remodelling. Acta Physiologica 2010; 200(1):11–22. https://doi.org/10.1111/j.1748-1716.2010.02102.x PMID: 20175764
10. Van Bilsen M, Daniels A, Brouwers O, Janssen BJA, Derks WJA, Brouns AE et al. Hypertension Is a Conditional Factor for the Development of Cardiac Hypertrophy in Type 2 Diabetic Mice. PLOS ONE 2014; 9(1):e85078. https://doi.org/10.1371/journal.pone.0085078 PMID: 24416343
11. Ko KY, Wu YW, Liu CW, Cheng MF, Yen RF, Yang WS. Longitudinal evaluation of myocardial glucose metabolism and contractile function in obese type 2 diabetic *db/db* mice using small-animal dynamic 18F-FDG PET and echocardiography. Oncotarget 2017; 8(50):87795–87808. https://doi.org/10.18632/oncotarget.21202 PMID: 29152121
12. Brainard RE, Watson LJ, DeMartino AM, Brittian KR, Readnower RD, Boakye AA et al. High Fat Feeding in Mice Is Insufficient to Induce Cardiac Dysfunction and Does Not Exacerbate Heart Failure. PLOS ONE 2013; 8(12):e83174. https://doi.org/10.1371/journal.pone.0083174 PMID: 24367585
13. Dong F, Zhang X, Yang X, Esberg LB, Yang H, Zhang Z et al. Impaired cardiac contractile function in ventricular myocytes from leptin-deficient *ob/ob* obese mice. Journal of Endocrinology 2006; 188(1):25–36. https://doi.org/10.1677/joe.1.06241 PMID: 16394172
14. Zhang L, Ussher JR, Oka T, Cadete VJJ, Wagg C, Lopaschuk GD. Cardiac diacylglycerol accumulation in high fat-fed mice is associated with impaired insulin-stimulated glucose oxidation. Cardiovascular Research 2011; 89(1):148–156. https://doi.org/10.1093/cvr/cvq266 PMID: 20729341
15. Naresh NK, Butcher JT, Lye RJ, Chen X, Isakson BE, Gan LM et al. Cardiovascular magnetic resonance detects the progression of impaired myocardial perfusion reserve and increased left-ventricular mass in mice fed a high-fat diet. Journal of Cardiovascular Magnetic Resonance 2016; 18(1):53–63. https://doi.org/10.1186/s12968-016-0273-y PMID: 27609091
16. Hafstad AD, Lund J, Hadler-Olsen E, Höper AC, Larsen TS, Aasum E. High- and Moderate-Intensity Training Normalizes Ventricular Function and Mechanoenergetics in Mice With Diet-Induced Obesity. Diabetes 2013; 62(7):2287–2294. https://doi.org/10.2337/db12-1580 PMID: 23493573
17. Jeong EM, Chung J, Liu H, Go Y, Gladstein S, Farzaneh-Far A et al. Role of Mitochondrial Oxidative Stress in Glucose Tolerance, Insulin Resistance, and Cardiac Diastolic Dysfunction. J Am Heart Assoc 2016; 5(5):e003046. https://doi.org/10.1161/JAHA.115.003046 PMID: 27151515
18. Calligaris SD, Lecanda M, Solis F, Ezquer M, Gutiérrez J, Brandan E et al. Mice Long-Term High-Fat Diet Feeding Recapitulates Human Cardiovascular Alterations: An Animal Model to Study the Early Phases of Diabetic Cardiomyopathy. PLOS ONE 2013; 8(4):e60931. https://doi.org/10.1371/journal.pone.0060931 PMID: 23593350
19. Russo SB, Baicu CF, Van Laer A, Geng T, Kasiganesan H, Zile MR et al. Ceramide synthase 5 mediates lipid-induced autophagy and hypertrophy in cardiomyocytes. J Clin Invest 2012; 122(11):3919–3930. https://doi.org/10.1172/JCI63888 PMID: 23023704
20. Nicholson A, Reifsnyder PC, Malcolm R, Lucas CA, MacGregor GR, Zhang W et al. Diet induced obesity in two C57BL/6 substrains with intact or mutant Nicotinamide Nucleotide Transhydrogenase (Nnt) gene. Obesity (Silver Spring) 2010; 18(10):1902–1905.
21. Lindsey ML, Kassiri Z, Virag JAI, de Castro Brás LE, Scherrer-Crosbie M. Guidelines for measuring cardiac physiology in mice. American Journal of Physiology-Heart and Circulatory Physiology 2018; 314(4):H733–H752. https://doi.org/10.1152/ajpheart.00339.2017 PMID: 29351456

22. Grupp IL, Subramaniam A, Hewett TE, Robbins J, Grupp G. Comparison of normal, hypodynamic, and hyperdynamic mouse hearts using isolated work-performing heart preparations. American Journal of Physiology-Heart and Circulatory Physiology 1993; 265(4):H1401–H1410.
23. Larsen TS, Belke DD, Sas R, Giles WR, Severson DL, Lopaschuk GD et al. The isolated working mouse heart: methodological considerations. Pflügers Archiv 1999; 437(6):979–985. https://doi.org/10.1007/s004240050870 PMID: 10370078
24. Galiñanes M, Hearse DJ. Species differences in susceptibility to ischemic injury and responsiveness to myocardial protection. Cardioscience 1990; 1(2):127–143. PMID: 2102801
25. Aasum E, Hafstad AD, Severson DL, Larsen TS. Age-Dependent Changes in Metabolism, Contractile Function, and Ischemic Sensitivity in Hearts From *db/db* Mice. Diabetes 2003; 52(2):434–441. PMID: 12540618
26. How OJ, Aasum E, Kunnathu S, Severson DL, Myhre ESP, Larsen TS. Influence of substrate supply on cardiac efficiency, as measured by pressure-volume analysis in *ex vivo* mouse hearts. Am J Physiol Heart Circ Physiol 2005; 288(6):H2979. https://doi.org/10.1152/ajpheart.00084.2005 PMID: 15764683
27. Lund J, Hafstad AD, Boardman NT, Rossvoll L, Rolim NP, Ahmed MS et al. Exercise training promotes cardioprotection through oxygen-sparing action in high fat-fed mice. Am J Physiol Heart Circ Physiol 2015; 308(8):H823–H829. https://doi.org/10.1152/ajpheart.00734.2014 PMID: 25637547
28. Schnelle M, Catibog N, Zhang M, Nabeebaccus AA, Anderson G, Richards DA et al. Echocardiographic evaluation of diastolic function in mouse models of heart disease. Journal of Molecular and Cellular Cardiology 2018; 114:20–28. https://doi.org/10.1016/j.yjmcc.2017.10.006 PMID: 29055654
29. How OJ, Aasum E, Severson DL, Chan WYA, Essop MF, Larsen TS. Increased Myocardial Oxygen Consumption Reduces Cardiac Efficiency in Diabetic Mice. Diabetes 2006; 55(2):466–473. PMID: 16443782
30. Ussher JR, Koves TR, Jaswal JS, Zhang L, Ilkayeva O, Dyck JRB et al. Insulin-Stimulated Cardiac Glucose Oxidation Is Increased in High-Fat Diet-Induced Obese Mice Lacking Malonyl CoA Decarboxylase. Diabetes 2009; 58(8):1766–1775. https://doi.org/10.2337/db09-0011 PMID: 19478144
31. Abdurrachim D, Ciapaite J, Wessels B, Nabben M, Luiken JJFP, Nicolay K et al. Cardiac diastolic dysfunction in high-fat diet fed mice is associated with lipotoxicity without impairment of cardiac energetics *in vivo*. Biochimica et Biophysica Acta (BBA)—Molecular and Cell Biology of Lipids 2014; 1841 (10):1525–1537.
32. Zeng H, Vaka VR, He X, Booz GW, Chen JX. High-fat diet induces cardiac remodelling and dysfunction: assessment of the role played by SIRT3 loss. J Cell Mol Med 2015; 19(8):1847–1856. https://doi.org/10.1111/jcmm.12556 PMID: 25782072
33. Roberts NW, González-Vega M, Berhanu TK, Mull A, García J, Heydemann A. Successful metabolic adaptations leading to the prevention of high fat diet-induced murine cardiac remodeling. Cardiovascular Diabetology 2015; 14(1):127–141.
34. Nguyen S, Shao D, Tomasi LC, Braun A, de Mattos ABM, Choi YS et al. The effects of fatty acid composition on cardiac hypertrophy and function in mouse models of diet-induced obesity. The Journal of Nutritional Biochemistry 2017; 46(Supplement C):137–142.
35. Nilsson J, Ericsson M, Joibari MM, Anderson F, Carlsson L, Nilsson SK et al. A low-carbohydrate high-fat diet decreases lean mass and impairs cardiac function in pair-fed female C57BL/6J mice. Nutr Metab (Lond) 2016; 13:79–90.
36. Park SY, Cho YR, Kim HJ, Higashimori T, Danton C, Lee MK et al. Unraveling the Temporal Pattern of Diet-Induced Insulin Resistance in Individual Organs and Cardiac Dysfunction in C57BL/6 Mice. Diabetes 2005; 54(12):3530–3540. PMID: 16306372
37. Yan J, Young ME, Cui L, Lopaschuk GD, Liao R, Tian R. Increased Glucose Uptake and Oxidation in Mouse Hearts Prevent High Fatty Acid Oxidation but Cause Cardiac Dysfunction in Diet-Induced Obesity. Circulation 2009; 119(21):2818. https://doi.org/10.1161/CIRCULATIONAHA.108.832915 PMID: 19451348
38. Wang S, Luo M, Zhang Z, Gu J, Chen J, Payne KM et al. Zinc deficiency exacerbates while zinc supplement attenuates cardiac hypertrophy in high-fat diet-induced obese mice through modulating p38 MAPK-dependent signaling. Toxicology Letters 2016; 258:134–146. https://doi.org/10.1016/j.toxlet.2016.06.020 PMID: 27346292
39. Barouch LA., Berkowitz DE, Harrison RW, O'Donnell CP, Hare JM. Disruption of Leptin Signaling Contributes to Cardiac Hypertrophy Independently of Body Weight in Mice. Circulation 2003; 108(6):754–759. https://doi.org/10.1161/01.CIR.0000083716.82622.FD PMID: 12885755
40. Dludla PV, Essop MF, Gabuza KB, Muller CJF, Louw J, Johnson R. Age-dependent development of left ventricular wall thickness in type 2 diabetic (*db/db*) mice is associated with elevated low-density lipoprotein and triglyceride serum levels. Heart and Vessels 2017; 32(8):1025–1031. https://doi.org/10.1007/s00380-017-0978-3 PMID: 28393273

41. Aasum E, Cooper M, Severson DL, Larsen TS. Effect of BM 17.0744, a PPARa ligand, on the metabolism of perfused hearts from control and diabetic mice. Can J Physiol Pharmacol 2005; 83(2):183–190. https://doi.org/10.1139/y04-139 PMID: 15791292

42. Aasum E, Belke DD, Severson DL, Riemersma RA, Cooper M, Andreassen M et al. Cardiac function and metabolism in Type 2 diabetic mice after treatment with BM 17.0744, a novel PPAR-a activator. American Journal of Physiology-Heart and Circulatory Physiology 2002; 283(3):H949–H957. https://doi.org/10.1152/ajpheart.00226.2001 PMID: 12181123

43. How OJ, Larsen TS, Hafstad AD, Khalid A, Myhre ESP, Murray AJ et al. Rosiglitazone treatment improves cardiac efficiency in hearts from diabetic mice. Archives of Physiology and Biochemistry 2007; 113(4–5):211–220. https://doi.org/10.1080/13813450701783281 PMID: 18158644

44. Hafstad AD, Khalid AM, How OJ, Larsen TS, Aasum E. Glucose and insulin improve cardiac efficiency and postischemic functional recovery in perfused hearts from type 2 diabetic (*db/db*) mice. American Journal of Physiology-Endocrinology and Metabolism 2007; 292(5):E1288–E1294. https://doi.org/10.1152/ajpendo.00504.2006 PMID: 17213470

45. Khalid AM, Hafstad AD, Larsen TS, Severson DL, Boardman N, Hagve M et al. Cardioprotective effect of the PPAR ligand tetradecylthioacetic acid in type 2 diabetic mice. American Journal of Physiology-Heart and Circulatory Physiology 2011; 300(6):H2116–H2122. https://doi.org/10.1152/ajpheart.00357.2010 PMID: 21421822

46. Coleman DL. Obese and diabetes: Two mutant genes causing diabetes-obesity syndromes in mice. Diabetologia 1978; 14(3):141–148. PMID: 350680

47. Panagia M, Schneider JE, Brown B, Cole MA, Clarke K. Abnormal function and glucose metabolism in the type-2 diabetic *db/db* mouse heart. Can J Physiol Pharmacol 2007; 85(3–4):289–294. https://doi.org/10.1139/y07-028 PMID: 17612636

48. Li RJ, Yang J, Yang Y, Ma N, Jiang B, Sun QW et al. Speckle tracking echocardiography in the diagnosis of early left ventricular systolic dysfunction in type II diabetic mice. BMC Cardiovascular Disorders 2014; 14(1):141–148.

49. Belke DD, Larsen TS, Gibbs EM, Severson DL. Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (*db/db*) mice. American Journal of Physiology-Endocrinology and Metabolism 2000; 279(5):E1104–E1113. https://doi.org/10.1152/ajpendo.2000.279.5.E1104 PMID: 11052966

50. Buchanan J, Mazumder PK, Hu P, Chakrabarti G, Roberts MW, Yun UY et al. Reduced Cardiac Efficiency and Altered Substrate Metabolism Precedes the Onset of Hyperglycemia and Contractile Dysfunction in Two Mouse Models of Insulin Resistance and Obesity. Endocrinology 2005; 146(12):5341–5349. https://doi.org/10.1210/en.2005-0938 PMID: 16141388

51. Van den Bergh A, Flameng W, Herijgers P. Type II diabetic mice exhibit contractile dysfunction but maintain cardiac output by favourable loading conditions. European Journal of Heart Failure 2006; 8(8):777–783. https://doi.org/10.1016/j.ejheart.2006.03.001 PMID: 16716661

52. Mori J, Patel VB, Abo Alrob O, Basu R, Altamimi T, DesAulniers J et al. Angiotensin 1–7 Ameliorates Diabetic Cardiomyopathy and Diastolic Dysfunction in *db/db* Mice by Reducing Lipotoxicity and Inflammation. Circ Heart Fail 2014; 7(2):327–339. PMID: 24389129

53. Hamdani N, Hervent AS, Vandekerckhove L, Matheeussen V, Demolder M, Baerts L et al. Left ventricular diastolic dysfunction and myocardial stiffness in diabetic mice is attenuated by inhibition of dipeptidyl peptidase 4. Cardiovascular Research 2014; 104(3):423–431. https://doi.org/10.1093/cvr/cvu223 PMID: 25341892

54. Andersson KB, Birkeland JAK, Finsen AV, Louch WE, Sjaastad I, Wang Y et al. Moderate heart dysfunction in mice with inducible cardiomyocyte-specific excision of the SERCA2 gene. Journal of Molecular and Cellular Cardiology 2009; 47(2):180–187. https://doi.org/10.1016/j.yjmcc.2009.03.013 PMID: 19328205

55. Boardman NT, Aronsen JM, Louch WE, Sjaastad I, Willoch F, Christensen G et al. Impaired left ventricular mechanical and energetic function in mice after cardiomyocyte-specific excision of SERCA2. American Journal of Physiology-Heart and Circulatory Physiology 2014; 306(7):H1018–H1024. https://doi.org/10.1152/ajpheart.00741.2013 PMID: 24486508

56. Land S, Louch WE, Niederer SA, Aronsen JM, Christensen G, Sjaastad I et al. Beta-Adrenergic Stimulation Maintains Cardiac Function in SERCA2 Knockout Mice. Biophysical Journal 2013; 104(6):1349–1356. https://doi.org/10.1016/j.bpj.2013.01.042 PMID: 23528094

57. Belke DD, Swanson EA, Dillmann WH. Decreased Sarcoplasmic Reticulum Activity and Contractility in Diabetic *db/db* Mouse Heart. Diabetes 2004; 53(12):3201–3208. PMID: 15561951

58. Stølen TO, Høydal MA, Kemi OJ, Catalucci D, Ceci M, Aasum E et al. Interval Training Normalizes Cardiomyocyte Function, Diastolic Ca2+ Control, and SR Ca2+ Release Synchronicity in a Mouse Model of Diabetic Cardiomyopathy. Circ Res 2009; 105(6):527–536. PMID: 19679837

59. Kuzmiak-Glancy S, Covian R, Femnou AN, Glancy B, Jaimes R, Wengrowski AM et al. Cardiac performance is limited by oxygen delivery to the mitochondria in the crystalloid-perfused working heart. American Journal of Physiology-Heart and Circulatory Physiology 2017; 314(4):H704–H715. https://doi.org/10.1152/ajpheart.00321.2017 PMID: 29127235

PAPER II

Diet-induced obese mouse hearts show metabolic adaptation that enhances tolerance to acute high fatty acid exposure and reduces susceptibility to ischemic injury

Neoma T. Boardman*, T. M. Pedersen*, L. Rossvoll, A.D. Hafstad, E. Aasum

Cardiovascular Research Group, Department of Medical Biology, Faculty of Health Sciences, UiT-The Arctic University of Norway, Norway

* N. T. Boardman and T. M. Pedersen contributed equally to this work.

Corresponding Author:

Ellen Aasum
Cardiovascular Research Group
Department of Medical Biology
Faculty of Health Sciences
UiT-The Arctic University of Norway
MH-building
9037 Tromsø
Norway
Phone: +4777646486
Email: ellen.aasum@uit.no

Highlights

- Acute elevation of fatty acids differently affects obese and normal hearts
- Obese hearts do not display fat-induced oxygen waste, oxidative stress or mitochondrial detriment
- Elevation of fatty acids improves ischemic tolerance in perfused hearts from obese mice

Abstract

An acute supply of high fatty acid (FA) levels to the normal heart is known to induce unfavorable effects, such as myocardial oxygen wasting, mechanical inefficiency and oxidative stress. Chronic supply of high FAs contributes in the development of obesity-/diabetes-related cardiomyopathy, however it is not clear how an acute FA load affects these hearts.

Feeding C57Bl/6J mice an obesogenic diet for 20 weeks, resulted in an obese model with insulin resistance and a mild cardiac dysfunction. In isolated hearts, the effects of high (1.8 mM) and low (0.4 mM) FA levels on left ventricular (LV) function, myocardial oxygen consumption (MVO_2), mechanical efficiency, oxidative stress, mitochondrial respiration and protein acetylation was assessed in hearts from control (CON) and diet-induced obese (DIO) mice. Finally, LV functional recovery and infarct size was determined following ischemia-reperfusion in hearts from CON and DIO mice.

In CON hearts, high FAs induced oxygen wasting and mechanical inefficiency. High FA also elevated ROS content, reduced mitochondrial OxPhos and enhanced mitochondrial protein acetylation. DIO hearts on the other hand, tolerated the high FA load, as they did not display unfavorable changes. In addition we found that high FA perfusion increased functional recovery and reduced infarct size in these hearts.

This study therefore highlights that there are marked differences in the response to an acute high FA-load in normal and obese hearts, which suggests that obese/diabetic hearts undergo adaptive changes that make them able to handle an acute FA load.

Keywords: heart perfusion, pressure-volume, oxygen consumption, cardiac efficiency, acetylation, mitochondrial respiration

1. Introduction

Obesity is closely associated with the development of type 2 diabetes and cardiovascular diseases. Both obesity and diabetes are known to increase the risk of developing a specific form of cardiac dysfunction, independent of coronary artery disease and hypertension. This cardiomyopathy is likely induced by the hormonal and metabolic derangements associated with obesity, where chronic dyslipidemia and long-term exposure to high circulating fatty acid (FA) levels contribute to the progression of cardiac dysfunction.

A chronic high FA supply leads to metabolic remodeling with changes in myocardial substrate utilization, making the heart more reliant on FA oxidation for energy production. This metabolic shift is complex and includes translational changes and allosteric control of FA transport proteins and enzymes, as well as post-translational modifications of enzymes. With regard to the latter, recent studies have also identified changes in protein lysine acetylation as an important contributor to these metabolic alterations [1]. If the FA uptake exceeds FA oxidation rate, myocardial lipotoxicity, with myocardial lipid and lipid metabolite accumulation, will occur. In addition, high FAs can be associated with increased reactive oxygen species (ROS) production. These changes will account for cardiac myocyte apoptosis, myocardial fibrosis [2] and perturbed mitochondrial function [3]. In addition, obese and diabetic hearts are known to be less efficient, as they show an increase in myocardial oxygen consumption (MVO_2) [4-6]. This elevated MVO_2 is likely linked to impaired mitochondrial energetics and altered Ca^{2+} handling [7].

Obesity and diabetes are known to worsen the long-term outcome of ischemic heart disease in patients [8]. This can be linked to impaired myocardial reperfusion (due to vascular dysfunction), reduced coronary reserve and/or decreased myocardial ischemic tolerance. Although higher myocardial susceptibility to ischemic injury has been described in pre-clinical models, there are also studies reporting unchanged or even enhanced ischemic tolerance [9-12]. This may partly be due to differences in the severity of the metabolic disease in these models. In addition, obesity and diabetes are complex disorders associated with changes in circulating hormonal and substrate levels, which may also influence the ischemic outcome. Accordingly, variation in experimental conditions may also contribute to the reported inconsistencies in myocardial ischemic tolerance when studied *in vitro* [13, 14].

Acute myocardial infarction is accompanied by increased circulating FAs, due to an adrenergic activation of lipolysis in adipose tissue [15]. Therefore, an ischemic heart will not only be challenged by myocardial hypoxia, but also by an acute high FA load. In normal hearts, high levels of FAs have been reported to induce oxygen wasting with subsequent mechanical inefficiency [16]. High FAs also

enhance mitochondrial ROS production and impair redox balance, and can alter Ca^{2+} homeostasis [17, 18]. These FA-mediated changes could all potentially aggravate ischemia-reperfusion injury. Accordingly, several experimental studies report that exposing normal hearts to high levels of FAs can reduce post-ischemic functional recovery [19-23]. To what extent an acute increase in FA concentration affects obese or diabetic hearts is, however, less clear. In 1997 Dennis Paulson suggested that the presence of FAs increased the degree of ischemic injury in type 1 diabetic hearts [13]. Regardless of this, and despite the increasing use of obese and type 2 diabetic preclinical models, glucose is still commonly used as the sole substrate, and only a few *in vitro* studies have added high levels of FAs to the perfusate. Desrois and co-workers reported unchanged ischemic tolerance in glucose-perfused hearts from Goto-Kakizaki diabetic rats when they were compared to controls [24], while when high FA levels were present during ischemia, diabetic hearts showed a lower tolerance than their controls [25]. In support of this, a direct comparison of hearts from DIO rats perfused in the absence and presence of high FAs suggested a FA-mediated reduction in ischemic tolerance following obesity [19]. Notably, these studies compared high FAs to conditions where FAs were totally absent. Thus, to our knowledge, this is the first study that aims to examine the effects of high FAs on ischemia-reperfusion in a model of pre-diabetes, by comparing it to a condition with lower FAs.

We hypothesized that cardiac metabolic remodeling following obesity may alter the tolerance to an acute high FA load, and by comparing the effects of a high FA exposure in isolated hearts from normal and obese mice, the finding in this study indicated that obese hearts are less vulnerable to disadvantageous FA-mediated changes. The study also contributes to increase our awareness of how experimental conditions influence the ischemic tolerance, which is crucial in order to foster our understanding within this field.

2. Materials and methods

2.1 Animals

C57BL/6J male mice (5-6 weeks) were purchased from Charles River Laboratories (Germany). Obesity and insulin resistance were induced by feeding mice an obesogenic diet for 20 weeks (diet-induced obese, DIO), as previously described [26, 27]. Age-matched mice fed a regular chow diet served as controls (CON). All mice were housed in a room with a constant temperature of 23°C and 55% humidity, with a 12:12-h reversed light:dark-cycle, and were given ad libitum access to water and their respective diets. To evaluate insulin resistance, plasma glucose (glucometer, FreeStyle Lite, Alameda, CA), and insulin (commercial kits from DRG Diagnostics, Marburg, Germany) was determined in blood samples obtained from the saphenous vein from fasted (4 hours) mice . Homeostatic model assessment (HOMA) was calculated from fasting blood glucose and insulin levels. Plasma free fatty acids were determined in blood samples taken from the saphenous vein of fed animals prior to euthanasia, using commercial kits from Wako Chemicals (Neuss, Germany). Animal experiments were approved by the Norwegian National Animal Research Authority, which conforms to the National Institute of Health guidelines (NIH publication No. 85-23, revised 1996) and European Directive 2010/63/EU.

2.2 Assessment of left ventricular (LV) function, fatty acid oxidation and oxygen consumption

Left ventricular (LV) function, fatty acid (FA) oxidation rate and myocardial oxygen consumption (MVO_2) were assessed in isolated perfused hearts. All hearts were perfused in a recirculating mode, with a modified Krebs-Henseleit bicarbonate buffer supplemented with glucose (5 mM) and palmitate (bound to 3% BSA) with either low (0.4 mM) or high (1.8 mM) FA concentration. LV pressure and volume was assessed in working hearts using a conductance catheter (1 F) inserted through the apex [16]. FA oxidation rates were also assessed in working hearts using [9,10-^{3}H] palmitate prebound to albumin [28]. MVO_2 was assessed using fiber-optic oxygen probes (FOXY-AL300; Ocean Optics, Duiven, Netherlands) inserted into the perfusion line just above the aortic cannula and into the pulmonary artery to obtain the arterial-venous difference in PO_2. Mechanical efficiency was defined as the ratio between cardiac work (calculated as cardiac power) and MVO_2 in working hearts. Work-independent MVO_2 was also assessed by changing to Langendorff perfusion mode and inserting a vent through the apex. Under these conditions the hearts were paced (7 Hz), and MVO_2 was measured before and after KCl-induced cardiac arrest, representing O_2 cost for unloaded hearts ($MVO_{2\ unloaded}$) and for basal metabolism ($MVO_{2\ BM}$), as previously described [29].

Oxygen cost for processes associated with excitation-contraction coupling (MVO_{2ECC}) was calculated as the difference between $MVO_{2\ unloaded}$ and $MVO_{2\ BM}$.

2.3 Mitochondrial respiration

Mitochondrial respiration was assessed in isolated cardiac mitochondria from Langendorff perfused hearts subjected to a low or high FA load for 30 minutes. Respiration was determined by high-resolution respirometry using an oxygraph (O2-k, Oroboros Instruments, Austria). Pyruvate (5mM) and malate (2mM) or palmitoyl-CoA (25µM), L-carnitine (5mM) and malate (2mM) served as substrates. Uncoupled respiration (V_0) was assessed in the presence of substrates but without ADP. Coupled respiration (V_{max}, OxPhos) was defined as the peak respiration after adding 100 µmol/L ADP. Respiration rates were adjusted to total protein, and respiratory control ratio (RCR) was calculated as the ratio between V_{max} and V_0. A rate-independent coupling parameter, based on the ratio of molecules of ADP phosphorylated to each oxygen molecule consumed (ADP/O), was also calculated.

2.4 Mitochondrial protein acetylation

Mitochondrial proteins were lysed in buffer containing: 75mM Tris-HCL, 3.8% SDS, 4M Urea and cOmplete™ Protease Inhibitor Cocktail (Sigma-Aldrich). Laemmli buffer was added and samples were boiled (95°C, 5 minutes). Protein (20 µg) was loaded onto a 4-15% Criterion gel (Bio-Rad) and transferred onto a nitrocellulose membrane (GE Healthcare) following electrophoresis. Membranes were blocked (5% milk, 1 hour), followed by incubation with acetylated-Lysine antibody or VDAC antibody (Cell Signalling Technology) overnight at 4°C, and thereafter washed and incubated with anti-Rabbit antibody for 1 hour. Immunopositive bands were developed in Chemoluminescent Peroxidase Substrate-3 (Sigma) for Acetylated-Lysine and LumiGlo (Cell Signalling Technology) for VDAC, and visualized using a GE ImageQuant LAS 4000 (GE healthcare). Densitometry of bands was performed using Image Studio Protein Lite (LI-COR Biosciences) and load was normalized using VDAC as a loading control.

2.5 Myocardial ROS

Following 30 minutes of Langendorff perfusion, LV tissue was embedded in O.C.T. compound, frozen in cooled isopentane and stored at -70°C. The samples were cryo-sectioned, stained with dihydroethidium (DHE) for evaluation of superoxide generation, and evaluated under an epifluorescence microscope [26]. 10-15 images from each heart were obtained for quantification using Image J software.

2.6 Susceptibility to ischemic injury

Ischemic tolerance was examined in Langendorff perfused hearts. LV function was recorded using an intraventricular fluid-filled balloon connected to a pressure transducer [27]. The volume of the balloon was adjusted to give an end-diastolic pressure of 5–10 mmHg. After 20 minutes of stabilization, the hearts were subjected to global ischemia followed by 90 min reperfusion. At the end of reperfusion, hearts were frozen at -20°C, prior to slicing and staining using a 1% 2,3,5-triphenyl-2H-tetrazolium chloride solution. Infarct size was determined using ImageJ software (National Institutes of Health, Bethesda, MD).

2.7 Statistical analysis

Data are presented as means ± SE. Differences between two groups were analyzed using an unpaired Student's *t*-test. Multiple comparisons were performed by two-way analysis ANOVA followed by Holm-Sidak method as the post hoc test. Differences of $P < 0.05$ were considered significant.

3. Results

Diet-induced obese (DIO) mice exhibited higher body weight and increased perirenal fat deposits (Table 1), elevated free fatty acids (FAs) plasma levels, and a moderate increase in fasted blood glucose. These mice also showed marked insulin resistance, as indicated by increased HOMA-IR, which was primarily due to higher insulin levels (Table 1).

Table 1. Animal characteristics of control (CON) and diet-induced obese (DIO) mice.

	CON	**DIO**
n	*71*	*73*
Body weight (g)	31.7 ± 0.3	47.0 ± 0.5 #
Tibia length (mm)	18.3 ± 0.1	18.1 ± 0.1
Perirenal fat mass (g)	0.34 ± 0.03	1.46 ± 0.06 #
Liver weight (g)	1.17 ± 0.02	2.05 ± 0.10 #
Blood glucose $_{fasted}$ (mmol/L)	5.7 ± 0.2	6.9 ± 0.2 #
Plasma insulin $_{fasted}$ (µg/L)	0.9 ± 0.1	3.2 ± 0.3 #
HOMA-IR	5.5 ± 0.5	25.2 ± 2.3 #
Plasma free FA (µmol/L)	371 ± 32	571 ± 37 #
Heart weight (mg)	155 ± 2	157 ± 2
Heart weight/tibia length	8.5 ± 0.1	8.7 ± 0.1

Data are means ± SE. Number of animals used for assessment of blood glucose, plasma insulin and the calculation of the homeostatic model assessment (HOMA-IR) was 49 and 43 in CON and DIO, respectively. Number of animals used for assessment of free fatty acids (FA) in non-fasted animals was 31 and 25 in CON and DIO, respectively. #$P < 0.05$ vs. CON.

3.1 Left ventricular function

Left ventricular (LV) function was assessed in isolated perfused working hearts (Table 2). When perfused with a low FA concentration, hearts from DIO mice are characterized by diastolic dysfunction, as indicated by elevated LV end-diastolic pressure, reduced LV relaxation (Tau), and augmented end-diastolic pressure-volume relationship (EDPVR). These hearts developed a mild systolic dysfunction, with a small reduction in cardiac output and cardiac work (assessed as calculated cardiac power), but without change in prerecruitable stroke work index (PRSWi) (Table 2 and Figure 1A). Subjecting CON and DIO to a high FA load (a 6-fold increase in FA concentration), did not alter cardiac function in either CON or DIO hearts (Table 2 and Figure 1A).

Table 2. Left ventricular function assessed in isolated perfused working hearts from control (CON) and diet-induced obese (DIO) mice, perfused with low and high fatty acid (FA) levels.

	CON Low FA	**CON High FA**	**DIO Low FA**	**DIO High FA**
n	*9*	*8*	*11*	*11*
Heart rate (bpm)	432 ± 9	461 ± 9 *	423 ± 9	412 ± 9
Cardiac output (mL/min)	16.7 ± 0.8	15.5 ± 0.8	14.1 ± 0.7 #	14.1 ± 0.7
Coronary flow (mL/min)	3.8 ± 0.2	4.5 ± 0.2	3.9 ± 0.2	3.7 ± 0.2 #
LVEDP (mmHg)	6.7 ± 0.5	5.6 ± 0.5	9.0 ± 0.5 #	10.1 ± 0.5 #
LVDP (mmHg)	66.8 ± 1.4	67.2 ± 1.5	65.0 ± 1.4	62.9 ± 1.4
dP/dt_{max} (mmHg/sec)	4923 ± 226	4657 ± 119	4457 ± 252	4642 ± 126
dP/dt_{min} (mmHg/sec)	-4069 ± 241	-3828 ± 96	-3718 ± 243	-3531 ± 99
Tau $_{Glanz}$ (mesc)	16.9 ± 0.6	15.7 ± 0.4	19.0 ± 0.7 #	20.0 ± 0.6 #
EDPVR (mmHg/mL)	0.15 ± 0.03	0.15 ± 0.03	0.30 ± 0.03 #	0.33 ± 0.04 #
PRSWi	66.8 ± 1.4	67.2 ± 1.5	65.0 ± 1.5	62.8 ± 1.4

Data are means ± SE. Steady state parameters were obtained in hearts perfused with a preload and afterload of 10 and 50 mmHg respectively. All hearts were paced at 10% higher than their intrinsic heart rate, and intraventricular pressure (LV) was assessed using a conductance catheter inserted through the apex. dP/dt_{max} and dP/dt_{min}; maximum positive and negative first-time derivative of LV pressure, LVED; LV end-diastolic pressure, LVDP; LV developed pressure, Tau; LV relaxation time constant. Load-independent parameters were assessed as from a family of pressure-volume loops created by a temporary preload occlusion in 7-10 unpaced hearts from each group: EDPVR; end-diastolic pressure-volume relationship, PRSWi, and prerecruitable stroke work index. *$P < 0.05$ vs low FA. #$p < 0.05$ vs. CON in the same perfusion group.

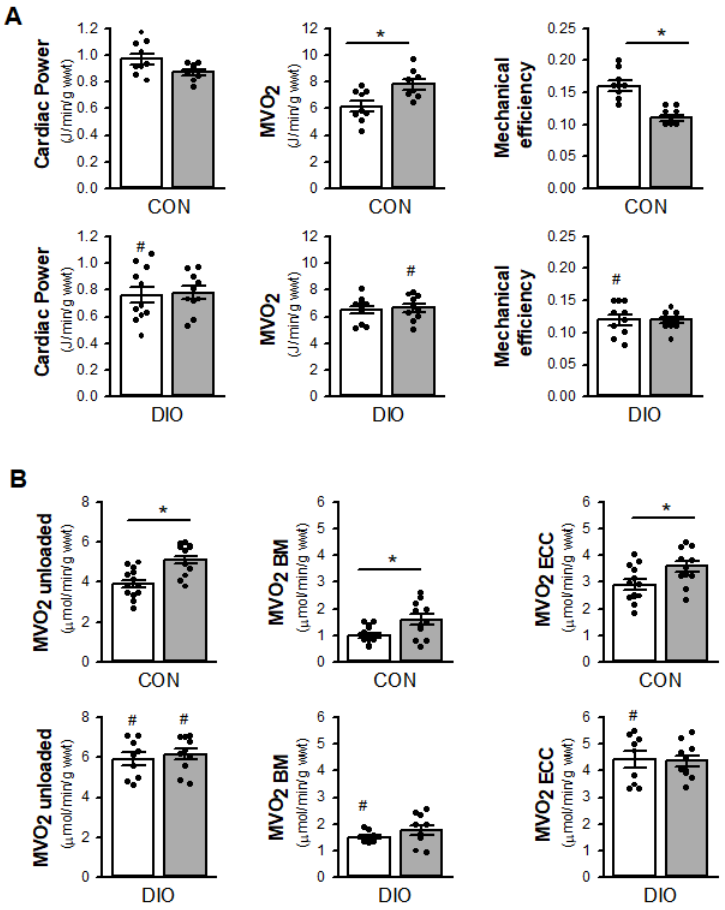

Figure 1. A: Cardiac power, myocardial oxygen consumption (MVO_2) and mechanical efficiency (ratio between cardiac power and MVO_2) measured in isolated perfused working hearts from control (CON) and diet-induced obese (DIO) mice. Hearts were perfused with a low (open bars) or high FA concentration (closed bars). Cardiac power was calculated from cardiac output and developed pressure (see Table 2). **B:** Myocardial oxygen consumption (MVO_2) in electrically paced (7 Hz) Langendorff perfused hearts prior to and after KCl-induced cardiac arrest, representing the oxygen cost of the heart in a unloaded condition ($MVO_{2\ unloaded}$) and for basal metabolism ($MVO_{2\ BM}$), respectively. The oxygen cost for excitation-contraction coupling ($MVO_{2\ ECC}$) was calculated from the difference between $MVO_{2\ unloaded}$ and $MVO_{2\ BM}$. Data are means ± SE. *p < 0.05 vs. low FA. #p < 0.05 vs. CON in the same perfusion group.

3.2 Myocardial FA oxidation rate, oxygen consumption and mechanical efficiency

Myocardial FA oxidation rates were higher in DIO hearts compared to CON hearts under low FA conditions (0.41 ± 0.02 vs 0.19 ± 0.02 in DIO (n=8) and CON (n=7), respectively). As expected, an elevation of the FA concentration increased FA oxidation rates in both CON (0.50 ± 0.04, p<0.05 vs. low FA, n=10) and DIO hearts (0.54 ± 0.04, p<0.05 vs. low FA, n=11).

Under low FA conditions, DIO hearts displayed higher myocardial oxygen consumption (MVO_2) compared to CON hearts. Thus, along with the lower cardiac power, these hearts have reduced

mechanical efficiency (Figure 1A). Elevated MVO_2 was also observed when hearts where subjected to an unloaded condition (Figure 1B) which illustrates an obesity-induced elevation of the O_2 cost for non-mechanical processes. This increased O_2 cost was associated with processes related to basal metabolism ($MVO_{2\,BM}$) as well as the excitation-contraction (EC) coupling ($MVO_{2\,ECC}$) (Figure 1B).

The high FA load markedly increased MVO_2 in CON hearts. As this was not associated with any change in cardiac function, mechanical inefficiency was due to FA-induced oxygen wasting (Figure 1A). High FAs also increased unloaded MVO_2 in CON, which was due to higher O_2 cost for both BM and EC coupling. In contrast to CON hearts, the high FA load did neither alter MVO_2 under working conditions (Figure 1A) nor unloaded conditions (Figure 1B) in DIO hearts. Thus, in these hearts high FAs do not induce oxygen wasting or affect mechanical efficiency (Figure 1A).

3.3 Mitochondrial respiration

Mitochondrial respiration was assessed using pyruvate (and malate) or palmitate and L-carnitine (and malate) as substrates for respiration. Mitochondria from DIO hearts perfused with a low FA concentration showed reduced coupled respiration (V_{max}) compared to controls both when pyruvate and palmitate served as substrate (Figure 2). Under the same conditions, uncoupled respiration (V_0), was also significantly reduced, such that the respiratory control ratio (RCR) was not different between CON and DIO , regardless of substrate. Correspondingly, the ADP/O ratio was not different between CON and DIO (pyruvate; 2.23 ± 0.06 vs. 2.37 ± 0.06; palmitate; 2.08 ± 0.06 vs. 2.09 ± 0.04, in CON and DIO, respectively).

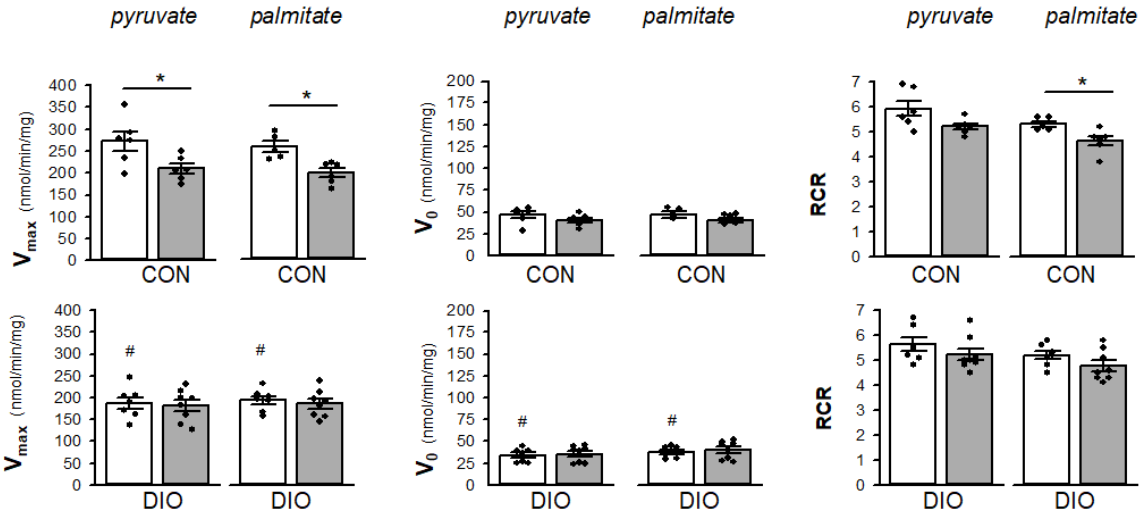

Figure 2. Mitochondrial respiration measured in cardiac mitochondria isolated from control (CON, n=5-6) and diet-induced obese (DIO, n=7-8) hearts perfused with a low (open bars) or high FA concentration (closed bars)

prior to the isolation procedure. The respiratory medium contained either pyruvate (5mM) and malate (2mM) or palmitoyl-CoA (25µM), L-carnitine (5mM) and malate (2mM). V_0 respiration is defined as the respiratory state before ADP is added and V_{max} is defined as the respiration peak after adding 100 µmol/L ADP. Respiratory control ratio (RCR) was calculated as the ratio between V_{max} and V_0. Mitochondrial respiration rates were normalized to protein. Data are means ± SE . *p < 0.05 vs. low FA. #p < 0.05 vs. CON in the same perfusion group.

When exposing the CON hearts to a high FA load prior to mitochondrial isolation, V_{max} was significantly reduced, accompanied by a small but not significant reduction in V_0. As the reduction in V_{max} was more pronounced than V_0, RCR was lowered in mitochondria from hearts perfused with high FAs (palmitate; p<0.05, pyruvate; p=0.067) (Figure 2), while ADP/O was unchanged (2.32 ± 0.03 and 2.16 ± 0.02, pyruvate and palmitate, respectively). In contrast to CON, high FA load did not impair either coupled (V_{max}) or uncoupled (V_0) respiration in mitochondria from DIO hearts, regardless of substrate conditions, leaving RCR unaltered (Figure 2). Nor was ADP/O altered by high FAs in DIO (2.32 ± 0.06 and 2.11 ± 0.04, pyruvate and palmitate, respectively).

3.4 Mitochondrial protein acetylation and myocardial ROS content

The effect of different FA concentrations on the lysine acetylation of mitochondrial proteins was also examined. In these experiments, we included hearts perfused with the same BSA-containing buffer, but without FA (NoF). Mitochondria from DIO hearts showed a near 2-fold higher protein acetylation than those from CON hearts, when perfused in absence of FAs (Figure 3A). Increasing FA concentration in the perfusate, resulted in a dose-dependent increase in protein acetylation in CON hearts (Figure 3B). However, the presence of FAs did not alter acetylation in DIO hearts. Gene expression analysis of tissue from these hearts revealed that perfusion with high FA load did not alter the mRNA expression of sirtuin 3 or GCN5L1 (data not shown).

Under low FA conditions we found elevated ROS levels in LV tissue from DIO compared to CON hearts (Figure 3C). However, subjecting hearts to the high FA load significantly increased ROS levels in CON hearts, whilst DIO hearts showed a resistance to FA-induced increase in ROS.

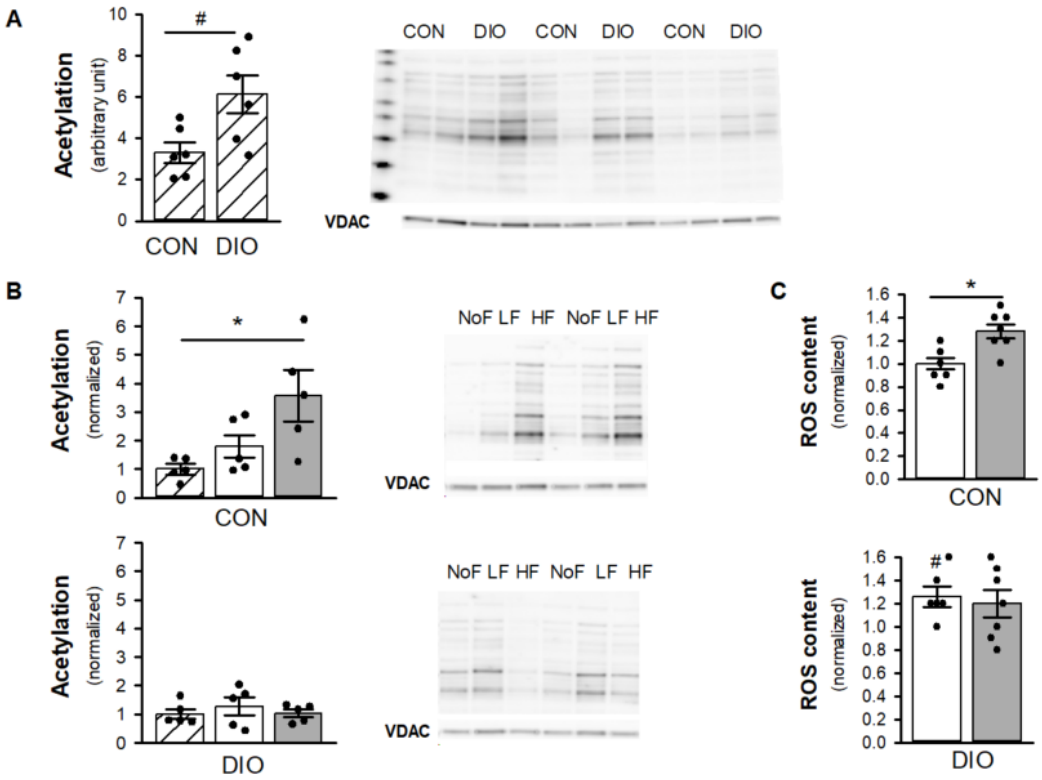

Figure 3. Overall mitochondrial protein lysine-acetylation and myocardial ROS content in control (CON) and diet-induced obese (DIO) mouse hearts. **A:** Protein acetylation in mitochondria isolated from CON and DIO hearts perfused without fatty acids (NoF, n=6). **B:** Protein acetylation in mitochondria isolated from hearts following perfusion without fatty acids (NoF, hatched bars, n=6), with low fatty acid (LF, open bars, n=6) or high fatty acid (HF, closed bars, n=6) concentration. **C:** Myocardial ROS content (n=6-7) in CON and DIO hearts was assessed as fluorescence intensity in left ventricular cryosections with dihydroethidium staining, and the data was normalized to low FA-perfused CON hearts. Data were normalized to NoF. Data are means ± SE. *p < 0.05 vs.low FA. #p < 0.05 vs. CON in the same perfusion group.

3.5 Susceptibility to ischemic injury

Ischemic tolerance was examined in Langendorff perfused hearts, as this perfusion mode is a robust technique for examining myocardial ischemic tolerance. It should be noted, however, that this mode is less sensitive for picking up LV functional changes [30]. Accordingly, using this perfusion mode we did not find differences in pre-ischemic function between CON and DIO hearts when perfused under low FA conditions (Table 3), even though dysfunction was evident in DIO hearts in the working mode. On the other hand, a significantly lower heart rate was observed in high FA-perfused DIO hearts when compared to CON. However, as the lower heart rate was accompanied by higher LV developed pressure (LVDP), the resulting pre-ichemic rate-pressure product (RPP) was similar to the other experimental groups (Table 3).

Table 3. Pre- and post-ischemic left ventricular function in isolated Langendorff perfused hearts from control (CON) mice and diet-induced obese (DIO) mice, perfused with low and high fatty acid (FA) levels.

	CON Low FA (n=14)			**CON High FA** (n=14)		
	pre-ischemia	post-ischemia	%recovery	pre-ischemia	post-ischemia	%recovery
Heart rate (bpm)	296 ± 8	284 ± 9	*96 ± 3*	320 ± 10	256 ± 17	*89 ± 4*
CF (mL/min)	3.0 ± 0.2	2.0 ± 0.2	*65 ± 4*	3.1 ± 0.2	2.0 ± 0.2	*63 ± 4*
LVDP (mmHg)	134 ± 7	76 ± 5	*58 ± 4*	126 ± 7	61 ± 8	*49 ± 6*
dP/dt_{max} (mmHg/sec)	4847 ± 340	2764 ± 283	*58 ± 4*	4416 ± 333	2301 ± 367	*53 ± 7*
dP/dt_{min} (mmHg/sec)	-3377 ± 160	-1969 ± 159	*59 ± 4*	-3132 ± 133	-1617 ± 217	*51 ± 6*
RPP (mmHg*bpm)	39702 ± 2486	20968 ± 1638	*55 ± 1*	40123 ± 2409	17979 ± 2192	*45 ± 5*

	DIO Low FA (n=15)			**DIO High FA** (n=12**)**		
	pre-ischemia	post-ischemia	%recovery	pre-ischemia	post-ischemia	%recovery
Heart rate (bpm)	293 ± 10	261 ± 15	*89 ± 5*	279 ± 12 #	292 ± 11 * #	*106 ± 4 * #*
CF (mL/min)	3.5 ± 0.2	2.6 ± 0.2 #	*74 ± 5*	3.5 ± 0.2	2.4 ± 0.2	*70 ± 4*
LVDP (mmHg)	147 ± 11	64 ± 10	*42 ± 6*	152 ± 7 #	80 ± 6	*53 ± 6*
dP/dt_{max} (mmHg/sec)	5413 ± 483	2259 ± 388	*41 ± 6*	5514 ± 420	2899 ± 161	*55 ± 7*
dP/dt_{min} (mmHg/sec)	-3712 ± 237	-1641 ± 225	*42 ± 5*	-3878 ± 251 #	-2019 ± 116	*54 ± 6*
RPP (mmHg*bpm)	42034 ± 3465	16528 ± 2661	*38 ± 6 #*	42042 ± 2414	23069 ± 1226	*56 ± 3 **

Data are means ± SE. Left ventricular (LV) function was assessed using a fluid-filled balloon where the end-diastolic pressure was adjusted to be between 5 and 10 mmHg. Post-ischemic functional recovery is calculated as % of pre-ischemic values. CF; coronary flow, LVDP; LV developed pressure, dP/dt_{max} and dP/dt_{min}; maximum positive and negative first-time derivative of LV pressure, respectively, RPP; rate-pressure product (LVDP x heart rate). *P < 0.05 vs low FA. #p < 0.05 vs. CON in the same perfusion group.

The hearts were subjected to 25 minutes of no-flow ischemia followed by reperfusion, with post-ischemic LV function assessed until maximum recovery. Functional recovery (expressed as % of the pre-ischemic values) showed that under low FA conditions, DIO hearts exhibited impaired recovery of RPP (Figure 4, Table 3), which was mainly due to a lower recovery of LVDP (Table 3). Corroborating this, infarct size was also greater in DIO hearts under these conditions.

Exposing CON hearts to high FA levels did not significantly impair functional recovery, nor was infarct size increased (Figure 4, Table 3). In contrast, and to our surprise, FA load was found to significantly improve post-ischemic recovery in DIO hearts, which was evident both due to a significant improvement in heart rate, as well as RPP. In line with an improved post-ischemic functional recovery, infarct size was also reduced following the high FA load in DIO hearts (Figure 4). Notably, although DIO hearts showed lower ischemic tolerance when compared to CON hearts when perfused under low FA conditions, there were no significant differences both with regard to post-ichemic functional recovery and infarct size between DIO and CON under high FA-perfusion (Figure 4).

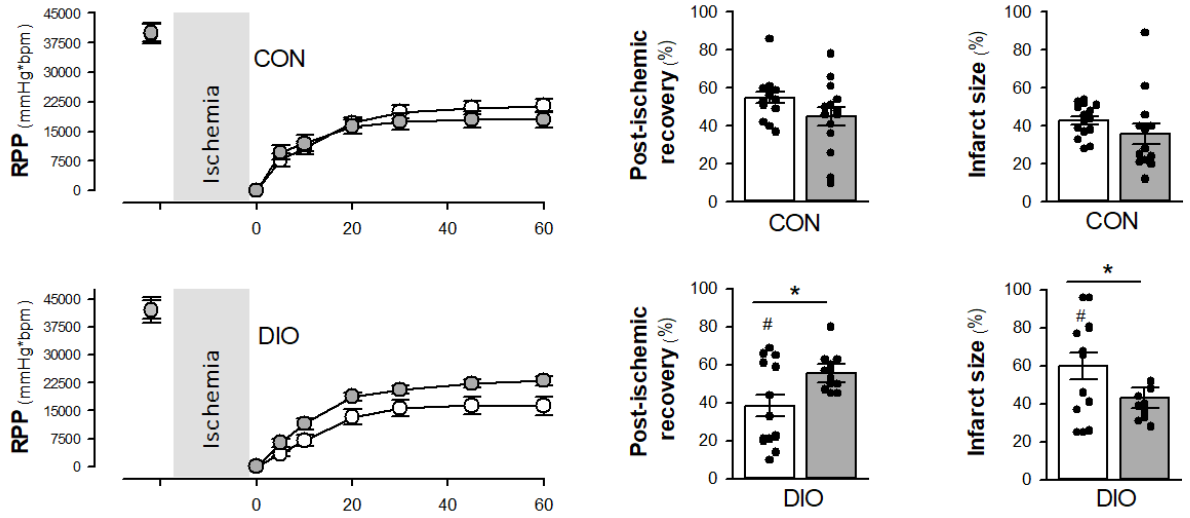

Figure 4. Rate-pressure product (RPP), the calculated post-ischemic recovery (% of pre-ischemic RPP values) and infarct size in isolated perfused hearts from control (CON) and diet-induced obese (DIO) mice. Hearts were subjected to perfusion with a low (open circle and bars) or high FA concentration (closed circle and bars). Data are means ± SE of 12-15 in each group. *p < 0.05 vs. low FA. #p < 0.05 vs. CON in the same perfusion group.

4. Discussion

As both physiological and pathophysiological stresses can lead to high circulating levels of fatty acids (FAs), we examined how a high, compared to a low, FA exposure affected hearts from normal and diet-induced obese (DIO) mice. While high FA load increased myocardial reactive oxygen species (ROS), reduced mitochondrial function and augmented mitochondrial protein acetylation in normal hearts, these effects were not found in obese hearts. We also found that FA-mediated oxygen wasting and mechanical inefficiency was present in normal hearts, but not in hearts from obese mice. Finally, while high FAs did not affect ischemic tolerance in controls, we found, to our surprise, increased ischemic tolerance when DIO hearts were exposed to high FAs. These findings clearly show that a high FA load affects normal and obese hearts differently, which may suggest that dyslipidemia and the chronic FA exposure may render DIO hearts less vulnerable to the disadvantageous effects of an acute FA load.

4.1 High FA load in normal hearts

In normal hearts, elevation of FAs has been reported to be associated with a range of cardiac effects. High FA supply decreases mechanical efficiency, due to an increase in myocardial oxygen consumption (MVO_2) [5, 16, 31]. This FA-mediated oxygen wasting is commonly attributed to the obligatory increase in myocardial FA oxidation, as the oxidation of FAs requires more oxygen for the same amount of ATP produced, compared to glucose. There is, however, evidence that the increased O_2 cost for FA oxidation *per se* is negligible, as inhibition of myocardial FA oxidation does not abolish the increase in MVO_2 when hearts are perfused with high FAs [31, 32]. The FA-mediated increase in MVO_2 is primarily linked to increased work-independent MVO_2 [16, 31], due to an increase in the O_2 cost of both excitation-contraction (EC) coupling and basal metabolism (BM) [31]. Accordingly, FA-mediated O_2 wasting is likely linked to changes in mitochondrial energetics and/or changes that increase the energy expenditure for processes associated with EC coupling.

In cardiomyocytes, FAs have been shown to decrease the amplitude and decay rate of the Ca^{2+} transients [17]. This is likely due to modifications of Ca^{2+} handling proteins such as the ryanodine receptor (RyR) and sarcoplasmic Ca^{2+}ATPase (SERCA). FAs also increase ROS production and alter the redox balance [17, 33]. Thus, as the majority of energy used in EC coupling is related to myocardial Ca^{2+} homeostasis, the higher O_2 cost of EC coupling following high FAs, is likely mediated by a redox-linked modification of Ca^{2+} handling proteins [18], although the exact underlying mechanisms remain to be uncovered.

We demonstrate that high FAs increased mitochondrial protein acetylation. As this occurred despite an increase in FA oxidation rate, the augmented acetylation is likely driven by a mismatch between the supply and oxidation of FAs, causing an accumulation of acetyl-CoA. Increased lysine acetylation has been associated with Complex I impairment [34], which could explain the FA-mediated decrease in oxidative phosphorylation (OxPhos). In support of this, Boudina *et al.* reported that skinned cardiac fibers from hearts perfused with high FAs (when compared to no FAs), showed reduced OxPhos when pyruvate (but not when palmitate) served as substrate [6]. The present study shows that perfusion with a high FA concentration (compared to low) decreased both pyruvate- and palmitate-driven OxPhos, and reduced the mitochondrial coupling efficiency.

4.2 High FA load in DIO hearts

The diet-induced obese (DIO) mice used in the present study resemble a pre-diabetic state that is typically characterized by obesity, elevated circulating free FA levels and insulin resistance [26, 27, 30]. In accordance with our previous studies, left ventricular (LV) pressure-volume recordings revealed that these hearts primarily develop diastolic dysfunction [26, 27, 30]. Under normal/low FA

conditions they also display mechanical inefficiency, due to an increased MVO_2, which is also present when they are perfused in an unloaded condition [26, 27].

The increased O_2 consumption may be related to a range of factors, such as altered substrate utilization, lipotoxicity, altered mitochondrial energetics and increased oxidative stress, as well as structural remodeling and inefficient Ca^{2+} transport, which has been reviewed in detail by Hafstad *et al.* [35]. Accordingly, DIO hearts showed increased ROS, higher FA oxidation rates and impaired mitochondrial respiration, together with augmented O_2 cost for EC coupling and BM. Although some of the changes induced by chronic FA exposure seem to resemble effects caused by the acute high FA load in normal hearts, they are not comparable, as long-term exposure to high circulating lipid levels causes a metabolic remodeling, making these heart uniquely adapted to handle high FAs, as discussed in the following section.

Although a high FA load neither improved nor impaired the LV function of perfused DIO hearts when compared to a low FA condition, there are reports showing that FAs improve cell shortening and Ca^{2+} transients in cardiomyocytes from obese and type 2 diabetic mice when compared to conditions where FAs are absent [17, 33]. Likewise, increased LV function has also been reported when FAs were added to the perfusate in isolated hearts from experimental models of diet-induced obesity [36] and type 2 diabetes [33]. This effect was particularly evident in hearts and cardiomyocytes subjected to metabolic (hyperglycemia) and/or adrenergic stress, where the improved function was linked to a FA-mediated increase in the content of reduced glutathione (GSH) and augmented mitochondrial ROS scavenging capacity [33, 37]. These findings suggest that FAs may be crucial in order to ensure a sufficient supply of reducing equivalents to prevent unfavorable ROS production and impaired energetics [33], and that the metabolic inflexibility in obese/diabetic hearts may render them energy starved when omitting FAs in experimental settings of increased work [38].

In accordance with a study by Cole *et al.* [5] using hearts from DIO rats, high FAs (compared to low FAs) did not alter LV mechanical efficiency in DIO mice in the present study. We have also shown that high FAs neither altered unloaded MVO_2 nor the O_2 cost for EC coupling and BM in DIO hearts, in accordance with a previous study on hearts from type 2 diabetic *db/db* mice [29]. Contrary to control hearts, high FAs did not increase mitochondrial protein acetylation in DIO hearts. This suggests that DIO hearts have higher capacity to handle the increase in acetyl-CoA that follows the sudden increase in FA load, and may have contributed to preserve mitochondrial respiration and coupling efficiency in high FA-perfused DIO hearts. Additionally, we did not find high FAs to exacerbate myocardial ROS accumulation, which corroborates findings in cardiomyocytes from *ob/ob* hearts, showing a resistance to a FA-mediated increase in mitochondrial ROS emission [17].

4.3 The effect of acute FA load on ischemic tolerance in normal and obese hearts

Acute myocardial infarction is reported to be accompanied by increased circulating FA levels [15], which is due to a hyper-adrenergic state, leading to increased lipolysis in white adipose tissue. Thus, the exposure to an acute high FA load poses an additional challenge in the hypoxic state, where FA-mediated O_2 wasting could be particularly detrimental. Accordingly, high FA-perfused rat hearts have been reported to have impaired post-ischemic functional recovery when compared to hearts perfused without FAs [19-23]. Similarly, a recent study, which also used normal rat hearts, showed that increasing the FA concentration from 0.4 to 1.2 mM, reduced post-ischemic function and increased infarct size [20]. In contrast to this, high FAs did not exacerbate ischemic injury in normal hearts in the present study. Although the reason for this discrepancy is not clear, it may be related to differences in species, or more likely the severity of the ischemic insult. As the aim of this study primarily was to examine the effects of high FA load in obese hearts, the duration of ischemia may have been too short to unmask the disadvantageous effects of high FA levels in the control hearts. Although clinical evidence indicates that obesity/diabetes sensitizes the human heart to ischemia-reperfusion injury, *in vivo* studies on myocardial ischemic tolerance in preclinical models remain somewhat ambiguous. It should be noted that despite the fact that FAs are the preferred substrate for the heart, the majority of *in vitro* studies using diabetic or DIO models have used glucose as the sole substrate, and decreased [39-41], unchanged [42-44] and even increased [45-48] ischemic tolerance have been reported. Although this inconsistency has been linked to the severity of the model [44, 47, 49], it does not seem to be the sole explanation as the divergence remains in older or long-term fed animals [43, 45, 50, 51]. The present and previous studies have reported decreased ischemic tolerance when obese and/or diabetic hearts are perfused with low levels of FAs [27, 28], which suggests that under these conditions, these hearts have higher susceptibility to ischemic injury. On the other hand, and to our surprise, the present study also shows that high FA levels did not worsen, but rather improved the ischemic outcome in DIO hearts. This contrasts du Toit *et al.*, reporting decreased ischemic tolerance with high FAs in DIO rat hearts when compared to a condition of no FAs [19]. On the other hand, hearts from type 1 diabetic rats have been reported to show improved post-ischemic functional recovery when high levels of FAs are present as compared to absent [52]. The present study is, to our knowledge, the first to examine the effect of high FAs on ischemic tolerance in hearts from obese mice, by comparing this to a more physiological condition with low FA levels. Our findings and the conflicting previous observations, highlight a need for more awareness and focus on experimental conditions in order to foster our understanding within this field. In addition, due to metabolic inflexibility and high dependence on FA oxidation, we should

question when or if omitting FAs in experiments is justifiable, when studying obesity/diabetes related cardiac disease.

4.4 Concluding remarks

Several physiological and pathophysiological conditions are known to be associated with increased circulating levels of FAs. Under physiological conditions, FA-induced ROS and acetylation may have signaling roles in the adaptation, whereby the FA oxidation capacity is enhanced and the mitochondrial machinery changed, so that it can match the shift to the high FA supply. Although this gives rise to an increase in MVO_2 (due to reduced mitochondrial efficiency and/or enhanced energy expenditure), it does not necessarily have any functional consequences, unless these hearts are also challenged by pathophysiological stress, such as myocardial infarction, as summarized in Figure 5.

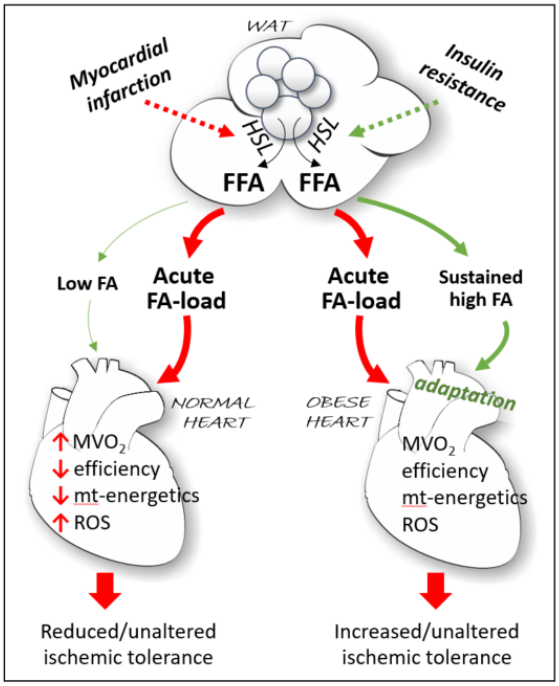

Figure 5. Fatty acid (FA)-mediated changes in normal and obese hearts. Under an acute physiological or pathophysiological stress, such as myocardial infarction, the heart will be subjected to an acute FA-load (red arrows), due to adrenergic activation of hormone-sensitive lipase (HSL) and subsequent increased lipolysis in white adipse tissue (WAT). In normal hearts, this high FA load is considered unfavorable due to FA-mediated changes in myocardial oxygen consumption (MVO_2), mechanical efficiency, ROS production and mitochondrial (mt-) energetics. In obesity and insulin resistance, a lack of the insulin-induced inhibition of the HSL in WAT, leads to sustained elevation in circulating FA levels (green arrows). The sustained high FA concentration will contribute to metabolic remodeling of these hearts, which we propose also will lead to adaptive changes, making these hearts capable of handling an acute FA-load without the subsequent FA-mediated alterations. Finally, while high FA load can reduce ischemic tolerance in normal hearts, the high FAs will not represent an additive stress in obese hearts.

In obesity and type 2 diabetes, dyslipidemia is likely to induce similar myocardial changes, however, as FA load is persistent, adaptive changes may eventually become maladaptive and contribute to the development of cardiomyopathy. Nevertheless, emerging data suggests that in these hearts, FAs are crucial for maintaining redox status and providing reducing equivalents, and hence are central in redox regulation of mitochondrial energetics and calcium handling. We suggest that this metabolic

remodeling makes these hearts capable of handling an acute FA load, so that upon myocardial infarction, the accompanying high FAs will not represent an additive stress (Figure 5). Although the therapeutic potential of targeting FAs remains to be determined, this study points towards a need of focusing on the importance of FAs as being more than just substrate to the heart. It also indicates that in contrast to in normal hearts, an acute FA load does not cause an aggravation of injury in obese hearts, and that other factors might contribute to the worsened ischemic outcome in obese patients.

5. Conclusion

In conclusion, this study confirms that exposure of normal hearts to high (as compared to low) FA levels, not only increases FA oxidation rate, but also induces oxygen wasting, mechanical inefficiency, increased ROS content, hyper-acetylation and reduced oxidative phosphorylation. However, hearts from diet-induced obese mice showed a resistance to these FA-mediated changes. In addition, we found that high FA levels do not impair, but rather increase functional recovery and reduce infarct size, normalizing ischemic tolerance in these hearts. We therefore suggest that although chronic dyslipidemia may have a causative role in diabetes-/obesity-mediated cardiomyopathy, these hearts undergo adaptive changes that make them able to handle an acute FA load.

Acknowledgements

We acknowledge Trine Lund and Thomas Andreasen for their technical assistance, and Matt Kerr at the University of Oxford for guidance related to the protein acetylation analysis.

Grants

This work was supported by grants from the Norwegian Council on Cardiovascular Disease, Novo Nordisk, Norwegian Diabetes Association, Foundation the Research Council of Norway and the Northern Norway Regional Health Authority (Helse Nord RHF, UNIKARD), as well as UiT - The Arctic University of Norway.

Disclosure and conflicts of interest

None of the authors have competing financial interests or other disclosures to declare. The authors declare no conflict of interest.

References

[1] D. Thapa, M. Zhang, J.R. Manning, D.A. Guimaraes, M.W. Stoner, R.M. O'Doherty, S. Shiva, I. Scott, Acetylation of mitochondrial proteins by GCN5L1 promotes enhanced fatty acid oxidation in the heart, Am J Physiol Heart Circ Physiol 313(2) (2017) H265-H274. doi:10.1152/ajpheart.00752.2016.

[2] K. Huynh, B.C. Bernardo, J.R. McMullen, R.H. Ritchie, Diabetic cardiomyopathy: mechanisms and new treatment strategies targeting antioxidant signaling pathways, Pharmacol Ther 142(3) (2014) 375-415. doi:10.1016/j.pharmthera.2014.01.003.

[3] E.J. Anderson, A.P. Kypson, E. Rodriguez, C.A. Anderson, E.J. Lehr, P.D. Neufer, Substrate-specific derangements in mitochondrial metabolism and redox balance in the atrium of the type 2 diabetic human heart, J Am Coll Cardiol 54(20) (2009) 1891-1898. doi:10.1016/j.jacc.2009.07.031.

[4] O.J. How, E. Aasum, D.L. Severson, W.Y. Chan, M.F. Essop, T.S. Larsen, Increased myocardial oxygen consumption reduces cardiac efficiency in diabetic mice, Diabetes 55(2) (2006) 466-473. doi: 10.2337/diabetes.55.02.06.db05-1164.

[5] M.A. Cole, A.J. Murray, L.E. Cochlin, L.C. Heather, S. McAleese, N.S. Knight, E. Sutton, A.A. Jamil, N. Parassol, K. Clarke, A high fat diet increases mitochondrial fatty acid oxidation and uncoupling to decrease efficiency in rat heart, Basic Res Cardiol 106(3) (2011) 447-457. doi:10.1007/s00395-011-0156-1.

[6] S. Boudina, S. Sena, B.T. O'Neill, P. Tathireddy, M.E. Young, E.D. Abel, Reduced mitochondrial oxidative capacity and increased mitochondrial uncoupling impair myocardial energetics in obesity, Circulation 112(17) (2005) 2686-2695. doi:10.1161/CIRCULATIONAHA.105.554360.

[7] D.D. Belke, E.A. Swanson, W.H. Dillmann, Decreased sarcoplasmic reticulum activity and contractility in diabetic db/db mouse heart, Diabetes 53(12) (2004) 3201-3208. doi:10.2337/diabetes.53.12.3201.

[8] P.H. Stone, J.E. Muller, T. Hartwell, B.J. York, J.D. Rutherford, C.B. Parker, Z.G. Turi, H.W. Strauss, J.T. Willerson, T. Robertson, et al., The effect of diabetes mellitus on prognosis and serial left ventricular function after acute myocardial infarction: contribution of both coronary disease and diastolic left ventricular dysfunction to the adverse prognosis. The MILIS Study Group, J Am Coll Cardiol 14(1) (1989) 49-57. doi: 10.1152/ajpheart.00752.2016.

[9] J. Russell, E.F. Du Toit, J.N. Peart, H.H. Patel, J.P. Headrick, Myocyte membrane and microdomain modifications in diabetes: determinants of ischemic tolerance and cardioprotection, Cardiovasc Diabetol 16(1) (2017) 155. doi:10.1186/s12933-017-0638-z.

[10] H.J. Whittington, G.G. Babu, M.M. Mocanu, D.M. Yellon, D.J. Hausenloy, The diabetic heart: too sweet for its own good?, Cardiol Res Pract 2012 (2012) 845698. doi:10.1155/2012/845698.

[11] T. Miki, T. Itoh, D. Sunaga, T. Miura, Effects of diabetes on myocardial infarct size and cardioprotection by preconditioning and postconditioning, Cardiovasc Diabetol 11 (2012) 67. doi:10.1186/1475-2840-11-67.

[12] N. Apaijai, S.C. Chattipakorn, N. Chattipakorn, Roles of obese-insulin resistance and anti-diabetic drugs on the heart with ischemia-reperfusion injury, Cardiovasc Drugs Ther 28(6) (2014) 549-562. doi:10.1007/s10557-014-6553-6.

[13] D.J. Paulson, The diabetic heart is more sensitive to ischemic injury, Cardiovasc Res 34(1) (1997) 104-112. doi:10.1016/s0008-6363(97)00018-7.

[14] D. Feuvray, G.D. Lopaschuk, Controversies on the sensitivity of the diabetic heart to ischemic injury: the sensitivity of the diabetic heart to ischemic injury is decreased, Cardiovascular Research 34(1) (1997) 113-120. doi:10.1016/s0008-6363(97)00037-0.

[15] M.F. Oliver, V.A. Kurien, T.W. Greenwood, Relation between serum-free-fatty acids and arrhythmias and death after acute myocardial infarction, Lancet 1(545) (1968) 710-714.

[16] O.J. How, E. Aasum, S. Kunnathu, D.L. Severson, E.S. Myhre, T.S. Larsen, Influence of substrate supply on cardiac efficiency, as measured by pressure-volume analysis in ex vivo mouse hearts, Am.J.Physiol Heart Circ.Physiol 288(6) (2005) H2979-H2985. doi: 10.1152/ajpheart.00084.2005.

[17] J. Fauconnier, D.C. Andersson, S.J. Zhang, J.T. Lanner, R. Wibom, A. Katz, J.D. Bruton, H. Westerblad, Effects of palmitate on Ca(2+) handling in adult control and ob/ob cardiomyocytes: impact of mitochondrial reactive oxygen species, Diabetes 56(4) (2007) 1136-1142. doi:10.2337/db06-0739.

[18] G.M. Kuster, S. Lancel, J. Zhang, C. Communal, M.P. Trucillo, C.C. Lim, O. Pfister, E.O. Weinberg, R.A. Cohen, R. Liao, D.A. Siwik, W.S. Colucci, Redox-mediated reciprocal regulation of SERCA and Na+-Ca2+ exchanger contributes to sarcoplasmic reticulum Ca2+ depletion in cardiac myocytes, Free Radic Biol Med 48(9) (2010) 1182-1187. doi:10.1016/j.freeradbiomed.2010.01.038.

[19] E.F. du Toit, W. Smith, C. Muller, H. Strijdom, B. Stouthammer, A.J. Woodiwiss, G.R. Norton, A. Lochner, Myocardial susceptibility to ischemic-reperfusion injury in a prediabetic model of dietary-induced obesity, Am.J Physiol Heart Circ.Physiol 294(5) (2008) H2336-H2343. doi:10.1152/ajpheart.00481.2007.

[20] C. Dalgas, J.A. Povlsen, B. Lofgren, S.B. Erichsen, H.E. Botker, Effects of fatty acids on cardioprotection by pre-ischaemic inhibition of the malate-aspartate shuttle, Clin Exp Pharmacol Physiol 39(10) (2012) 878-885. doi:10.1111/j.1440-1681.2012.05749.x.

[21] S. Gambert, C. Vergely, R. Filomenko, D. Moreau, A. Bettaieb, L.H. Opie, L. Rochette, Adverse effects of free fatty acid associated with increased oxidative stress in postischemic isolated rat hearts, Mol Cell Biochem 283(1-2) (2006) 147-152. doi:10.1007/s11010-006-2518-9.

[22] Q. Liu, J.C. Docherty, J.C. Rendell, A.S. Clanachan, G.D. Lopaschuk, High levels of fatty acids delay the recovery of intracellular pH and cardiac efficiency in post-ischemic hearts by inhibiting glucose oxidation, J.Am.Coll.Cardiol. 39(4) (2002) 718-725.

[23] G.D. Lopaschuk, M. Saddik, R. Barr, L. Huang, C.C. Barker, R.A. Muzyka, Effects of high levels of fatty acids on functional recovery of ischemic hearts from diabetic rats, Am J Physiol 263(6 Pt 1) (1992) E1046-1053.

[24] M. Desrois, R.J. Sidell, D. Gauguier, C.L. Davey, G.K. Radda, K. Clarke, Gender differences in hypertrophy, insulin resistance and ischemic injury in the aging type 2 diabetic rat heart, J Mol Cell Cardiol 37(2) (2004) 547-555. doi:10.1016/j.yjmcc.2004.05.014.

[25] M. Desrois, K. Clarke, C. Lan, C. Dalmasso, M. Cole, B. Portha, P.J. Cozzone, M. Bernard, Upregulation of eNOS and unchanged energy metabolism in increased susceptibility of the aging type 2 diabetic GK rat heart to ischemic injury, Am J Physiol Heart Circ Physiol 299(5) (2010) H1679-1686. doi:10.1152/ajpheart.00998.2009.

[26] A.D. Hafstad, J. Lund, E. Hadler-Olsen, A.C. Hoper, T.S. Larsen, E. Aasum, High- and moderate-intensity training normalizes ventricular function and mechanoenergetics in mice with diet-induced obesity, Diabetes 62(7) (2013) 2287-2294. doi:10.2337/db12-1580.

[27] J. Lund, A.D. Hafstad, N.T. Boardman, L. Rossvoll, N.P. Rolim, M.S. Ahmed, G. Florholmen, H. Attramadal, U. Wisloff, T.S. Larsen, E. Aasum, Exercise training promotes cardioprotection through oxygen-sparing action in high fat-fed mice, Am.J.Physiol Heart Circ.Physiol 308(8) (2015) H823-H829. doi:10.1152/ajpheart.00734.2014.

[28] E. Aasum, A.D. Hafstad, D.L. Severson, T.S. Larsen, Age-dependent changes in metabolism, contractile function, and ischemic sensitivity in hearts from db/db mice, Diabetes 52(2) (2003) 434-441. doi:10.2337/diabetes.52.2.434.

[29] N. Boardman, A.D. Hafstad, T.S. Larsen, D.L. Severson, E. Aasum, Increased O2 cost of basal metabolism and excitation-contraction coupling in hearts from type 2 diabetic mice, Am.J.Physiol Heart Circ.Physiol 296(5) (2009) H1373-H1379. doi: 10.1152/ajpheart.01264.2008.

[30] T.M. Pedersen, N.T. Boardman, A.D. Hafstad, E. Aasum, Isolated perfused working hearts provide valuable additional information during phenotypic assessment of the diabetic mouse heart, PLoS One 13(10) (2018) e0204843. doi:10.1371/journal.pone.0204843.

[31] N.T. Boardman, T.S. Larsen, D.L. Severson, M.F. Essop, E. Aasum, Chronic and acute exposure of mouse hearts to fatty acids increases oxygen cost of excitation-contraction coupling, Am.J.Physiol Heart Circ.Physiol 300(5) (2011) H1631-H1636. doi:10.1152/ajpheart.01190.2010.

[32] J.F. Hutter, H.M. Piper, P.G. Spieckerman, Effect of fatty acid oxidation on efficiency of energy production in rat heart, Am.J Physiol 249(4 Pt 2) (1985) H723-H728. doi:10.1152/ajpheart.1985.249.4.H723.

[33] C.G. Tocchetti, V. Caceres, B.A. Stanley, C. Xie, S. Shi, W.H. Watson, B. O'Rourke, R.C. Spadari-Bratfisch, S. Cortassa, F.G. Akar, N. Paolocci, M.A. Aon, GSH or palmitate preserves mitochondrial energetic/redox balance, preventing mechanical dysfunction in metabolically challenged myocytes/hearts from type 2 diabetic mice, Diabetes 61(12) (2012) 3094-3105. doi: 10.2337/db12-0072.

[34] E.J. Vazquez, J.M. Berthiaume, V. Kamath, O. Achike, E. Buchanan, M.M. Montano, M.P. Chandler, M. Miyagi, M.G. Rosca, Mitochondrial complex I defect and increased fatty acid oxidation enhance protein lysine acetylation in the diabetic heart, Cardiovasc Res 107(4) (2015) 453-465. doi:10.1093/cvr/cvv183.

[35] A.D. Hafstad, N. Boardman, E. Aasum, How exercise may amend metabolic disturbances in diabetic cardiomyopathy, Antioxid Redox Signal 22(17) (2015) 1587-1605. doi:10.1089/ars.2015.6304.

[36] W. Smith, G.R. Norton, A.J. Woodiwiss, A. Lochner, E.F. du Toit, Dependence of Cardiac Systolic Function on Elevated Fatty Acid Availability in Obese, Insulin-Resistant Rats, J Card Fail 22(7) (2016) 560-568. doi:10.1016/j.cardfail.2016.04.012.

[37] S. Cortassa, V. Caceres, C.G. Tocchetti, M. Bernier, R. de Cabo, N. Paolocci, S.J. Sollott, M.A. Aon, Metabolic remodelling of glucose, fatty acid and redox pathways in the heart of type 2 diabetic mice, J Physiol. doi:10.1113/JP276824 (2018). doi:10.1113/JP276824.

[38] E. Bertero, V. Sequeira, C. Maack, Let's face the fats: palmitate restores cellular redox state in the diabetic heart, J Physiol. (2018). doi:10.1113/JP277473.

[39] M.F. Essop, W.Y. Anna Chan, A. Valle, F.J. Garcia-Palmer, E.F. Du Toit, Impaired contractile function and mitochondrial respiratory capacity in response to oxygen deprivation in a rat model of pre-diabetes, Acta Physiol (Oxf) 197(4) (2009) 289-296. doi:10.1111/j.1748-1716.2009.02024.x.

[40] B. Huisamen, A. Genis, E. Marais, A. Lochner, Pre-treatment with a DPP-4 inhibitor is infarct sparing in hearts from obese, pre-diabetic rats, Cardiovasc Drugs Ther 25(1) (2011) 13-20. doi:10.1007/s10557-010-6271-7.

[41] B. Littlejohns, P. Pas dois, S. Duggan, A.R. Bond, K. Heesom, C.L. Jackson, G.D. Angelini, A.P. Halestrap, M.S. Suleiman, Hearts from mice fed a non-obesogenic high-fat diet exhibit changes in their oxidative state, calcium and mitochondria in parallel with increased susceptibility to reperfusion injury, PLoS One 9(6) (2014) e100579. doi:10.1371/journal.pone.0100579.

[42] L. Lacerda, L.H. Opie, S. Lecour, Influence of tumour necrosis factor alpha on the outcome of ischaemic postconditioning in the presence of obesity and diabetes, Exp Diabetes Res 2012 (2012) 502654. doi:10.1155/2012/502654.

[43] M.V. Hjortbak, J. Hjort, J.A. Povlsen, R.V. Jensen, N.B. Stottrup, M.R. Laursen, N.R. Jespersen, B. Lofgren, H.E. Botker, Influence of diabetes mellitus duration on the efficacy of ischemic preconditioning in a Zucker diabetic fatty rat model, PLoS One 13(2) (2018) e0192981. doi:10.1371/journal.pone.0192981.

[44] H.J. Whittington, I. Harding, C.I. Stephenson, R. Bell, D.J. Hausenloy, M.M. Mocanu, D.M. Yellon, Cardioprotection in the aging, diabetic heart: the loss of protective Akt signalling, Cardiovasc Res 99(4) (2013) 694-704. doi:10.1093/cvr/cvt140.

[45] F. Edland, A. Wergeland, R. Kopperud, K.S. Asrud, E.A. Hoivik, S.L. Witso, A.E. R, L. Madsen, K. Kristiansen, M. Bakke, S.O. Doskeland, A.K. Jonassen, Long-term consumption of an obesogenic high fat diet prior to ischemia-reperfusion mediates cardioprotection via Epac1-dependent signaling, Nutr Metab (Lond) 13 (2016) 87. doi:10.1186/s12986-016-0147-1.

[46] D. Donner, J.P. Headrick, J.N. Peart, E.F. du Toit, Obesity improves myocardial ischaemic tolerance and RISK signalling in insulin-insensitive rats, Dis Model Mech 6(2) (2013) 457-466. doi:10.1242/dmm.010959.

[47] I. Webster, R. Salie, E. Marais, W.J. Fan, G. Maarman, B. Huisamen, A. Lochner, Myocardial susceptibility to ischaemia/reperfusion in obesity: a re-evaluation of the effects of age, BMC Physiol 17(1) (2017) 3. doi:10.1186/s12899-017-0030-y.

[48] K.B. Paelestik, N.R. Jespersen, R.V. Jensen, J. Johnsen, H.E. Botker, S.B. Kristiansen, Effects of hypoglycemia on myocardial susceptibility to ischemia-reperfusion injury and preconditioning in hearts from rats with and without type 2 diabetes, Cardiovasc Diabetol 16(1) (2017) 148. doi:10.1186/s12933-017-0628-1.

[49] J.A. Povlsen, B. Lofgren, C. Dalgas, R.I. Birkler, M. Johannsen, N.B. Stottrup, H.E. Botker, Protection against myocardial ischemia-reperfusion injury at onset of type 2 diabetes in Zucker diabetic fatty rats is associated with altered glucose oxidation, PLoS One 8(5) (2013) e64093. doi:10.1371/journal.pone.0064093.

[50] R.J. Sidell, M.A. Cole, N.J. Draper, M. Desrois, R.E. Buckingham, K. Clarke, Thiazolidinedione treatment normalizes insulin resistance and ischemic injury in the zucker Fatty rat heart, Diabetes 51(4) (2002) 1110-1117. doi:10.2337/diabetes.51.4.1110.

[51] I. Wensley, K. Salaveria, A.C. Bulmer, D.G. Donner, E.F. du Toit, Myocardial structure, function and ischaemic tolerance in a rodent model of obesity with insulin resistance, Exp Physiol 98(11) (2013) 1552-1564. doi:10.1113/expphysiol.2013.074948.

[52] L.M. King, R.J. Sidell, J.R. Wilding, G.K. Radda, K. Clarke, Free fatty acids, but not ketone bodies, protect diabetic rat hearts during low-flow ischemia, Am J Physiol Heart Circ Physiol 280(3) (2001) H1173-1181. doi:10.1152/ajpheart.2001.280.3.H1173.

PAPER III

Overexpression of NOX2 exacerbates AngII-mediated cardiac remodelling and dysfunction in male, but not female mice

T.M. Pedersen, S.S. Hansen, N.T. Boardman, E. Aasum, A.D. Hafstad

Cardiovascular Research Group, Department of Medical Biology, Faculty of Health Sciences, University of Tromsø – The Arctic University of Norway, Norway

Abstract

Although angiotensin II (AngII)-induced heart failure has been extensively studied preclinically, the effect of AngII on cardiac energetics and substrate utilization is less known. Hence, the present study aimed to examine the effects of treatment with a low (slow-pressor) dose of AngII on cardiac substrate utilization, myocardial oxygen consumption and efficiency. AngII is a known activator of NOX2, thus in addition to normal (wild type, WT) male mice, we also examined male and female mice with a cardiac-specific overexpression of NOX2 (TG). **Methods.** Mice were treated with saline or AngII (400 ng/kg/min) using micro-osmotic pumps for 2 weeks. Blood pressures (plethysmography) and *in vivo* cardiac function (echocardiography) were measured at baseline, 1 week and/or 2 weeks. *Ex vivo* cardiac function, myocardial substrate utilization and cardiac mechanical efficiency (cardiac power/MVO_2) was assessed in isolated perfused working hearts. **Results.** Both WT and TG male mice developed elevated systolic blood pressure and cardiac hypertrophy following AngII treatment. While left ventricular (LV) function was preserved in AngII-treated WT male mice, cardiac-specific overexpression of NOX2 caused an AngII-induced decrease in ejection fraction and fractional shortening, as well as increased E/E', signifying systolic and diastolic heart failure. Male TG mice also displayed a shift in cardiac metabolism, showing an increased glucose oxidation compared to WT. Female TG mice displayed increased systolic blood pressures after 2 weeks of AngII treatment, however neither WT nor TG female mice displayed pathologic traits following AngII treatment, i.e. no hypertrophy, cardiac dysfunction or shift in substrate utilization. **Conclusion.** A slow-pressor dose of 400 ng/kg/min of AngII causes hypertrophy with preserved function, cardiac substrate utilization and oxygen consumption in WT males. NOX2 overexpression exacerbates the pathology, with TG males showing cardiac failure and a shift in substrate utilization. Females seem to be protected from the detrimental effects of AngII and NOX2 overexpression.

Introduction

Activation of the renin-angiotensin system and subsequent increased levels of angiotensin II (AngII) is associated with the development of heart failure. AngII causes sodium and water retention, vasoconstriction, and therefore hypertension, but it also has a direct effect on cardiomyocytes, affecting fibrosis, cell growth, hypertrophy, apoptosis and autophagy [1-3]. Therefore, AngII plays an important role in cardiac remodelling and the development of cardiac dysfunction through both systemic and local processes.

In experimental studies of AngII-mediated heart failure, the AngII dose and treatment protocols are highly variable. High doses (i.e. pressor doses of >1000 ng/kg/min) typically cause an overt hypertension and hypertrophy [4-6], creating severe models of heart disease and possibly cachexia. Lower doses (particularly those of ≤ 500 ng/kg/min), will cause an initial prehypertensive period characterized by autopotentiation to AngII, and subsequent development of hypertension, depending on the duration of treatment [7; 8]. Consequently, the doses are labelled depending on their ability to induce a pressor response. Although the phenotypic changes are easily studied in models using pressor doses, lower doses (no-, sub- or slow-pressor doses) are required in order to study the initial effect of AngII, without introducing confounding disease factors that may mask the direct effects of AngII. Additionally, although the suppression of the renin-angiotensin system is an important medical therapy in heart failure patients, there is still progression of failure, even in optimally treated patents. Thus, investigating the development of early AngII-induced disease can be an important step in order to discover relevant treatment options and novel therapies.

Altered substrate metabolism and loss of metabolic flexibility is a common trait in heart failure. In addition, decreased cardiac efficiency is an early hallmark of the failing heart, often preceding development of severe cardiac dysfunction [9; 10]. Although it is well known that AngII alters structural remodelling in the cardiomyocyte, its effect on the cardiac metabolic remodelling is less clear. There is evidence showing that the prevention of metabolic alterations in the failing heart is beneficial [11], thus studying the metabolic adaptations and possible maladaptations in early AngII-induced disease could provide important insight into the therapeutic potential.

A common mediator of AngII-induced processes is production of reactive oxygen species (ROS) [3]. Although there are several sources of ROS in cardiomyocytes, studies have confirmed that nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) is

a crucial contributor to AngII-induced ROS production in the pathogenesis of heart failure [12-14].

The aim of the present study was therefore to examine the effects of a slow-pressor dose of AngII (400 ng/kg/min) on cardiac function, substrate utilization and efficiency. Additionally, in order to examine the role of NOX2 in the AngII-induced cardiac changes, we also included mice with cardiac-specific NOX2 overexpression. As there are sex differences in heart failure, transgenic mice of both sexes were included in this study.

Materials and methods

In the present study we used 11-week-old male C57BL/6J mice (Charles River Laboratories, Germany), in addition to age-matched male and female wild type (WT) and transgenic mice (TG) with a cardiac-specific overexpression of nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) (csNOX2, obtained from King's College in London). TG mice were created on a C57BL/6J background by cloning a 1.8 kb human NOX2 cDNA downstream of the myosin light chain-2 (MLC-2v) promoter, prior to injection into fertilized oocytes [14]. The expression of the NOX2 protein is approximately five times higher in csNOX2 TG mice than in WT, however basal NOX2 activity and cardiac phenotype is unaltered [14].

All mice were acclimatized to the animal facilities for one week and kept on a 12:12 hour reversed light: dark cycle, in a room with a constant temperature of 21ºC and 55 % humidity. The animals were given ad libitum access to a normal chow diet and water, and were otherwise treated in accordance with the guidelines on accommodation and care of animals given by the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes. Experiments were done in our laboratory at the University of Tromsø – The Arctic University of Norway, and were approved by the Animal Welfare Committee at the university and the Norwegian Food Safety Authority (FOTS id: 7435).

Following acclimatisation, the C57BL/6J were divided into two groups of 10 mice; one group was treated with 400 ng/kg/min angiotensin II (AngII) (A9525, Sigma Aldrich), which is considered to be a slow-pressor dose [7; 15], and the other group was treated with saline (vehicle). Micro-osmotic pumps (Model 1002, Alzet, California, USA) were inserted subcutaneously and the treatment lasted for 2 weeks. Animals were given a standard volume of 100 µl of saline or AngII according to body weight. Prior to pump implantation, animals

were given buprenorphine as an analgesic. In the follow up study, WT mice (6 males and 6 females) and csNOX2 TG mice (6 males and 6 females) were treated using the same AngII treatment regimen.

Echocardiography and blood pressure measurements. Echocardiographic measurements were done at baseline and repeated after two weeks. Animals were lightly anaesthetized with isoflurane (1.5-2% isoflurane), while lying in a supine position on a heated platform [16]. Measurements were obtained and analysed from parasternal short-axis M-mode (study 1), and, for a more comprehensive examination, apical four-chamber Doppler images (study 2), as previously described [16]. A blinded operator imaged all mice and performed the subsequent analyses.

Blood pressures were obtained using tail-cuff plethysmography (Coda High Throughput Non-Invasive Blood Pressure System, Kent Scientific, Connecticut, USA) on alert animals. Blood pressures were measured after two weeks of treatment (study 1) or weekly, in order to follow the development of hypertension (study 2).

Isolated heart perfusions. The day after the final echocardiographic and blood pressure measurements, animals were given an intraperitoneal injection of pentobarbital and heparin, and hearts were excised prior to being perfused in an isolated working heart mode, to assess left ventricular function, and measure myocardial substrate utilization, myocardial oxygen consumption (MVO_2) and mechanical efficiency (defined as the ratio between cardiac power and MVO_2). The animals were all weighed before being euthanized**,** and the heart weight was registered after perfusions were completed. Tibias were collected from all the animals, and samples for mRNA isolation and quantification were taken from the left ventricle after the perfusions.

A modified Krebs-Henseleit buffer containing 5 mM of glucose and 0.4 mM of palmitate was used. The buffer also contained ^{14}C-labelled glucose and ^{3}H-labelled palmitate prebound to albumin in order to measure fatty acid and glucose oxidation rates [17]. Preload and afterload pressures were kept at a constant standardized level throughout the protocol (10 mmHg and 55 mmHg respectively). A pressure catheter (Codman Microsensor, DePuy Synthes Co, Massachusetts, USA) was placed in the aortic line close to the heart, measuring pressure development in the ejected buffer. Data was obtained and analysed using LabChart 7Pro software (ADInstruments, Bella Vista, Australia).

Fibre-optic O_2 sensors (FOXY-AL 300; Ocean Optics, Duiven, Netherlands) were placed in the buffer flow above the aorta (i.e. in the buffer entering the coronary vessels) and in the pulmonary trunk (in the buffer leaving the heart), to obtain the arterial-venous difference in PO_2, in order to measure MVO_2 [18].

Real-time quantitative PCR. Left ventricular tissue from perfused hearts was immersed in RNAlater (Qiagen, Hilden, Germany), and total RNA was extracted according to the RNeasy Fibrous Tissue kit Protocol (Qiagen Nordic, Norway). Real-time qPCR analysis was performed on tissue samples using an ABI PRISM 7900 HT Fast real-time thermal cycler as previously described [19]. Details about primer/probes sequences are given in supplemented data.

Statistics. The results are presented as means ± standard error of means in tables, line charts and column bar graphs. In order to compare differences between groups or individuals, unpaired and paired Student's t-tests were performed, respectively.

Results

Study 1

Hypertrophy and *in vivo* cardiac function. Two weeks of angiotensin II (AngII) treatment (400 ng/kg/min) did not alter body weight in C57BL/6J males (24.3 ± 0.4 vs 24.4 ± 0.3 g in vehicle and AngII, respectively). Echocardiographic examination revealed that AngII treatment induced an increase in left ventricular (LV) mass and wall thickness, indicating AngII-induced cardiac hypertrophy. This was confirmed by a higher heart weight and an increase in the mRNA expression of brain natriuretic peptide (*bnp*) and atrial natriuretic factor (*anf*) in the AngII group (Figure 1).

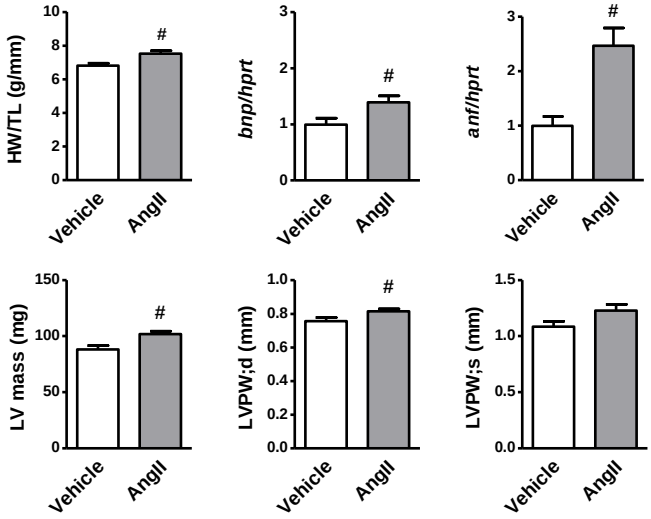

Figure 1. Heart weight, hypertrophic gene expression and echocardiographic left ventricular mass and wall thickness in hearts from C57BL/6J mice treated with saline (vehicle, n=7-10) or angiotensin II (AngII, n=7-10) for 2 weeks. HW/TL: heart weight to tibia length ratio, *bnp/hprt*: brain natriuretic peptide to hypoxanthine guanine phosphoribosyl transferase ratio, *anf/hprt*: atrial natriuretic factor to *hprt* ratio, LVPW;d and LVPW;s: left ventricular posterior wall in diastole and systole respectively. Data are means ± SEM. # $p < 0.05$ compared to vehicle.

In vivo echocardiography revealed an increase in diastolic LV internal diameter (LVID;d), and subsequently end-diastolic volume (EDV) was also increased. However, AngII treatment was not found to alter *in vivo* LV function, and ejection fraction (EF) and fractional shortening (FS) were unaltered (Table 1).

Table 1. *In vivo* left ventricular function assessed by transthoracic echocardiography in mice treated with angiotensin II (AngII) or saline (vehicle) for 2 weeks.

	Vehicle		**AngII**	
	Baseline	**2 weeks**	**Baseline**	**2 weeks**
n	7	7	7	7
Heart rate (BPM)	460 ± 21	451 ± 13	470 ± 9	458 ± 19
LVID;d (mm)	3.7 ± 0.1	3.9 ± 0.1	3.7 ± 0.1	4.0 ± 0.1*
LVEDV (µL)	57 ± 3	65 ± 4	59 ± 3	69 ± 2 *
FS (%)	37 ± 1	36 ± 1	36 ± 1	36 ± 2
EF (%)	67 ± 1	67 ± 2	66 ± 2	66 ± 3

Data are means ± SEM. LVID;d, left ventricular (LV) internal diameter in diastole; EDV and ESV, end-diastolic and end-systolic volumes; FS, fractional shortening; EF, ejection fraction. * $p < 0.05$ compared to baseline.

Substrate utilization and myocardial oxygen consumption. AngII treatment did not alter myocardial oxygen consumption (MVO_2) or mechanical efficiency (Figure 2). Also, myocardial glucose and fatty acid (FA) oxidation was not altered by AngII treatment (Figure 2). This was supported by unchanged gene expression of markers of metabolic reprogramming, such as peroxisome proliferator-activated receptor α (*pparα*), *cd36*, protein pyruvate dehydrogenase kinase 4 (*pdk4*) and hexokinase (*hk*) (Table S1).

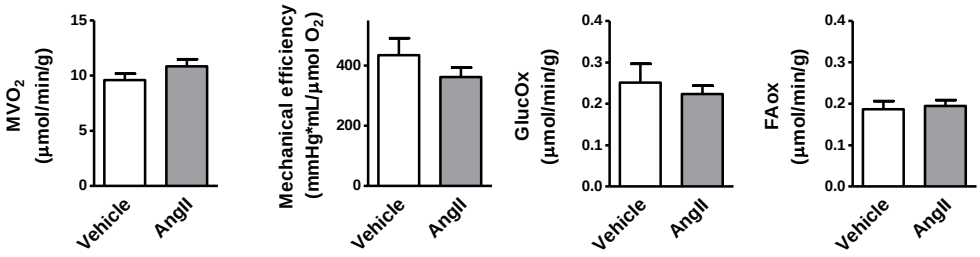

Figure 2. Myocardial oxygen consumption, mechanical efficiency and substrate utilization assessed in isolated perfused working hearts from C57BL/6J mice treated with saline (vehicle, n=6-7) or angiotensin II (AngII, n=9) for 2 weeks. MVO_2; myocardial oxygen consumption, GlucOx; glucose oxidation, FAox; fatty acid oxidation. Data are means ± SEM.

Taken together, 2 weeks of treatment with a slow-pressor dose of AngII induced hypertrophy with preserved left ventricular function and unaltered myocardial metabolism in heart from normal male mice.

Study 2

As AngII activation of NADPH oxidase 2 (NOX2) has been demonstrated to be central in the progression of several forms of heart failure [3], we also treated cardiac-specific NOX2 overexpressing (csNOX2 TG) male and female mice, to accelerate the development of AngII-induced cardiac dysfunction. Prior to AngII treatment, these animals show similar body weight, blood pressure, cardiac morphology and function to wild type (WT) mice in both sexes. AngII treatment did not cause any differences in body weight gain between WT and TG males (27.2 ± 0.8 vs 27.0 ± 0.7 g in WT and TG, respectively) or females (21.3 ± 0.3 vs 20.6 ± 0.3 g in WT and TG, respectively).

Blood pressure, hypertrophy and *in vivo* cardiac function. Two weeks of AngII treatment increased systolic blood pressure (SBP) in both WT and in TG males (Figure 3), however, the pressure increase was significant already after the first week treatment in the TG mice. The AngII treatment also caused an increase in the mean blood pressure (MBP) in the male TG (71.9 ± 1.6 to 89.7 ± 3.8 mmHg at baseline and 2 weeks respectively, $p<0.05$), but not in WT (77.2 ± 3.0 to 86.0 ± 2.6 mmHg). Notably, systolic and mean pressures did not differ significantly between groups after two weeks of treatment.

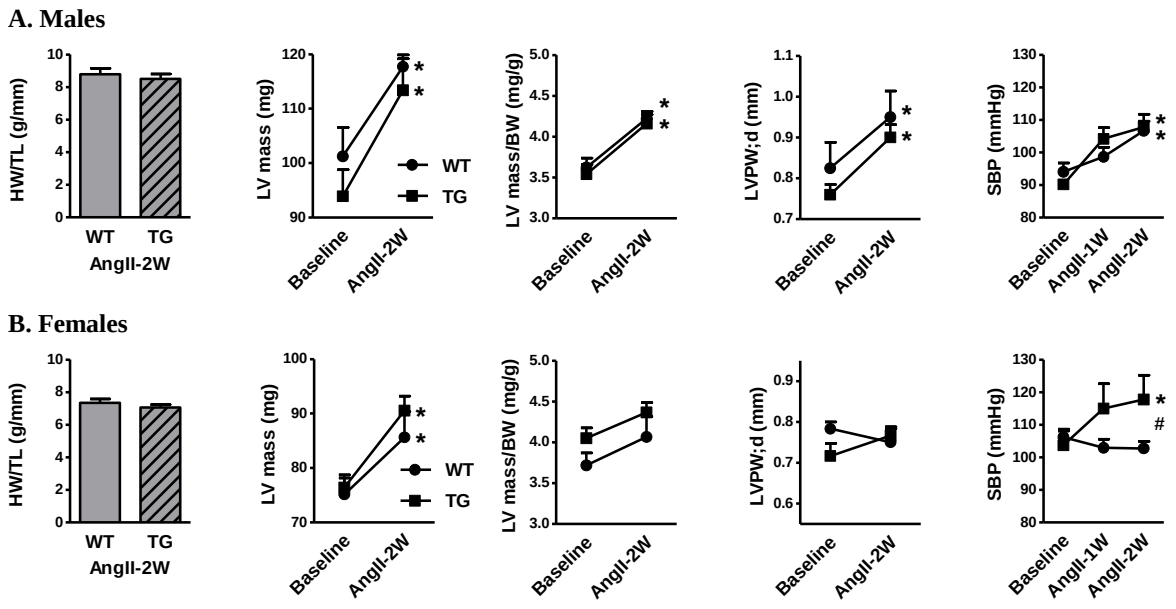

Figure 3. Heart weight, echocardiographic left ventricular (LV) mass and wall thickness, and systolic blood pressure in male (A) and female (B) wild type (WT) and cardiac-specific NOX2 transgenic (TG) mice prior to (baseline) and following 2 weeks of angiotensin II treatment (AngII-2W). HW/TL; heart weight to tibia length ratio, LV mass/BW; LV mass to body weight ratio, LVPW;d; left ventricular posterior wall in diastole, SBP; systolic blood pressure. Data are means ± SEM of n=4-6. * $p < 0.05$ compared to baseline, # $p < 0.05$ compared to WT.

In TG females SBP also increased after 2 weeks of AngII treatment. In contrast, AngII treatment did not alter SBP in the WT female mice (Figure 3). MBP was unaltered in both WT and TG female mice (87.4 ± 1.1 to 84.9 ± 2.0 mmHg in WT and 86.1 ± 4.9 to 99.1 ± 7.0 mmHg in TG).

In line with study 1, AngII treatment induced cardiac hypertrophy in WT and in TG male mice, as there was an increase in LV wall thickness, LV mass and LV mass adjusted to body weight (Figure 3). Although female mice also displayed elevated LV wall thickness and LV mass, this was attributed to an overall increase in body weight, resulting in unaltered LV mass/body weight ratio (Figure 3).

NOX2 overexpression in the heart did not exacerbate the AngII-induced hypertrophy (Figure 3), as indicated by unchanged heart weights (Figure 3) and gene expression of *bnp* and *anf* (Table S2). Moreover, there was no difference in LV wall thickness either (Figure 3). However, the ratio between end-diastolic volume and LV mass was elevated in TG male mice (0.59 ± 0.05 vs 0.74 ± 0.04 µl/mg in WT and TG, respectively, $p < 0.05$), signifying an eccentric hypertrophy with dilation and increased intracardial volume. Thus, although hypertrophy was not exacerbated per se, the hypertrophic *phenotype* was aggravated, signifying progression of disease in the transgenic males. In female mice, on the other hand, the volume/LV mass ratio did not differ between the two groups (0.73 ± 0.02 vs 0.76 ± 0.02 µl/mg in WT and TG, respectively), indicating a sex-dependent difference of the AngII-induced cardiac effects following NOX2 overexpression in the heart.

AngII treatment increased the systolic LV internal diameter (LVID;s) in TG but not WT male mice. This led to a decrease in FS in TG mice (Figure 4). Similarly, only TG male mice showed an increase in end-systolic volume (ESV) (Figure 4), resulting in a decreased EF (Table 2). Similar changes were not found in females (Figure 4 and Table 3), suggesting that females are less sensitive to the NOX2-mediated aggravation of AngII-induced systolic dysfunction.

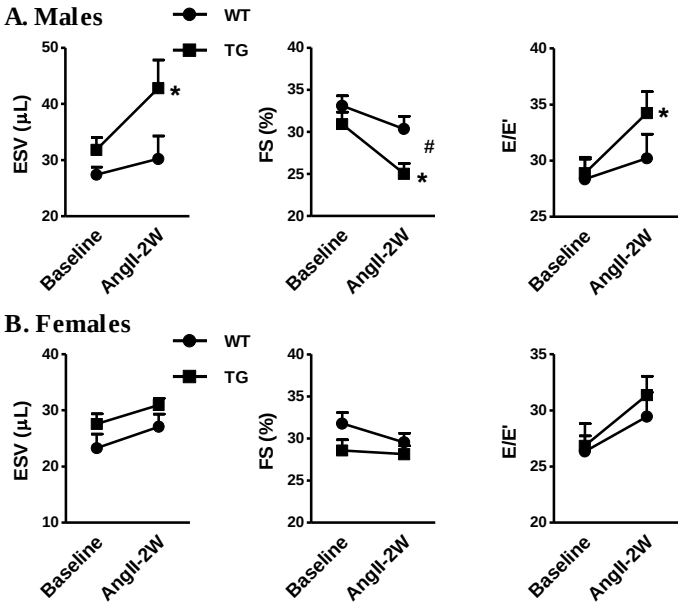

Figure 4. *In vivo* left ventricular function assessed by transthoracic echocardiography in male (A) and female (B) wild type (WT) and cardiac-specific NOX2 transgenic (TG) mice at baseline and following 2 weeks of angiotensin II treatment (AngII-2W). ESV; end-systolic volume, FS; fractional shortening, E/E'; ratio of velocity of early ventricular filling to early diastolic mitral annular velocity. Data are means ± SEM of n=4-6. * $p < 0.05$ compared to baseline, # $p < 0.05$ compared to WT.

AngII was also found to increase the E/E' ratio by 18.5 % in male TG mice (Figure 4). As elevation of the E/E' ratio indicates increased LV filling pressure and decreased compliance, these data suggest a more advanced disease state in TG males. There were no differences in diastolic function when comparing WT and TG females (Figure 4 and Table 3).

Table 2. *In vivo* left ventricular function assessed by transthoracic echocardiography in male wild type (WT) and cardiac-specific NOX2 transgenic (TG) mice prior to (baseline) and after 2 weeks of angiotensin II (AngII) treatment.

Males - AngII	**WT**		**TG**	
	Baseline	**2 weeks**	**Baseline**	**2 weeks**
n	4	6	6	5
Heart rate (BPM)	491 ± 12	530 ± 22	479 ± 6	515 ± 13 *
LVID;d (mm)	4.06 ± 0.05	3.98 ± 0.14	4.17 ± 0.06	4.31 ± 0.17
LVEDV (µL)	72 ± 2	70 ± 6	78 ± 3	84 ± 8
EF (%)	62 ± 2	58 ± 3	58 ± 2	50 ± 2 #*
E/A	1.29 ± 0.08	1.48 ± 0.16	1.35 ± 0.04	1.44 ± 0.15
Deceleration time (ms)	24 ± 1	20 ± 2	23 ± 2	19 ± 2

Data are means ± SEM. LVID;d, left ventricular (LV) internal diameter in diastole; EDV, end-diastolic volume; EF, ejection fraction; E/A; ratio of velocities of early to late ventricular filling, DT; deceleration time. * $p < 0.05$ compared to baseline, # $p < 0.05$ compared to WT.

Table 3. *In vivo* left ventricular function assessed by transthoracic echocardiography in female wild type (WT) and cardiac-specific NOX2 transgenic (TG) mice prior to (baseline) and after 2 weeks of angiotensin II (AngII) treatment.

Females - AngII	**WT**		**TG**	
	Baseline	**2 weeks**	**Baseline**	**2 weeks**
n	6	6	6	6
Heart rate (BPM)	515 ± 10	519 ± 11	484 ± 9	519 ± 10 *
LVID;d (mm)	3.70 ± 0.11	3.81 ± 0.09	3.80 ± 0.07	3.97 ± 0.04
LVEDV (μL)	58 ± 4	63 ± 4	62 ± 3	69 ± 2
EF (%)	61 ± 2	57 ± 2	56 ± 2	55 ± 2
MV E/A	1.76 ± 0.11	1.75 ± 0.08	1.83 ± 0.17	1.97 ± 0.19
MV Deceleration time (ms)	18 ± 2	18 ± 1	16 ± 2	15 ± 1

Data are means ± SEM. LVID;d, left ventricular (LV) internal diameter in diastole; EDV, end-diastolic volume; EF, ejection fraction; E/A; ratio of velocities of early to late ventricular filling, DT; deceleration time. * $p < 0.05$ compared to baseline.

Substrate utilization and myocardial oxygen consumption. NOX2 overexpression did not affect the MVO_2 in neither males nor females (Figure 5). As these hearts showed similar *ex vivo* function, there was no change in mechanical efficiency (Figure 5). On the other hand, the NOX2-mediated progression of heart failure in males was associated with a shift in myocardial substrate utilisation in the TG mice, as glucose oxidation rates were increased (Figure 5). Accordingly, there was a tendency for decreased FA oxidation rates in the AngII-treated TG ($p=0.149$) when compared to WT hearts (Figure 5). A similar metabolic switch was not present in female hearts (Figure 5).

Finally, a NOX2-mediated metabolic reprogramming was also evident by a significant increase in *pdk4* mRNA levels in TG males compared to WT, which was not present in csNOX2 TG females. Other markers of metabolic reprogramming (*pparα*, *cd36*, *ldh* and *hk*) were not different between WT and csNOX2 TG hearts following AngII treatment (Table S2).

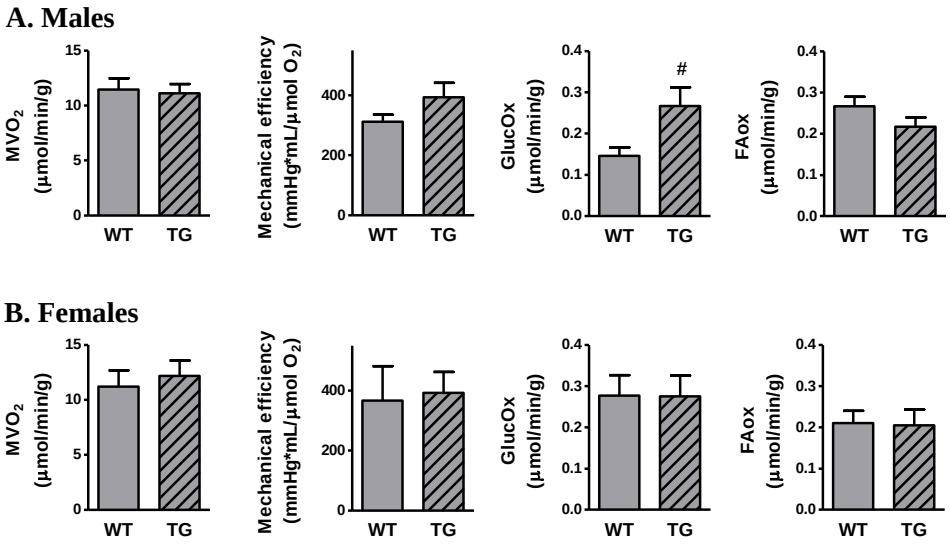

Figure 5. Myocardial oxygen consumption, mechanical efficiency and substrate utilization assessed in isolated perfused working hearts from male (A) and female (B) wild type (WT) and cardiac-specific NOX2 transgenic (TG) mice treated with angiotensin II for 2 weeks. MVO_2; myocardial oxygen consumption, GlucOx; glucose oxidation, FAox; fatty acid oxidation. Data are means ± SEM of n= 3-6. # $p < 0.05$ compared to WT.

In summary, 2 weeks of treatment with a slow-pressor dose of AngII in TG males aggravated the hypertrophic phenotype and blood pressure increase, induced systolic and diastolic failure and caused a metabolic shift. Treatment in TG females led to an increase in blood pressure, however, they did not show signs of hypertrophy, dysfunction or metabolic alteration.

Discussion

The present study shows that a slow-pressure dose of angiotensin II (AngII) induces cardiac hypertrophy, without affecting ventricular function or myocardial metabolism in normal male mice. Cardiac overexpression of nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) causes deterioration of the AngII-mediated hypertrophy, and induces ventricular dysfunction and a metabolic shift towards increased glucose oxidation in male mice. Female mice, however, seem to be protected from the NOX2-mediated detrimental cardiac effects of AngII.

While it is well documented that high doses of AngII lead to a rapid and overt pressor response accompanied by development of cardiac dysfunction [4-6], the functional effect of low doses has been less described. In the present study we have examined metabolic and functional changes in the hearts associated with treatment of mice using a slow-pressor dose of AngII (400 ng/kg/min, 2 weeks). This dose was used in order to mimic an early phase of

heart failure development, prior to the introduction of confounding effects that follow the complexity of overt hypertension. In accordance with previous studies using similar doses [15; 20-22], we found increased systolic blood pressure (SBP) in wild type (WT) males in this study. However, the increased blood pressure in this study was subtle compared to what was reported in the aforementioned studies (13% vs ~20-60%). Additionally, we could not detect an increase in the mean pressure (MBP) with this treatment. It should be recognized that there are possible limitations in sensitivity when using tail-cuff plethysmography, causing discrepancies compared to invasive pressure measurements. Nonetheless, the blood pressure changes reported in the present study were relatively minor, and it can be deduced that the impact of hypertension on overall cardiac health is of less importance, rather than if the elevation had been higher and detectable as both elevated systolic and mean pressures.

Reduced diastolic function both with or without systolic dysfunction has been reported following treatment with low doses of AngII [4; 21]. In contrast to these studies, we could not detect any *in vivo* ventricular dysfunction in the AngII-treated WT mice in this study. The mice displayed a hypertrophic phenotype, which was confirmed both by increased wall thickness, heart weight/tibia length ratio and increased gene expression of *bnp* and *anf*. Interestingly, previous studies using *sub-pressor* doses (100-200 ng/kg/min for 2-4 weeks) have also reported cardiac hypertrophy, and found development of fibrosis [4; 12; 23; 24], which also supports the direct cardiac effect of AngII. Correspondingly, in a study using transgenic mice with cardiac-specific overexpression of angiotensinogen, mice that did not develop hypertension, displayed both hypertrophy and fibrosis, further confirming the direct local effect of AngII [2].

Pathological hypertrophy and heart failure is known to be associated with changes in myocardial substrate utilization [10]. Although changes in myocardial substrate utilization has been reported following chronic administration of AngII [11; 25-27], there are discrepancies in terms of substrate preference. Studies using pressor doses (>1000 ng/kg/min for 2 weeks), generally report systemic insulin resistance, with a subsequent increased cardiac preference for fatty acids and diminished glucose and lactate oxidation [26; 27]. Lower doses (< 800 ng/kg/min for 2-4 weeks), however, seem to cause a reduction in fatty acid oxidation, with increased utilization of glucose [11]. Although the metabolic phenotype varies, the AngII-treated mice in these studies all displayed ventricular dysfunction, suggesting that a metabolic shift is only evident after the onset of cardiac failure. Correspondingly, in a study using transgenic mice with a cardiac-specific overexpression of angiotensinogen, Pellieux and

colleagues divided mice into two groups depending on the presence of heart failure [25]. Here they only found a reduced fatty acid oxidation rate (with unaltered glucose oxidation) in the group of mice that developed heart failure (i.e. pulmonary congestion). Moreover, they also showed that myocardial oxygen consumption (MVO_2) was elevated only in the hearts from mice with failure. In accordance with this, the present study shows that WT mice treated with the slow-pressor AngII dose, neither showed cardiac dysfunction nor changes in myocardial substrate utilization, MVO_2 or mechanical efficiency, further supporting the notion that the metabolic alterations occur only when the AngII-induced pathological condition has progressed far enough to manifest as cardiac dysfunction.

NOX2 and slow-pressor doses of AngII

Generation of reactive oxygen species (ROS) and oxidative stress are known to be important in the pathogenesis of heart failure, and several of the reported downstream effects of AngII are likely facilitated by NOX-mediated ROS production [3]. Bendall and colleagues were among the first to elucidate the role of NOX in AngII-induced hypertrophy [12], describing that a low dose of AngII (~200 ng/kg/day for 2 weeks) only induced hypertrophy in WT and not in mice lacking the major subunit of NOX2 ($gp91^{phox}$). The present study supports this by showing that cardiac overexpression of NOX2 causes an aggravated hypertrophic phenotype in male mice. The exacerbated effect of NOX2 following AngII treatment in the TG animals, also caused more rapid increase in blood pressures when compared to treated WT mice, which corroborates a previous study by Ebrahimian and co-workers, where the same AngII treatment regimen increased the SBP, which was associated with increased cardiac ROS production and NOX activity [20]. It should be noted, however, that the increase in blood pressures in the present study was subtle, even in the TG animals.

Zhang and colleagues [14] have previously studied the effects of AngII treatment in csNOX2 TG mice, using a lower dose of AngII (~200 ng/kg/day for 2 weeks). They report an aggravation of the hypertrophic response in TG mice compared to WT [14]. Notably, they saw an improved *in vivo* cardiac function in the TG mice, contrasting the present study, where we found that a slightly higher dose (400 ng/kg/day for 2 weeks) resulted in cardiac dysfunction in these mice. This discrepancy may be explained by a suggested transient NOX2-induced inotropic effect, causing increased contractility in the short term [14; 28]. The fact that Zhang *et al.* also demonstrated that when subjected to chronic stress (abdominal aortic banding), the TG mice displayed more severe dysfunction than WT mice [14], supports

that the negative effects of ROS outweigh the positive, when hearts are exposed to long-term treatment and/or higher doses of AngII [28].

Hearts from TG male mice displayed increased glucose oxidation and a tendency for decreased FA oxidation. This corroborates the metabolic shift previously reported in animal models with AngII-induced heart failure [11; 25]. Interestingly, Pellieux and collaborators have reported that AngII treatment led to a nuclear factor-κB-mediated decrease in palmitate oxidation and downregulation of key regulatory proteins of FA oxidation in rodent cardiomyocytes, and that these effects was impeded by inhibition of ROS production [29]. This would then support the notion that AngII-mediated ROS production in the heart causes downregulation of FA oxidation. Accordingly, the overexpression and subsequent increase in ROS production following AngII treatment in the csNOX2 TG animals in this study, could mediate downregulation of regulatory proteins and cause the observed substrate shift through the same signalling pathways that were described by Pellieux and colleagues [29]. Although the increased PDK4 mRNA levels in the TG males is contradictory to the enhanced glucose oxidation, the overexpression of NOX2 is likely to have caused additional oxidative stress, thus altering the cellular milieu and possibly counteracting these changes. Accordingly, oxidative stress has been associated with induction of translocation of GLUT4 to the plasma membrane and deacetylation of the pyruvate dehydrogenase complex, and thus, enhanced glucose utilization [26; 30-32]. Furthermore, we should also stress that altered gene expression do not necessarily match changes in *protein* levels, and there are reports showing a similar dissociation between myocardial mRNA levels and protein expression of regulatory proteins following AngII [25; 29].

Females and NOX2

It is well known that there are differences between males and females in symptoms and frequency of heart failure [33], and sex hormones and different antioxidative capacities have been suggested to explain discrepancies in AngII-mediated cardiovascular disease [20; 34; 35]. In this study we showed that in contrast to in WT male mice, WT females did not display AngII-induced blood pressure elevation nor heart failure development. We also showed that females were less sensitive to the NOX2-mediated aggravation of AngII-induced cardiac dysfunction, despite an AngII-induced hypertension.

Several studies have reported sex differences in the manifestation of cardiac disease. In accordance with this, the present study, as well as other studies, have shown an augmented

increase in blood pressure following AngII treatment in males, when compared to females [20; 35; 36]. Moreover, while gonadectomy has been reported to attenuate the AngII-mediated hypertension in males, the blood pressure was increased with ovariectomy in females [35], signifying that sex hormones greatly influence the hypertensive response. Oestrogen replacement therapy has also been shown to ameliorate heart failure in males and ovariectomized female rodents [37-39], and to lower the blood pressure in ovariectomized female rats [34]. Additionally, the lack of development of hypertrophy and cardiac dysfunction in females has been associated with decreased development of cardiac fibrosis and infarction [38; 40; 41], as well as a different and a more favourable geometric remodelling [33; 42; 43].

There is evidence of sex differences in ROS content, both in terms of their generation and the removal by antioxidants. Accordingly, female rodent hearts have been reported to have enhanced anti-oxidant capacity [20; 41]. Moreover, AngII-induced NOX-mediated increase in myocardial superoxide production is higher in ovariectomized female mice compared to in intact females [20]. In addition, the higher AngII-induced blood pressure in male rats has been associated with higher arteriolar production of superoxide, an effect that was attenuated by treating the animals with a NOX inhibitor [44], and males have increased arteriolar and cardiac protein expression of NOX2 subunits [36; 44]. Thus, protection from the AngII-mediated effects in the female mice in the present study may well be due to an enhanced oestrogen-mediated ability to modulate ROS production. Further evaluation of the effects of low doses of AngII and NOX2 overexpression in intact and ovariectomized females is needed to fully elucidate the role of oestrogens in this context.

Conclusion

This study shows that a slow-pressor dose of AngII induces hypertrophy and hypertension in male mice, and that cardiac-specific overexpression of NOX2 further leads to cardiac dysfunction and metabolic reprogramming. It also demonstrates that female mice are protected from the AngII-mediated effects, both in wild type and transgenic mice, as females do not develop hypertrophy or failure.

Acknowledgements

Trine Lund's expert technical assistance is gratefully acknowledged. The authors would also like to thank professor Ajay Shah's group, and especially Norman Catibog, at the BHF Centre of Research Excellence, Cardiovascular Division at King's College London, for their guidance with regard to echocardiography.

References

1. **Mazzolai L, Nussberger J, Aubert J, Brunner DB, Gabbiani G, Brunner HR and Pedrazzini T**. Blood pressure-independent cardiac hypertrophy induced by locally activated renin-angiotensin system. *Hypertension* 31: 1324-1330, 1998.

2. **Domenighetti AA, Wang Q, Egger M, Richards SM, Pedrazzini T and Delbridge LM**. Angiotensin II-mediated phenotypic cardiomyocyte remodeling leads to age-dependent cardiac dysfunction and failure. *Hypertension* 46: 426-432, 2005.

3. **Zablocki D and Sadoshima J**. Angiotensin II and oxidative stress in the failing heart. *Antioxidants & Redox Signaling* 19: 1095-1109, 2012.

4. **Zhong J, Basu R, Guo D, Chow FL, Byrns S, Schuster M, Loibner H, Wang XH, Penninger JM, Kassiri Z and Oudit GY**. Angiotensin-Converting Enzyme 2 suppresses pathological hypertrophy, myocardial fibrosis, and cardiac dysfunction. *Circulation* 122: 717-28, 2010.

5. **Hauck L, Grothe D and Billia F**. p21CIP1/WAF1-dependent inhibition of cardiac hypertrophy in response to Angiotensin II involves Akt/Myc and pRb signaling. *Peptides* 83: 38-48, 2016.

6. **Zhang Y, Yan H, Guang Gc and Deng Zr**. Overexpressed connective tissue growth factor in cardiomyocytes attenuates left ventricular remodeling induced by angiotensin II perfusion. *Clinical and Experimental Hypertension* 39: 168-174, 2017.

7. **Kawada N, Imai E, Karber A, Welch WJ and Wilcox CS**. A mouse model of angiotensin II slow pressor response: role of oxidative stress. *J Am Soc Nephrol* 13: 2860, 2002.

8. **Simon G, Abraham G and Cserep G**. Pressor and subpressor angiotensin II administration: Two experimental models of hypertension. *American Journal of Hypertension* 8: 645-650, 1995.

9. **Hafstad AD, Boardman NT and Aasum E**. How exercise may amend metabolic disturbances in diabetic cardiomyopathy. *Antioxidants & Redox Signaling* 22: 1587-1605, 2015.

10. **Karwi QG, Uddin GM, Ho KL and Lopaschuk GD**. Loss of metabolic flexibility in the failing heart. *Frontiers in Cardiovascular Medicine* 5: 68, 2018.

11. **Choi YS, de Mattos ABM, Shao D, Li T, Nabben M, Kim M, Wang W, Tian R and Kolwicz SC**. Preservation of myocardial fatty acid oxidation prevents diastolic dysfunction in mice subjected to angiotensin II infusion. *Journal of Molecular and Cellular Cardiology* 100: 64-71, 2016.

12. **Bendall JK, Cave AC, Heymes C, Gall C and Shah AM**. Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. *Circulation* 105: 293-296, 2002.

13. **Byrne JA, Grieve DJ, Bendall JK, Li JM, Gove C, Lambeth JD, Cave AC and Shah AM**. Contrasting roles of NADPH oxidase isoforms in pressure-overload versus angiotensin II-induced cardiac hypertrophy. *Circ Res* 93: 802-805, 2003.

14. **Zhang M, Prosser BL, Bamboye MA, Gondim ANS, Santos CX, Martin D, Ghigo A, Perino A, Brewer AC, Ward CW, Hirsch E, Lederer WJ and Shah AM**. Contractile function during angiotensin-II-activation: increased NOX2 activity modulates cardiac calcium handling via phospholamban phosphorylation. *Journal of the American College of Cardiology* 66: 261-272, 2015.

15. **Brand S, Amann K and Schupp N**. Angiotensin II-induced hypertension dose-dependently leads to oxidative stress and DNA damage in mouse kidneys and hearts. *Journal of Hypertension* 31: 2013.

16. **Pedersen TM, Boardman NT, Hafstad AD and Aasum E**. Isolated perfused working hearts provide valuable additional information during phenotypic assessment of the diabetic mouse heart. *PLOS ONE* 13: e0204843, 2018.

17. **Aasum E, Hafstad AD, Severson DL and Larsen TS**. Age-dependent changes in metabolism, contractile function, and ischemic sensitivity in hearts from *db/db* mice. *Diabetes* 52: 434-441, 2003.

18. **Boardman NT, Hafstad AD, Larsen TS, Severson DL and Aasum E**. Increased O_2 cost of basal metabolism and excitation-contraction coupling in hearts from type 2 diabetic mice. *American Journal of Physiology-Heart and Circulatory Physiology* 296: H1373-H1379, 2009.

19. **Hafstad AD, Boardman NT, Lund J, Hagve M, Khalid AM, Wisløff U, Larsen TS and Aasum E**. High intensity interval training alters substrate utilization and reduces oxygen consumption in the heart. *Journal of Applied Physiology* 111: 1235-1241, 2011.

20. **Ebrahimian T, He Y, Schiffrin EL and Touyz RM**. Differential regulation of thioredoxin and NAD(P)H oxidase by angiotensin II in male and female mice. *Journal of Hypertension* 25: 1263-1271, 2007.

21. **Glenn DJ, Cardema MC, Ni W, Zhang Y, Yeghiazarians Y, Grapov D, Fiehn O and Gardner DG**. Cardiac steatosis potentiates angiotensin II effects in the heart.

American Journal of Physiology-Heart and Circulatory Physiology 308: H339-H350, 2014.

22. **Wang Q, Wang H, Wang J, Venugopal J, Kleiman K, Guo C, Sun Y and Eitzman DT**. Angiotensin II-induced hypertension is reduced by deficiency of P-selectin glycoprotein ligand-1. *Scientific Reports* 8: 3223, 2018.

23. **Zhang H, Wu J, Dong H, Khan SA, Cu ML and Tsuda T**. Fibulin-2 deficiency attenuates angiotensin II-induced cardiac hypertrophy by reducing transforming growth factor-b signalling. *Clin Sci (Lond)* 126: 275-288, 2014.

24. **Regan JA, Mauro AG, Carbone S, Marchetti C, Gill R, Mezzaroma E, Valle Raleigh J, Salloum FN, Van Tassell BW, Abbate A and Toldo S**. A mouse model of heart failure with preserved ejection fraction due to chronic infusion of a low subpressor dose of angiotensin II. *American Journal of Physiology-Heart and Circulatory Physiology* 309: H771-H778, 2015.

25. **Pellieux C, Aasum E, Larsen TS, Montessuit C, Papageorgiou In, Pedrazzini T and Lerch R**. Overexpression of angiotensinogen in the myocardium induces downregulation of the fatty acid oxidation pathway. *Journal of Molecular and Cellular Cardiology* 41: 459-466, 2006.

26. **Mori J, Alrob OA, Wagg CS, Harris RA, Lopaschuk GD and Oudit GY**. ANG II causes insulin resistance and induces cardiac metabolic switch and inefficiency: a critical role of PDK4. *American Journal of Physiology-Heart and Circulatory Physiology* 304: H1103-H1113, 2013.

27. **Mori J, Basu R, McLean BA, Das SK, Zhang L, Patel VB, Wagg CS, Kassiri Z, Lopaschuk GD and Oudit GY**. Agonist-induced hypertrophy and diastolic dysfunction are associated with selective reduction in glucose oxidation: a metabolic contribution to heart failure with normal ejection fraction. *Circulation: Heart Failure* 5: 493-503, 2012.

28. **Ago T and Sadoshima J**. From contractile enhancement to pathological hypertrophy: angiotensin II-induced NOX2-mediated reactive oxygen species. *Journal of the American College of Cardiology* 66: 273-277, 2015.

29. **Pellieux C, Montessuit C, Papageorgiou I and Lerch R**. Angiotensin II downregulates the fatty acid oxidation pathway in adult rat cardiomyocytes via release of tumour necrosis factor-α. *Cardiovascular Research* 82: 341-350, 2009.

30. **Aikawa R, Nawano M, Gu Y, Katagiri H, Asano T, Zhu W, Nagai R and Komuro I**. Insulin prevents cardiomyocytes from oxidative stress-induced apoptosis through activation of PI3 Kinase/Akt. *Circulation* 102: 2873-2879, 2000.

31. **Wang M, Sun Gb, Sun X, Wang Hw, Meng Xb, Qin M, Sun J, Luo Y and Sun Xb**. Cardioprotective effect of salvianolic acid B against arsenic trioxide-induced injury in

cardiac H9c2 cells via the PI3K/Akt signal pathway. *Toxicology Letters* 216: 100-107, 2013.

32. **Chen Y, Chen C, Dong B, Xing F, Huang H, Yao F, Ma Y, He J and Dong Y**. AMPK attenuates ventricular remodeling and dysfunction following aortic banding in mice via the SIRT3/oxidative stress pathway. *European Journal of Pharmacology* 814: 335-342, 2017.

33. **Levinsson A, Dubé MP, Tardif JC and de Denus S**. Sex, drugs, and heart failure: a sex-sensitive review of the evidence base behind current heart failure clinical guidelines. *ESC Heart Fail* 5: 745-754, 2018.

34. **Hernández I, Delgado JL, Díaz J, Quesada T, Teruel MJ, Llanos MC and Carbonell LF**. 17β-Estradiol prevents oxidative stress and decreases blood pressure in ovariectomized rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 279: R1599-R1605, 2000.

35. **Xue B, Pamidimukkala J and Hay M**. Sex differences in the development of angiotensin II-induced hypertension in conscious mice. *American Journal of Physiology-Heart and Circulatory Physiology* 288: H2177-H2184, 2005.

36. **Tatchum-Talom R, Eyster KM and Martin DS**. Sexual dimorphism in angiotensin II-induced hypertension and vascular alterations. *Can J Physiol Pharmacol* 83: 413-422, 2005.

37. **van Eickels M, Grohé C, Cleutjens J, Janssen B, Wellens H and Doevendans A**. 17-β-Estradiol Attenuates the Development of Pressure-Overload Hypertrophy. *Circulation* 104: 1419-1423, 2001.

38. **Wang M, Tsai BM, Reiger KM, Brown JW and Meldrum DR**. 17-β-Estradiol decreases p38 MAPK-mediated myocardial inflammation and dysfunction following acute ischemia. *Journal of Molecular and Cellular Cardiology* 40: 205-212, 2006.

39. **Iorga A, Li J, Sharma S, Umar S, Bopassa JC, Nadadur RD, Centala A, Ren S, Saito T, Toro L, Wang Y, Stefani E and Eghbali M**. Rescue of pressure overload-induced heart failure by estrogen therapy. *Journal of the American Heart Association* 5: e002482, 2016.

40. **Cavasin MA, Tao Z, Menon S and Yang XP**. Gender differences in cardiac function during early remodeling after acute myocardial infarction in mice. *Life Sciences* 75: 2181-2192, 2004.

41. **Lagranha CJ, Deschamps A, Aponte A, Steenbergen C and Murphy E**. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. *Circ Res* 106: 1681-1691, 2010.

42. **Weinberg EO, Thienelt CD, Katz SE, Bartunek J, Tajima M, Rohrbach S, Douglas PS and Lorell BH**. Gender differences in molecular remodeling in pressure overload hypertrophy. *Journal of the American College of Cardiology* 34: 264-273, 1999.

43. **Fliegner D, Schubert C, Penkalla A, Witt H, Kararigas G, Dworatzek E, Staub E, Martus P, Noppinger PR, Kintscher U, Gustafsson JA and Regitz-Zagrosek V**. Female sex and estrogen receptor-β attenuate cardiac remodeling and apoptosis in pressure overload. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 298: R1597-R1606, 2010.

44. **Dantas AP, Franco MdoC, Silva-Antonialli MM, Tostes RCA, Fortes ZB, Nigro D and Carvalho MH**. Gender differences in superoxide generation in microvessels of hypertensive rats: role of NAD(P)H-oxidase. *Cardiovascular Research* 61: 22-29, 2004.

Supplemented data

Primers for real-time quantitative PCR

mRNA expression of the genes of interest was adjusted to the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (*hprt*). Forward and reverse primer and probe sequences (5'-3'): brain natriuretic peptide (*bnp*) – forward: CCA GTC TCC AGA GCA ATT CAA, reverse: GCC ATT TCC TCC GAC TTT T; atrial natriuretic factor (*anf*) – forward: CAC AGA TCT GAT GGA TTT CAA GA, reverse: CCT CAT CTT CTA CCG GCA TC; nicotinamide-adenine dinucleotide phosphate oxidase 2 (*nox2*) – forward: TGAATGCCAGAGTCGGGATTT, reverse: CCCCCTTCAGGGTTCTTGATTT; pyruvate dehydrogenase kinase 4 (*pdk4*) – forward: TTC ACA CCT TCA CCA CAT GC, reverse: AAA GGG CGG TTT TCT TGA TG; lactate dehydrogenase (*ldh*) – forward: CAT TGT CAA GTA CAG TCC ACA CT, reverse: TTC CAA TTA CTC GGT TTT TGG GA; peroxisome proliferator-activated receptor α (*pparα*) – forward: ACG ATG CTG TCC TCC TTG ATG, reverse: GTG TGA TAA AGC CAT TGC CGT; hexokinase (*hk*) – forward: GAA GGG GCT AGG AGC TAC CA, reverse: CTC GGA GCA CAC GGA AGT T; cluster of differentiation 36 (*cd36*) – forward: TTG TAC CTA TAC TGT GGC TAA ATG AGA, reverse: CTT GTG TTT TGA ACA TTT CTG CTT; *hprt* – forward: TCC TCC TCA GAC CGC TTT T , reverse: CCT GGT TCA TCA TCG CTA ATC.

Table S1. mRNA expression of genes in cardiac tissue from C57BL/6J mice treated with angiotensin II (AngII) or saline (vehicle) for 2 weeks.

	Vehicle	**AngII**
n	9-10	9-10
pparα	1.00 ± 0.12	0.97 ± 0.05
cd36	1.00 ± 0.06	0.96 ± 0.03
pdk4	1.00 ± 0.13	0.73 ± 0.08
ldh	1.00 ± 0.04	0.87 ± 0.03$^{\#}$
hk	1.00 ± 0.03	0.96 ± 0.05
nox2	1.00 ± 0.09	1.10 ± 0.11

Data are means ± SEM. *Pparα*, peroxisome proliferator-activated receptor α; *cd36*, cluster of differentiation 36; *pdk4*, pyruvate dehydrogenase kinase 4; *ldh*, lactate dehydrogenase; *hk*, hexokinase; *nox2*, nicotinamide-adenine dinucleotide phosphate oxidase 2. # $p < 0.05$ compared to vehicle.

Table S2. mRNA expression of genes in cardiac tissue from male and female wild type (WT) and cardiac-specific NOX2 transgenic (TG) mice treated with angiotensin II for 2 weeks.

	Males		**Females**	
	WT	**TG**	**WT**	**TG**
n	4-6	5-6	4-6	5-6
bnp	1.00 ± 0.11	0.86 ± 0.10	1.00 ± 0.09	0.92 ± 0.07
anf	1.00 ± 0.20	0.72 ± 0.22	1.00 ± 0.36	0.62 ± 0.12
pparα	1.00 ± 0.05	0.97 ± 0.03	1.00 ± 0.04	1.04 ± 0.07
cd36	1.00 ± 0.04	0.98 ± 0.02	1.00 ± 0.05	0.99 ± 0.07
pdk4	1.00 ± 0.07	1.57 ± 0.13$^{\#}$	1.00 ± 0.13	0.93 ± 0.09
ldh	1.00 ± 0.05	0.93 ± 0.04	1.00 ± 0.05	1.01 ± 0.06
hk	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.03	0.95 ± 0.06
nox2	1.00 ± 0.06	33.51 ± 1.08 $^{\#}$	1.00 ± 0.12	30.28 ± 1.53$^{\#}$

Data are means ± SEM. *Bnp*, brain natriuretic peptide; *anf*, atrial natriuretic factor; *pparα*, peroxisome proliferator-activated receptor α; *cd36*, cluster of differentiation 36; *pdk4*, pyruvate dehydrogenase kinase 4; *ldh*, lactate dehydrogenase; *hk*, hexokinase; *nox2*, nicotinamide-adenine dinucleotide phosphate oxidase 2. # $p < 0.05$ compared to WT.