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Cardiometabolic adaptations to altered fuel supply, Ca^{2+} handling and exercise

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List of papers

Paper 1 Boardman N.T., Hafstad A.D., Larsen T.S., Severson D.L., Aasum E. *Increased O_2 cost of basal metabolism and excitation-contraction coupling in hearts from type 2 diabetic mice.* Am J Physiol Heart Circ Physiol. 2009 May:296(5):H1373-9.

Paper 2 Boardman N.T., Larsen T.S., Severson D.L., Essop M.F., Aasum E. *Chronic and acute exposure of mouse hearts to fatty acids increases oxygen cost of excitation-contraction coupling.* Am J Physiol Heart Circ Physiol. 2011 Feb:

Paper 3 Hafstad A.D., Boardman N.T., Lund J, Hagve M, Khalid A.M., Wisløff U, Larsen T.S., Aasum E. *High intensity training alters substrate utlilization and reduces oxygen consumption in the heart.* Submitted March 2011.

Paper 4 Boardman N.T., Sejersted O, Sjaastad I, Larsen T.S., Christensen G, Aasum E. *Increased oxygen cost for contractile function and decreased oxygen cost for excitation-contraction coupling in mice with inducible cardiomyocyte specific excision of SERCA2.* Manuscript in progress.

Abbreviations

BM	basal metabolism
E-C	excitation-contraction
FA	fatty acid
HIT	high intensity training
ISO	isoproterenol
KO	knock-out
MIT	moderate intensity training
MVO_2	myocardial oxygen consumption
$MVO_{2\ unloaded}$	O_2 consumption in the unloaded heart
$MVO_{2\ BM}$	O_2 consumption for basal metabolism
$MVO_{2\ ECC}$	O_2 consumption for excitation-contraction coupling
NCX	Na^+-Ca^{2+} exchanger
PCr:ATP	phosphocreatine:adenosine-5'-triphosphate
P:O	phosphorous:oxidation
PVA	pressure volume area
ROS	reactive oxygen species
SR	sarcoplasmic reticulum
SERCA	sarco(endo)plasmic reticulum Ca^{2+}-ATPase
TCA	tricarboxylic acid cycle
TAG	triacylglycerol
UCP	uncoupling protein

Introduction

In the heart, the whole is more than the sum of its parts. Therefore understanding of the control and regulation of cardiac metabolism is an essential field within heart research (134). Cardiac metabolism and contraction are fundamentally integrated, thus without adequate fuel supply and/or utilization the heart is unable to meet the circulatory demands. The heart requires 3.5-5 kg of adenosine 5'-triphosphate (ATP) per day to maintain continuous pumping and as the heart has limited energy reserves, a constant renewal of ATP by the metabolic "machinery" within the myocardium is essential. The heart relies on ATP generated primarily by oxidative phosphorylation, and the rate of energy expenditure of the heart can therefore be assessed using myocardial oxygen consumption (MVO_2).

Efficiency is described by the relationship between the energy output and energy input (MVO_2) in the heart (13). MVO_2 can be designated for both mechanical and non-mechanical processes (132) (Figure 1). ATP is used for non-mechanical processes such as basal metabolism (BM) and excitation-contraction (E-C) coupling (120; 131; 132), the conversion of incoming electrical stimuli to a mechanical response (117), and for mechanical work, including external work (*i.e.* stroke work (SW), the work performed by the ventricle to eject the volume of blood within) as well as "internal" work/ potential energy (the energy generated within each cardiac cycle but not converted to external work) (132) (Figure 1).

Several physiological states and pathological conditions can also alter the oxygen cost for both mechanical and non-mechanical processes; these include changes in substrate supply to the heart (elevated fatty acid supply), altered Ca^{2+} handling, beta-adrenergic stimulation, diabetes, exercise hypertrophy and heart failure. Their effects on cardiac efficiency are the focus of the present thesis.

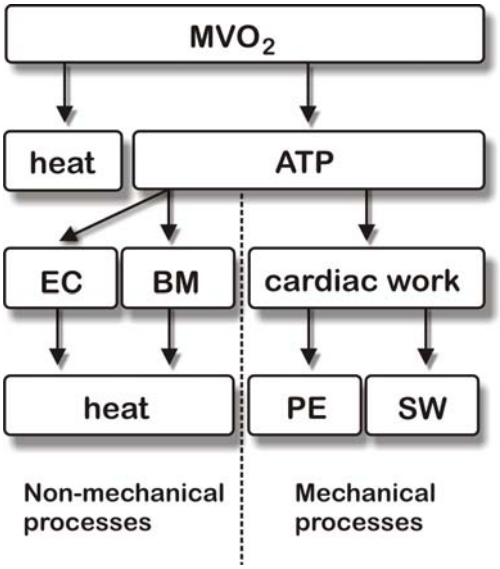

Figure 1. Energy flow diagram for myocardial oxygen consumption (MVO_2). ATP is designated for mechanical processes (cardiac work) that are comprised of potential energy (PE) and stroke work (SW). Non-mechanical processes within the myocardium include basal metabolism (BM), the amount of energy to maintain homeostasis in the quiescent heart and excitation-contraction (EC) coupling. Adapted from Suga (1990) (132).

Oxygen consumption in the heart

The close correlation between cardiac work and MVO_2 describes the increased energy expenditure in the form of MVO_2 as the work demand of the heart increases (120; 132). MVO_2 used for mechanical activity is often called work-dependent MVO_2, and includes both an internal and external work component. Several studies have shown that there is a linear relationship between MVO_2 and increasing cardiac work whether assessed only as external mechanical cardiac work (144) or total cardiac work (131; 132). A regression analysis of the relationships between MVO_2 and these parameters of cardiac work will provide information of changes in the efficiency of the heart. Contractile efficiency represents the additional MVO_2 required for a given increase in cardiac work (the inverse of the slope). It is the product of the efficiency by which O_2 consumption is converted to ATP synthesis (oxidative phosphorylation) and the efficiency by which ATP hydrolysis is converted to cardiac mechanical work (cross-bridge cycling) (132). Work-independent MVO_2 represents the O_2 required for non-mechanical processes, including E-C coupling and basal metabolism which are described in more detail below.

Excitation-contraction (E-C) coupling in the heart. E-C coupling is a term that includes the process of converting an electrical stimulus to a mechanical response, and thus primarily includes the Ca^{2+} handling associated with the Ca^{2+} transient (117), as described in more detail in Figure 2. The most energetically costly process of E-C coupling is the ATP driven pump of the SR, which accounts for approximately 15% of the total myocardial energy expenditure (98; 136). In spite of this, Ca^{2+} uptake by SERCA into the SR is more energetically efficient (2 Ca^{2+}:1 ATP) than Ca^{2+} extrusion via sarcolemmal Ca^{2+}-ATPase and the Na^{+}-Ca^{2+} exchanger (NCX) coupled to the Na^{+}-K^{+} ATPase driven pump (1 Ca^{2+}:1 ATP) (124).

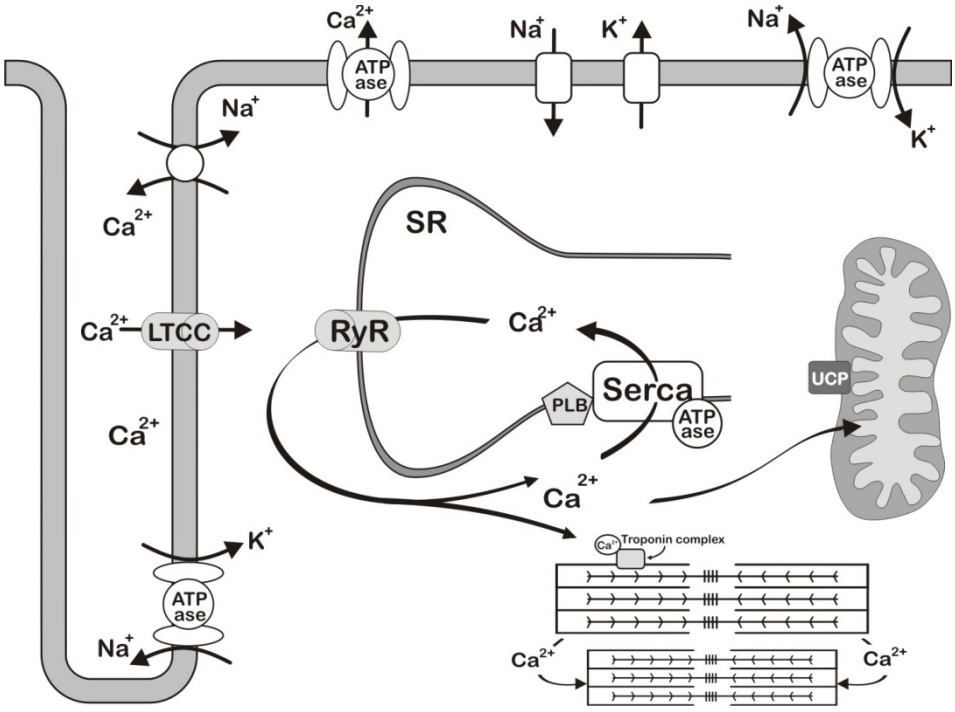

Figure 2. Ca^{2+} handling in the cardiomyocyte during excitation-contraction coupling. The electrical signal in the cardiomyocyte is initiated by a wave of depolarization travelling through the myocardium, causing the rapid entry of Na^{+} ions (initial depolarization) followed by opening of the L-type Ca^{2+} channels (LTCC) in the sarcolemma and Ca^{2+} entry into the cytosol. This Ca^{2+} influx stimulates Ca^{2+} induced Ca^{2+} release through the ryanodine receptor (RyR) in the sarcoplasmic reticulum (SR). Ca^{2+} binds to the troponin complex on the actin filament, opens the binding site for myosin attachment and thus initates cross-bridge formation. Cytosolic Ca^{2+} is taken up by the SR Ca^{2+}-ATPase (SERCA) into the SR, as well as transported out of the cell by the sarcolemmal Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger. In addition, the mitochondria can take up Ca^{2+} and act as a Ca^{2+} buffer within the cytosol. However, this process takes longer than that of the other Ca^{2+} transport mechanisms mentioned (98).

Several physiological and pathophysiological conditions have been shown to alter Ca^{2+} handling in the heart. It is well known that Ca^{2+} transients can be increased by β-adrenergic stimulation as well as an increased external concentration of Ca^{2+}, which will both result in an increased force produced by the myocardium. Physiological adaptations that occur following exercise training have been shown to affect the dynamics of Ca^{2+} cycling within the heart as well as improve myofilament Ca^{2+} sensitivity (64; 65; 147). Changes in Ca^{2+} homeostasis are known to occur in type 2 diabetes including altered Ca^{2+} transients (102) and increased Ca^{2+} leakage from the ryanodine receptors in the SR (11; 130). Recent evidence has also linked a reduced energetic state in the heart to reduced SR Ca^{2+} content and increased leakiness from the SR (75). Reduced activity and/or expression of SERCA2 in the cardiomyocyte will have major implications on contractile function. Cardiomyopathy and pathological cardiac remodeling, *i.e.* heart failure and diabetes, have been shown to be associated with a reduction in the capacity (122) or presence (5; 30) of myocardial SERCA2. Thus given the central role of Ca^{2+} handling (including SERCA2 activity) in cardiac function this may lead to altered oxygen cost for Ca^{2+} handling in E-C coupling.

Basal metabolism. The basal metabolism (BM) of the heart represents the rate of energy expenditure in the quiescent myocardium, and accounts for approximately 20-35% of total cardiac metabolism (44). The BM rate in heart tissue is several fold higher than that found in any other tissue, however the absolute or relative values of the oxygen cost of BM in the heart vary tremendously within the literature, most likely due to differences in species, type of cardiac preparation and method of assessment of energy consumption, which has been skillfully reviewed by Gibbs and Loiselle (2001) (44). The primary energy requirements of BM are designated for non-mitochondrial purposes (10%) and maintaining the mitochondrial membrane potential to protect against proton leaks (20-30%); the remaining energy expenditure (60-70%) is devoted to ATP production in the mitochondria for protein synthesis, maintaining transmembrane ionic balance across the sarcolemma (Na^+-K^+-ATPase, Ca^{2+}-ATPase), and resting actomyosin ATPase (28; 44; 112).

Cardiac substrate utilization

The heart can be regarded as an omnivore as it can use a variety of substrates (fuels) for ATP synthesis including fatty acid (FA), glucose, lactate and certain amino acids. The heart has therefore a high degree of plasticity with respect to substrate selection, encompassing daily changes between the fed and fasted state, as well as adaptations to various physiological (β-adrenergic stimulation, exercise) and pathophysiological conditions (pathological hypertrophy, heart failure, diabetes). The main substrates used by the heart are FA and glucose; the reciprocal changes in glucose and FA oxidation depending on their availability are the basis of the "glucose-fatty acid cycle", also known as the Randle cycle (107; 108). Other factors that influence substrate utilization in the heart include hormones, cardiac workload and oxygen availability, as well as transcriptional changes of key metabolic enzymes and transporters (80; 99).

Fatty acid utilization. When plasma levels of circulating FA (bound to albumin) are high, the uptake of FA by the myocardium is also elevated. Most FA transport across the sarcolemma is mediated by FA transport and binding proteins (including fatty acid transport protein (FABT), fatty acid translocase (FAT/CD36) and the fatty acid binding proteins (FABP) located on the inner and outer side of the cell membrane (Figure 3). FA is also released from triglyceride (TG) in the form of triacylglycerol (TAG) bound to circulating lipoproteins (chylomicrons and VLDL) that are broken down by cardiac lipoprotein lipase to provide an additional source of FA for cardiac metabolism (46; 80; 142). When FA has entered the cardiomyocyte, it must first be converted to acyl-CoA by fatty acid acyl-CoA synthetase (FACS). The acyl-CoA can be converted to TAG or transferred into the mitochondria. For mitochondrial uptake, cytosolic fatty acyl-CoA is first converted to an acyl-carnitine derivate by carnitine-palmitoyltransferase-1 (CPT-1) and then transferred by an acyl-carnitine translocase into the mitochondria matrix where acyl-CoA is re-generated by CPT-2. Thus, the CPT-1 step is regarded generally as the rate limiting step of FA oxidation in the heart. Within the mitochondria, β-oxidation of the acyl-CoA will yield multiple acetyl-CoA molecules for entry into the tricarboxylic (TCA) cycle, and subsequent ATP production by the electron transport chain and oxidative phosphorylation (80; 99). High levels of acetyl-CoA formed by β-oxidation will also activate pyruvate dehydrogenase-kinase-4 (PDK4) which has an inhibitory effect on pyruvate dehydrogenase (PDH), thereby limiting glucose oxidation (46; 80). If FA uptake exceeds demand, excess acetyl-CoA provides negative feedback via malonyl-CoA and prevents further FA uptake into the mitochondria. Furthermore, acyl-CoA that does not enter the mitochondria for β-oxidation can be stored as TAG or structural lipids in the myocardial membrane.

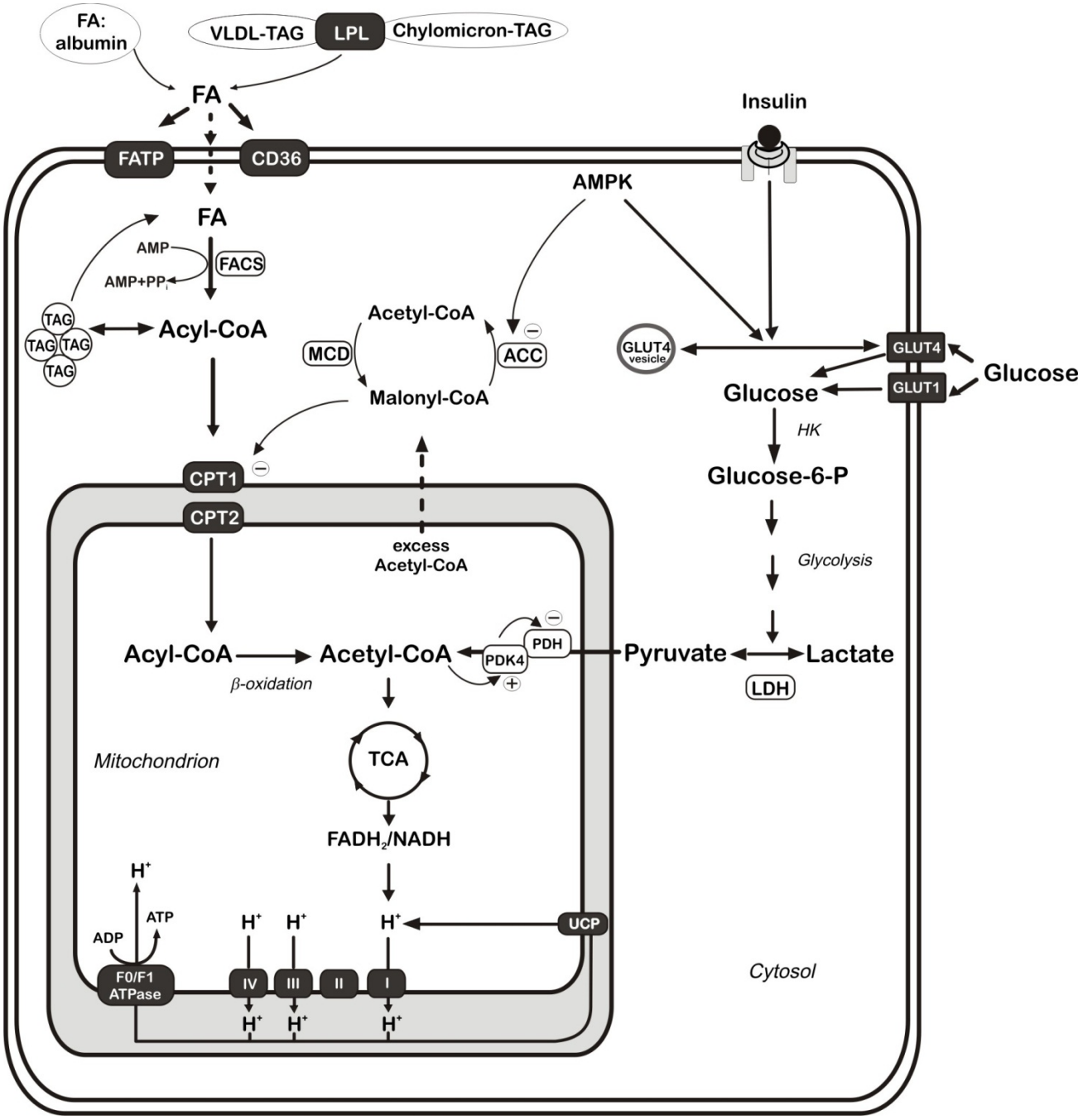

Figure 3. Glucose and fatty acid uptake and oxidation in the cardiomyocyte. Fatty acids are delivered to the cardiomyocyte attached to albumin or as triacylglycerol (TAG) bound to chylomicrons or VLDL. They can enter via FA transport protein (FATP) and FAT/CD36 or by diffusion across the membrane. Once converted to acyl-CoA, they can pass through carnitine-palmitoyltransferase (CPT-1 and CPT-2) located in the mitochondrial membrane. Following β-oxidation, acetyl-CoA can enter the tricarboxylic (TCA) cycle to form $FADH_2$/NADH which can enter the electron transport chain (ETC). H^+ formed from the ETC is pumped into the mitochondrial matrix, which contributes to the mitochondrial membrane potential. Activation of uncoupling proteins (UCP) can enhance proton leak. Glucose uptake is mediated by glucose transporters (GLUT 1 and 4). Under aerobic conditions, pyruvate formed from glycolysis, forms acetyl-CoA in the mitochondria and can enter the TCA cycle and ETC. Adapted from Lopaschuk *et al.* (80).

Glucose utilization. Glucose uptake into the cardiomyocyte occurs by facilitated transport regulated by sarcolemmal glucose transporters (GLUT1 and GLUT4) (Figure 3). The dominant and insulin-sensitive transporter GLUT4 moves between intracellular vesicles and the sarcolemma by insulin-mediated translocation, and is also stimulated by the AMP-activated protein kinase (AMPK) in response to increased contraction and hypoxia (121). Intracellular glucose is phosphorylated by hexokinase (HK) to form glucose-6-phosphate, which apart from entering glycolysis, can also be used in glycogenesis and can enter the pentose phosphate and hexosamine biosynthetic pathways. In the glycolytic pathway, fructose-6-phosphate is converted to fructose 1,6-biphosphate by the enzyme phosphofructokinase (PFK), an important regulator of glycolytic flux stimulated by increased contraction, hypoxia, fed state, and catecholamines. When ATP levels are high, PFK is inhibited by cytosolic citrate released from the TCA cycle. The end product of glycolysis is pyruvate, which under anaerobic conditions may be reduced to lactate; under aerobic conditions, pyruvate is decarboxylated to acetyl-CoA by the enzyme PDH in the mitochondrial membrane prior to entering the TCA cycle. The enzyme PDH is stimulated by insulin, increased heart work and catecholamines; all conditions where the glycolytic rates are also high. Inhibition of PDH occurs via PDK4 which increases its activity when levels of acetyl-CoA derived from FA oxidation are high (99).

Physiological and pathophysiological changes in cardiac metabolism

Several physiological conditions (fasting, post-operatively, exercise) and pathophysiological conditions (diabetes/obesity) are associated with changes in levels of circulating FA and/or catecholamines which can affect substrate utilization, MVO_2 and cardiac efficiency (56; 71; 80; 103; 133).

Acute elevation of FA supply to the heart. Elevated circulating FA levels are known to occur following fasting and following operative procedures due to increased β-adrenergic stimulation and/or postoperative insulin resistance, both of which act to mobilize FA from adipose tissue. Under these conditions, FA becomes the main substrate utilized by the heart (3; 71). Elevated FA is not only associated with elevated myocardial FA oxidation, but also with an increase in MVO_2 that is larger than would be expected by changes in cardiac work, leading to what is often called oxygen waste (56; 72; 87). Several mechanisms have been suggested to induce FA-induced oxygen waste which is addressed in more detail in the Discussion section of this thesis. In brief, the phosphorous:oxygen (P:O) ratios of oxidative phosphorylation can offer a partial explanation for

the increased MVO_2 following acute elevation of FA. The P:O ratio represents the amount of ATP (mols) formed from each mol of oxygen (terminal electron acceptor) utilized by the mitochondrial electron transport chain, and will vary depending on the type of energy substrate utlilized (99). There is an ATP yield of 32 for a mol of glucose oxidized, with a corresponding P:O ratio of 2.58. By comparison, the ATP yield is 105 for palmitate but with a lower P:O ratio of 2.33. Hence, although FA clearly generates a higher energy yield it comes at the expense of a larger oxygen requirement (61; 99). Elevated FA oxidation has been associated with increased mitochondrial production of reactive oxygen species (ROS). Both FA and ROS have been suggested to activate mitochondrial uncoupling proteins (UCP) which can increase proton conductance across the mitochondrial membrane (19) causing a reduced ATP production (19; 34; 35). Enhanced mitochondrial uncoupling may therefore contribute to impaired myocardial function and elevated O_2 consumption (16). Under conditions of elevated FA, futile cycling of FA intermediates from TAG and back into the TAG pool is an energy consuming process that may further contribute to FA-induced O_2 waste (93). Finally, recent studies have reported changes in Ca^{2+} handling following the elevation of FA (37), in addition to altered Ca^{2+} handling when UCP is elevated (140) and altered SR Ca^{2+} cycling in conditions where FA has been elevated over a prolonged period (11; 102).

Diabetes. Diabetes is associated with an increased prevalence of heart disease, increased morbidity and mortality rate (40; 48; 63). Heart failure in diabetics is due to coronary heart disease caused by accelerated atherosclerosis, and/or development of a specific diabetic cardiomyopathy (defined as the development of dysfunction independent of known coronary disease and/or hypertension) (125). Although the mechanisms behind the pathogenesis of diabetic cardiomyopathy are multifactoral and complex, there is evidence that metabolic changes play an important role in the development of mechanical dysfunction (2; 4; 26; 94). In support of this animal and human studies have revealed that alterations in myocardial metabolism may occur prior to major ventricular dysfunction (2; 32; 79; 145). A continuous elevation of plasma lipid levels and FA availability to the heart over time will lead to an adaptive increase in FA oxidation due to both the elevated FA supply as well as to transcriptional changes, as FA is known to activate the transcription factor peroxisome proliferator-activated receptor (PPAR) α responsible for the regulation of genes coding for proteins increasing FA transport and metabolism in the diabetic heart (1; 9; 46; 92). Another hallmark of the type 2 diabetic heart is decreased cardiac efficiency (15; 26; 57; 85). Previous studies from our laboratory, using type 2 diabetic *db/db* hearts have demonstrated by regression analysis of the relationship between MVO_2 and cardiac work that decreased cardiac efficiency in these hearts was due to

increased oxygen cost for non-mechanical processes (49; 57). Clinical studies have also documented an altered substrate utilization (elevated rates of FA oxidation) and decreased cardiac efficiency (103), as well as impaired energetic state (lowered PCr:ATP ratios) (32; 119) in obese and/or type 2 diabetic subjects. A higher O_2 cost may be of particular importance during conditions of limited O_2 availability for the heart and reduced cardiac efficiency may play a particular role with regard to the increased susceptibility to ischemia often found in diabetes (2; 7; 48; 49). Several of the same mechanisms as previously discussed for the FA-induced increase in MVO_2 may contribute to the elevated MVO_2 in diabetic hearts including a switch in substrate utilization to give a lower P:O ratio (80; 99), ROS-mediated mitochondrial uncoupling (18) and metabolic futile-cycling (93; 114; 115). In addition, increased SR Ca^{2+} leak (11; 130) can increase Ca^{2+} recycling and thus the oxygen cost of E-C coupling.

Exercise. Although chronic exercise training leads to a variety of systemic changes in the circulatory system and on the heart, the specific cardiometabolic effects of exercise are not clear. There are few and inconsistent reports with regard to exercise-induced changes in substrate utilization (22; 24) whereas the effect of exercise on cardiac energetics has not been previously reported. Isolated cardiomyocytes from rodents subjected to high intensity interval training show increased contractility, improved Ca^{2+} handling and increased myofilament Ca^{2+} sensitivity (65; 66; 147) which can imply improvement of cardiac contractile efficiency and/or reduced oxygen cost for processes associated with E-C coupling. Moderate intensity exercise has been linked to reduced mitochondrial ROS production (128) and mitochondrial uncoupling (14), processes that may also affect cardiac efficiency in terms of altered O_2 consumption. Finally, exercise-induced cardiac hypertrophy is associated with a shift in the myosin heavy chain (MHC) expression from β to α isoform (62; 101); the α isoform has higher ATPase activity and thus is energetically less efficient (54; 100). As the work of the heart will vary depending upon the type, intensity, duration and regularity of the exercise performed, there are reasons to believe that this may also affect the cardiometabolic status following exercise.

Aims of the study

The general purpose of this thesis was to elucidate how cardiac substrate metabolism and/or Ca^{2+} handling influence cardiac energetics in hearts under altered physiological and pathophysiological conditions. Specific attention was paid to examination of cardiac efficiency, in particular the changes in oxygen cost for processes associated with basal metabolism (BM) and excitation-contraction (E-C) coupling in these hearts.

Specific aims:

1. Establish a technique for measurement of oxygen cost for BM ($MVO_{2\ BM}$) and E-C coupling ($MVO_{2\ ECC}$) in isolated perfused mouse hearts.
2. Examine changes in $MVO_{2\ BM}$ and $MVO_{2\ ECC}$ in hearts from type 2 diabetic (*db/db*) *vs.* non-diabetic mice.
3. Elucidate the role of fatty acid oxidation rate *vs.* fatty acid load on myocardial oxygen waste.
4. Elucidate the cardiometabolic effects of exercise training, with a special focus on the role of exercise intensity.
5. Examine the cardiometabolic effects of myocardial SERCA deletion using conditional myocardial SERCA knock-out (KO) mice.

Methodological Considerations

Assessment of MVO_2 and cardiac efficiency

The isolated perfused *(ex vivo)* heart is an important tool for characterizing the cardiac phenotype. It is important to remember that as the heart has been removed from its natural milieu, *ex vivo* characterization will not give the complete *in vivo* picture of heart function (8). Nevertheless, isolated heart perfusions have great value with regard to describing changes within the heart as factors such as loading conditions, heart rate, substrate supply and drug administration are easily controlled, and the heart is without neuro-hormonal influences. In some cases, the *ex vivo* perfusion setting allows for earlier detection of contractile abnormalities that are difficult to detect *in vivo* due to variable *in vivo* hemodynamics and/or neuro-hormonal influences (49; 57; 141). In the present thesis, cardiac work, contractile properties, MVO_2, cardiac efficiency and myocardial substrate utilization has been assessed in isolated hearts that have been perfused in the antegrade perfusion (working) mode (Figure 4A) and in the retrograde perfusion (non-working Langendorff) mode (Figure 4B).

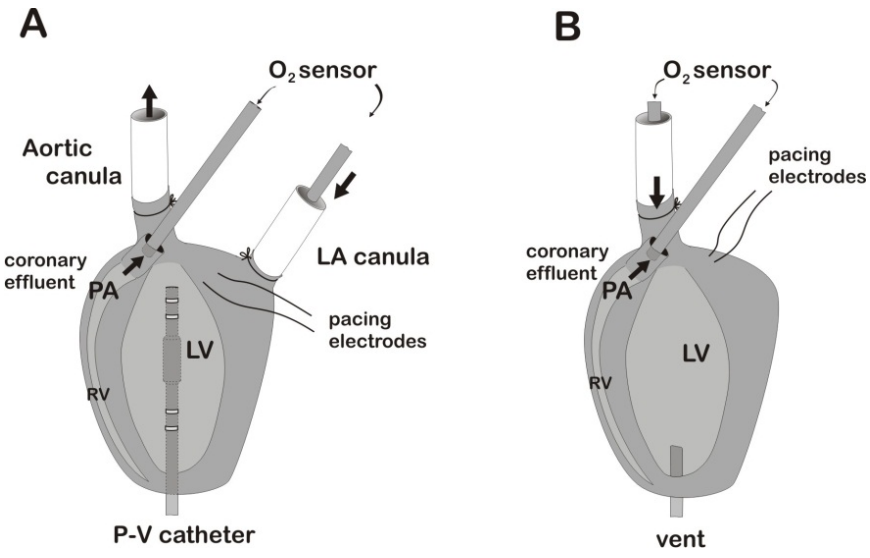

Figure 4. Instrumentation of an isolated perfused working heart (panel A) where PO_2 is measured using oxygen sensors placed in the left atrium (LA) canula and pulmonary artery (PA). Left ventricle (LV) pressure and volume were measured using a P-V catheter. In a retrograde perfused unloaded heart PO_2 is measured by oxygen sensors placed in the aortic cannula and PA (panel B). The heart is mechanically unloaded by venting the LV. In both models, electrodes are attached on the left atrium for electrical pacing of the heart.

Cardiac efficiency, as proposed by Bing in 1949 (13), is defined as the relationship between the energy produced (cardiac work) and energy consumed (MVO_2). This concept of cardiac efficiency requires a cardiac work term that correlates as closely as possible to MVO_2. In 1979, Suga described a linear relationship between such a work term and MVO_2, which he defined as the pressure-volume work (pressure volume area, PVA) (131). PVA was measured using a conductance catheter inserted through the apex of the left ventricle of a working heart (paper 1, 3, 4). This catheter contains a micromanometer for pressure recordings in addition to electrodes for measurement of volume within the left ventricle. Volume is calculated from the total conductance (G) by the following formula: $V_t = (1/\alpha) * (L2/\rho) * [G(t)-Gp]$, where L is the inner electrode distance and ρ is the resistivity of the perfusate. The α factor is calculated by the ratio between the directly measured stroke volume and the stroke volume obtained by the catheter. The instantaneous conductance by the myocardium, G_p, gives rise to the estimated volume within the ventricle wall (called parallel volume). Using the conductance catheter, pressure-volume loops are obtained by plotting left ventricular pressure against the corresponding volume throughout a cardiac cycle (the P-V loop). A temporary reduction of preload pressure that causes a passing reduction of ventricular filling forms a family of PV loops that are used to define the end systolic pressure volume relationship (ESPVR) and the end diastolic pressure volume relationship (EDPVR) (Figure 5A). These are used to describe systolic and diastolic properties of the heart, as well as to determine the theoretical value of the volume in the heart when zero pressure is generated (V_0). The PVA includes the work exerted by the heart on its environment, stroke work (SW, defined by the P-V loop) and the potential energy triangle, limited by the ESPVR, EDPVR, and the descending limb of the P-V loop (Figure 5B). Thus, PVA can be calculated using the following formula:

$PVA = SW + [P_{es} \bullet (V_{es} - V_0)/2] - [P_{ed} \bullet (V_{es}-V_0)/4]$ (148).

The MVO_2 was obtained using fiber-optic O_2 probes for the measurement of the partial pressure of oxygen (PO_2) of the buffer entering ($PO_{2\ buffer}$) and the buffer exiting the heart ($PO_{2\ effluent}$), representing the arterial-venous difference in PO_2. MVO_2 is calculated by the Fick's principle, according to the following equation: $MVO_2 = [PO_{2\ buffer}-PO_{2\ effluent}] *$ Bunsen solubility coefficient of O_2 * coronary flow (Figure 4). It is worth noting that constant on-line measurement of the PO_2 provides accurate assessment of MVO_2 as despite the use of a constant gas mixture (95% O_2), oxygenation of the perfusate can vary due to an altered flow pattern in the surface oxygenator. This variation can be of considerable importance when, for example, the arterial-venous PO_2 difference becomes small. Although using arterial-venous difference is a recognized method for measuring

MVO_2 in isolated perfused hearts, the possibility of transepicardial O_2 flux (44) cannot be excluded and may be a methodological limitation.

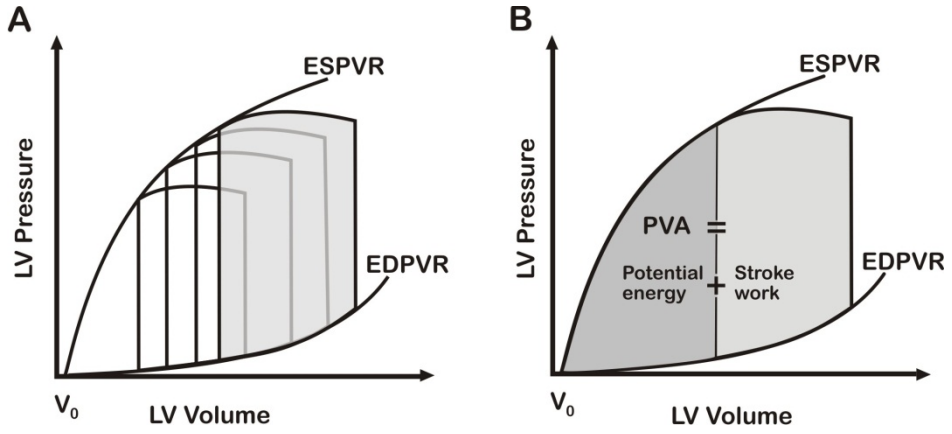

Figure 5. A reduction in preload pressure creates a family of loops that define the end systolic and end-diastolic pressure volume relationships (panel A). The pressure-volume area (PVA) is the sum of the stroke work and the potential energy triangle (panel B).

Regression analysis of the PVA:MVO_2 relationship (131) allows us to evaluate two aspects that affect cardiac efficiency (Figure 6): whereas changes in the slope can reflect the contractile efficiency of the heart (*i.e.* how much work-dependent O_2 is converted to mechanical energy), the extrapolated y-intercept of the relationship (when PVA is 0) can reflect the O_2 required for non-mechanical (work-independent) processes (unloaded MVO_2). Thus cardiac inefficiency can be exhibited by i) a parallel increase of the PVA:MVO_2 relationship (*i.e.* inotropic stimulation) due to an increase in work-independent oxygen consumption, ii) by a change in the slope due to an increase in work-dependent MVO_2 (*i.e.* increased wall stress), or in some cases iii) by both.

As the y-intercept is obtained by extrapolation of the PVA:MVO_2 relationship, it represents an indirect value for unloaded MVO_2 and not a direct measurement. Alternatively, work-independent MVO_2 ($MVO_{2\ unloaded}$) can be directly measured in isolated hearts by reducing the workload through retrograde perfusion where PVA is zero. This is obtained using retrogradely (Langendorff) perfused mouse hearts where the heart is unloaded by inserting a small cannula (a vent) in the left ventricle to drain any remaining perfusate (Figure 4B).

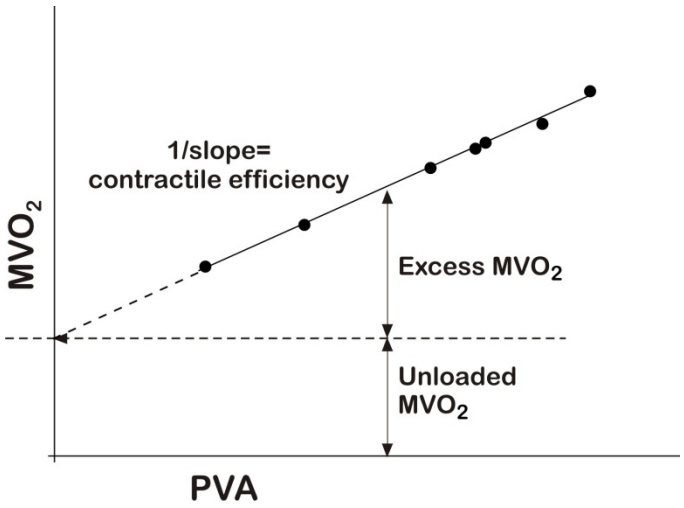

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Figure 6. Regression analysis of the PVA and MVO_2 relationship allows for the determination of contractile efficiency and the energy required for non-mechanical processes in the heart.

Although residual cross-bridge interaction remains in the myocardium as the myocytes remain intact and in contact with each another, the O_2 consumed for the unloaded contraction with zero PVA is considered to be of a negligible amount relative to the unloaded MVO_2 (132). In spite of this other studies have measured unloaded MVO_2 in isolated cardiomyocytes or in muscles slices from the heart (124; 135; 136; 149) where compounds including 2,3-butanedione monoxime (BDM) and blebbistatin, known to affect myofilament interaction by stopping any residual, energy-consuming crossbridge formation, have been added (33; 38; 135; 149). BDM has been reported to reduce the Ca^{2+} sensitivity of the myofilaments without affecting the intracellular Ca^{2+} transient (76; 149) however, there are also several reports that demonstrate that BDM may in fact alter total Ca^{2+} handling (83; 104; 138). In the present thesis, pilot experiments were performed where BDM (10 mM) was added to the perfusion buffer in order to further "unload" the heart. We found that BDM reduced $MVO_{2\ unloaded}$ by more than 50%, without altering $MVO_{2\ BM}$. The dramatic reduction in $MVO_{2\ ECC}$ seen in these pilot studies could indicate that BDM affected total Ca^{2+} handling. As this would lead to an underestimation of the O_2 cost for E-C coupling in our experimental protocol, the use of BDM was therefore not further explored. In further pilot experiments, we have also evaluated the use of blebbistatin (10 μM), a compound reported to specifically inhibit actin-myosin interaction in cardiac muscle without altering the Ca^{2+} transients (33; 38). Our experiments revealed that blebbistatin stopped contractions and caused an immediate fall in $MVO_{2\ unloaded}$. However, as the fall

in MVO_2 was approximately 60% this raised the uncertainty of whether blebbistatin could have altered E-C coupling in isolated heart perfusions. For this reason, in addition to its sensitivity to ultra-violet light (38) and the challenge of blebbistatin contamination in the perfusion system (not water soluble), it was not further explored.

Measurement of the O_2 cost for BM was obtained by electrically arresting the heart through the elevation of the extracellular concentration of potassium chloride (KCl) to approximately 16 mM. Elevated KCl results in depolarization of the sarcolemma membrane and arrests the heart through the cessation of action potentials and thereby of mechanical contractions. As $MVO_{2\ unloaded}$ represents the O_2 required for the unloaded contraction with zero PVA (132) and this was directly measured in isolated hearts during retrograde perfusion, the difference between $MVO_{2\ unloaded}$ and $MVO_{2\ BM}$ was defined as the O_2 cost for E-C coupling ($MVO_{2\ ECC}$).

Assessment of myocardial substrate utilization

Myocardial substrate flux rates were assessed using radioisotope techniques by adding trace amounts of labeled radioactive substrates to the perfusion buffer, where the end-products (^3H_2O or $^{14}CO_2$) are quantitatively collected at regularly timed intervals. In the present thesis, glucose oxidation was determined using [U-^{14}C]-glucose where $^{14}CO_2$ is released during the pyruvate dehydrogenase step and in the TCA cycle. Gaseous $^{14}CO_2$ was trapped when the gas was bubbled from a closed (airtight) perfusion system through hyamine hydroxide, while ^{14}C-labelled bicarbonate was measured by injecting a sample of the perfusate into a sealed test tube containing sulphuric acid where the $^{14}CO_2$ released as a consequence of the acidification is trapped on filter paper with hyamine hydroxide (8). Palmitate oxidation was measured using [9,10-^{3}H]-palmitate, where ^3H_2O is released at the cytochrome C step in the respiratory chain, and separated from the tritiated palmitate by Folch's extraction (41). The use of radioactive isotope techniques is a relatively inexpensive method to assess metabolic rates in isolated hearts that does not require the use of advanced equipment. As with all *ex vivo* experiments it cannot completely reproduce the complex *in vivo* situation (96). For example, only 2 labeled substrates in the same experiment (to detect ^3H_2O and $^{14}CO_2$) can be measured simultaneously and there is a potential contribution of endogenous substrates in the heart when using this technique.

Mouse models

The inbred mouse strains C57BL/6J and BalbC/cJ have been used in this thesis for all experiments in normal, control mice with the exception of the C57BL/KsJ-lepr$^{db+/+}$(*db/*+) mice which were always used as a control in comparison with the *db/db* type 2 diabetic mouse model (described below). The choice of mouse model for studies carried out in normal mice was based on several reasons. The C57BL/6J mouse shares the same background as the *db/*+ and *db/db* mouse strain thus in paper 1 it was the appropriate model for establishing the method that was later used in *db/*+ and *db/db* mice. In paper 2, early pilot studies performed in BalbC/cJ mice made this strain the natural choice for the continuation of experiments. A report by Lightfoot *et al.* (2001) showed that C57BL/6J mice, used again in paper 3, had a lower aerobic capacity as compared to other mouse strains (77), however, as this strain has been previously described to run willingly (77) and respond to training (65) they were included in the exercise protocol.

The type 2 diabetic (db/db) mouse. In paper 1 and 2, C57BL/KsJ-lepr^{db}/lepr^{db} (*db/db*) mice were used as a monogenic model of obesity and type 2 diabetes. These mice have a mutation on the leptin receptor gene (chromosome 4) (29) which in the homozygote mice (*db/db*) causes hyperphagia and the development of obesity, insulin resistance, hyperinsulinemia, hyperglycemia and dyslipidemia, while their heterozygote littermates (*db/*+) are phenotypically normal. In addition to having very severe and fast progressing type 2 diabetes, the *db/db* mouse develops contractile dysfunction without the presence of atherosclerosis which has resulted in its use as a model for diabetic cardiomyopathy. The majority of studies evaluating cardiac metabolism and function in *db/db* hearts have been performed with *ex vivo* perfusions. Despite a difference in cardiac substrate availability in *db/db* and *db/*+ mice *in situ*, these hearts are most often perfused *ex vivo* with buffer containing fixed levels of glucose and palmitate and have been shown to display altered substrate utilization, where FA utilization is elevated whereas glucose oxidation and glycolysis are reduced (2; 9; 26).

The myocardial Serca2 knockout mouse. Reduction in myocardial sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) has been regarded to play an important role in development of heart failure (86; 97; 105; 122). To study the consequences of a reduction in SERCA2, colleagues at the University of Oslo, Norway, have generated a genetically modified mouse with an inducible cardiac specific excision of the Atp2a2 (*Serca2*) gene. These *Serca2$^{flox/flox}$* Tg (αMHC-MerCreMer) (SERCA2 KO) mice and their WT mice *Serca2$^{flox/flox}$* (SERCA2 FF) have been described previously (5; 6; 126).

The SERCA2 KO mice do not present any abnormalities until cardiomyocyte-specific excision of *Serca2* is induced by tamoxifen. Tamoxifen, however, does not affect the SERCA2 FF control mice (5; 6). *In situ* assessment of heart function has revealed that SERCA2 KO mice maintain near normal function at 4 weeks following SERCA2 excision, despite less than 5% of cardiac SERCA2 protein abundance as compared to that found in SERCA2 FF control mice. At this stage, there were no signs of cardiac hypertrophy or heart failure as assessed by echocardiography, whereas end stage heart failure had developed by 7 weeks (5; 82). Myocytes isolated from SERCA2 KO hearts exhibited reduced fractional shortening, smaller amplitude and longer decay rate of Ca^{2+} transients as compared to controls (5; 82; 129), all of which progressively worsened until end stage heart failure at 7 weeks (82). These changes were accompanied by increased dependence on other mechanisms to maintain Ca^{2+} homeostasis, such as increased Ca^{2+} influx through L-type Ca^{2+} channels and the enhanced presence of plasma membrane Ca^{2+}-ATPase and NCX in the sarcolemma (5; 82; 129). These compensatory mechanisms result in Na^{+} accumulation over time, contributing to the development of heart failure in these mice (82).

Exercise training protocol in mice

The aim of paper 3 was to determine the cardiometabolic effects of long term exercise training at both high intensity (HIT) levels versus those of moderate intensity (MIT). Treadmill running allows control of the intensity and volume of the workload (duration, speed, inclination, distance) (58). Moreover, exercise intensity can be further controlled through regular assessment of $VO_{2\,max}$, where the running speed can be adjusted to maintain a constant relative intensity throughout the entire protocol (58). Mice have commonly been used to study the cardiovascular effects of exercise training although the majority of exercise studies in mice have applied continuous moderate intensity treadmill running (12; 39; 113). In recent years, HIT has become of a topic of interest for both the professional and amateur athlete, as well as for the researcher, based on the argument that HIT achieves higher aerobic fitness earlier than MIT (53; 146). HIT also seems more effective for achieving structural and functional adaptations within the heart, such as exercise-induced hypertrophy (52; 67). Based on this, a training protocol, slightly modified from that described by Kemi *et al.* (2002) (68), was designed and is further described in paper 3.

Summary of Results

Paper 1

Previous studies have demonstrated reduced cardiac efficiency in type 2 diabetic (*db/db*) mice. Regression analysis of the relationship between cardiac work (measured as pressure-volume area, PVA) and myocardial oxygen consumption (MVO_2) has revealed that the decreased efficiency is due to increased work-independent MVO_2 as indicated by an elevated y-intercept of this relationship. In paper 1, our aims were to compare the estimated (extrapolated) value for work-independent MVO_2 from the PVA:MVO_2 relationship to a directly measured value of MVO_2 in the same hearts now perfused in an unloaded retrograde mode ($MVO_{2\ unloaded}$). As the unloaded MVO_2 is representative of the oxygen cost for basal metabolism ($MVO_{2\ BM}$) and excitation-contraction coupling ($MVO_{2\ ECC}$), our aim was to separately determine O_2 cost for each of these processes in normal and *db/db* mouse hearts. We found that the estimated value of work-independent MVO_2 corresponded well with the directly measured MVO_2. In addition, we measured $MVO_{2\ BM}$ in KCl-arrested hearts and determined $MVO_{2\ ECC}$ as the difference between $MVO_{2\ unloaded}$ and $MVO_{2\ BM}$. The procedure was validated by demonstrating that elevations in perfusate FA and/or Ca^{2+} concentrations resulted in predicted changes in either $MVO_{2\ BM}$ and/or $MVO_{2\ ECC}$. The main finding of this study is that we have demonstrated for the first time using this technique that elevated $MVO_{2\ unloaded}$ in *db/db* mice was due to both a higher $MVO_{2\ BM}$ and $MVO_{2\ ECC}$.

Paper 2

It is well known that the administration of catecholamines as well as an elevation of fatty acids *in situ* induces cardiac O_2 waste (23; 87-89) which has more recently been shown to be due to an increase in unloaded MVO_2 (56; 72; 133). In paper 2 we have examined the O_2 waste associated with both high FA supply and catecholamines in normal and type 2 diabetic hearts. We found that an acute elevation of FA induced an acute increase in $MVO_{2\ unloaded}$ in normal hearts, due to an increase in $MVO_{2\ BM}$ as well as for $MVO_{2\ ECC}$. Isoproterenol stimulation, on top of a high FA supply, led to an additive increase in $MVO_{2\ unloaded}$, due to increased $MVO_{2\ ECC}$. The acute FA-induced O_2 waste seen in normal hearts was shown to be dependent on processes initiated by the presence of FA and not to the increased FA oxidation *per se*, as we found that the increase in FA oxidation rate following pharmacological stimulation (GW610742) under normal fat conditions was equivalent to that obtained with hearts exposed to HF, yet only HF increased $MVO_{2\ unloaded}$. Likewise, reducing FA oxidation rate (dichloroacetate, DCA) in hearts that remained exposed to high FA supply did not reduce $MVO_{2\ unloaded}$. In hearts from type 2 diabetic (*db/db*) mice,

isoproterenol but not acute elevation of FA supply led to a further increase in $MVO_{2\ unloaded}$. This may suggest that diabetic hearts are adapted to chronic (*in vivo*) exposure to a high fat environment and are thereby resistant to the O_2 wasting effect following an acute elevation in FA supply.

Paper 3

High intensity training (HIT) has been shown to have a more profound effect on cardiovascular function and aerobic capacity than isocaloric low and moderate intensity training (MIT). The specific effects of exercise on myocardial metabolism and energetics remain unclear. In paper 3, the cardiometabolic effects of exercise were evaluated with a specific focus on the role of exercise intensity. Although both exercise training regimens resulted in the same degree of cardiac hypertrophy, HIT was found to have a greater effect with regard to improvement of aerobic capacity and running speed as compared to MIT. Furthermore, only HIT was found to alter cardiac substrate utilization (increased glucose oxidation and decreased FA oxidation) as well as increase cardiac efficiency due to decreased $MVO_{2\ BM}$. HIT also increased cardiac mitochondrial biogenesis and elevated maximal respiratory capacity. Based on these findings we concluded that the metabolic effects of exercise on the heart were intensity-dependent, and high intensity was shown to be necessary for inducing changes in cardiac substrate utilization and energetics.

Paper 4

Although several studies have examined myocardial Ca^{2+} dynamics and functional characteristics in the SERCA2 KO mouse (5; 82; 129), cardiac substrate metabolism and ventricular energetics for this model have not been described. Thus, work-dependent and work-independent myocardial oxygen consumption (MVO_2) as well as substrate metabolism was measured in isolated perfused hearts from SERCA2 KO mice, four weeks after the induction of *Serca2* excision. Although these hearts showed no signs of hypertrophy and normal substrate utilization, they clearly exhibited reduced systolic and diastolic function. Regression analysis of the PVA:MVO_2 relationship revealed that KO hearts displayed reduced contractile efficiency. Moreover, we found that unloaded MVO_2 was reduced in KO hearts due to a 30% reduction in oxygen cost for Ca^{2+} handling ($MVO_{2\ ECC}$).

General discussion

Exposure to variations in metabolic milieu will cause the heart to adapt to accommodate ATP synthesis at whatever cost necessary within its new environment. These adaptations will include changes in myocardial substrate utilization and oxygen consumption (MVO_2), which may result in rapid/acute metabolic changes or create signals for long term adaptation to occur. Although these changes initially may be essential for the heart to maintain optimal function, they may over time in some conditions contribute to development of dysfunction and/or be energetically disadvantageous to the heart. Thus, the line between changes regarded as a metabolic adaptation to those regarded as metabolic maladaptation is not clear. For instance, in type 2 diabetic hearts, altered metabolism, which clearly is essential in the acute adaptation of the heart to diabetes, most likely also contributes over the long term to development of contractile dysfunction and unfavorable cardiac energetics (151). There are also reasons to believe that cardiometabolic changes are essential in the adaptations of the heart to long term exercise and/or altered Ca^{2+} handling.

In this thesis a recurrent theme is to elucidate how cardiac substrate metabolism and/or Ca^{2+} handling influence cardiac energetics under altered physiological and pathophysiological conditions in the heart. A specific focus was given to examining cardiac efficiency, specifically the changes in oxygen cost for processes associated with basal metabolism (BM) and excitation-contraction (E-C) coupling in these hearts.

Measurement of unloaded MVO_2

Assessment of cardiac efficiency by regression analysis of the PVA:MVO_2 relationship is advantageous, as it may point to the underlying mechanisms in the energetically inefficient myocardium. This concept of cardiac efficiency recognizes that cardiac ATP can be destined for either mechanical activity or for non-mechanical processes. While changes in the slope of this relationship indicate changes in contractile efficiency (work-dependent MVO_2), changes in the extrapolated y-intercept indicate an altered work-independent MVO_2, reflecting the oxygen consumption in a heart not performing mechanical work. We have in the present thesis (paper 1) shown that MVO_2 in an isolated, retrogradely perfused mouse heart ($MVO_{2\ unloaded}$) corresponded well with the extrapolated value. By further subjecting these hearts to electrical arrest we could also determine the oxygen cost of BM ($MVO_{2\ BM}$) and from this calculate the oxygen cost for processes associated with E-C coupling ($MVO_{2\ ECC}$). In accordance with the values reported in other species we have found that in mouse hearts the oxygen cost for BM accounts for approximately 22% of the total $MVO_{2\ unloaded}$ (44; 45; 133).

Conditions including fasting, diabetes/obesity, or post-operatively are known to be associated with elevated levels of circulating fatty acid (FA) and/or catecholamines. Previous studies have shown that increased FA supply to hearts both *in situ* (72; 87) and *ex vivo* (50; 56) results in increased MVO_2 and decreased cardiac efficiency. Regression analysis of the PVA:MVO_2 relationship revealed that the FA-induced decrease in cardiac efficiency was due to an increased work-independent oxygen consumption (increase in the extrapolated value of the y-intercept of the regression line) and not to any change in contractile efficiency (49; 56; 72). We confirmed by direct measurement in mechanically unloaded hearts, that high FA exposure increased $MVO_{2\ unloaded}$. Furthermore, the increase in $MVO_{2\ unloaded}$ was due not only to an increase in oxygen cost for BM (paper 1 and 2) but also to increased oxygen cost for E-C coupling (paper 2), which is discussed in the following section. Similarly to the effect of elevated levels of FA, a rise in catecholamine and extracellular Ca^{2+} concentration has been shown to cause a parallel upward shift of the PVA:MVO_2 relationship (73; 133; 148) reflecting increased work-independent MVO_2. In accordance with this, we have found $MVO_{2\ unloaded}$ to be increased following both elevated Ca^{2+} and isoproterenol (paper 1 and 2). Both interventions are known to enhance cycling of Ca^{2+} during E-C coupling (43; 133; 137) and in accordance with this increase $MVO_{2\ ECC}$ (paper 1 and 2). These findings suggest (paper 2) that the isoproterenol-induced myocardial oxygen waste that has previously been observed *in situ* (23; 88; 89) is due indirectly to FA-induced increase in the oxygen cost of BM and E-C coupling, in addition to a direct catecholamine-induced increase in the oxygen cost for E-C coupling.

The cardiometabolic effect of an acute elevation of fatty acid supply to the heart

The underlying mechanisms for the acute FA-induced increase in MVO_2 have yet to be fully deciphered. As elevations in FA supply are normally accompanied by increased rates of myocardial FA oxidation, the increased MVO_2 may be related to increased FA oxidation rates due to the lower ATP to oxygen (P:O) ratio for FA as compared to carbohydrate oxidation (99). However, as the exclusive switch from carbohydrate to FA as the main energy source would only result in an approximate 12% increase in MVO_2, it is clear that the 30-50% increase in MVO_2 that we have observed following elevated FA (paper 1 and 2) cannot be explained solely by differences in the P:O ratio. It is therefore clear that additional mechanisms must also be involved in FA-induced O_2 wasting. This notion is further supported by the finding that stimulation of the FA oxidation rate (by GW610742) to the same extent as that obtained following increased FA supply, did not alter $MVO_{2\ unloaded}$ (paper 2) and likewise, that inhibition of FA oxidation by DCA did not reduce the FA-induced increase in MVO_2 unloaded (paper 2). Thus, it is reasonable to conclude that it is not the increase in FA oxidation rate *per se*, but the presence of a high FA load that leads to FA-induced

oxygen waste. Other mechanisms that may also contribute to oxygen waste will be discussed briefly, including i) mitochondrial uncoupling ii) FA-induced changes in metabolic pathways and iii) FA-induced changes in Ca^{2+} handling.

i) FA-induced mitochondrial uncoupling. Uncoupling proteins 2 and 3 (UCP2, UCP3) are found in the myocardium and are believed to increase proton conductance of the mitochondrial membrane and/or contribute to FA anion transport across the mitochondrion, both potentially leading to mitochondrial uncoupling (19-21). Furthermore, it has been shown that FA or FA derivatives/metabolites can increase production of ROS (37) and that superoxide and lipid peroxidation products can activate uncoupling proteins (34; 36; 123). Himms-Hagen and Harper hypothesized that UCP3 was essential for maintaining high rates of FA oxidation when FA were in over-supply (55). This has since been rejected by the same group who has instead demonstrated the role of UCP3 under conditions where FA is elevated such as during starvation/fasting (123). Under these conditions ROS production increases during catabolism of FA, however, subsequent stimulation of mitochondrial uncoupling can lower the proton-motive force, thus reducing the rate of FA-induced ROS production (34; 36). Although it remains unclear to what extent these processes alter ATP levels (20; 140) increased uncoupling activity will increase the oxygen consumption of the heart and thus potentially contribute to a reduction in cardiac efficiency. An alternative mechanism suggested to contribute to increased MVO_2, is the hypothesis that UCP3 can export FA-peroxide anions out of the mitochondria during elevated FA levels, thus reducing the accumulation of toxic FA oxidation products within the mitochondria (47) although the extent to which this increases uncoupling is unknown (21).

Genipin (Gardenia fruit extract) which has been used in traditional Chinese medicine to treat type 2 diabetes has recently been described as a membrane soluble inhibitor of UCP2 (84; 140; 153). Genipin has not been reported to have been used in isolated perfused hearts therefore we performed pilot experiments where the effect of different concentrations of genipin (100-1000 μm) on $MVO_{2\ unloaded}$ was examined. We were unable to demonstrate any effect on $MVO_{2\ unloaded}$ in hearts perfused with either normal or high FA concentrations, or in *db/db* hearts. The lack of effect seen by genipin is not fully understood although we speculate it is either due to ineffective exposure of the UCP inhibitor within the cells, and/or that the effect of inhibition of UCP2 does not significantly affect MVO_2 under the experimental conditions used. In addition, pilot experiments were performed where the effect of the antioxidant *N-(2-mercaptopropionyl)-glycine* (MPG) on $MVO_{2\ unloaded}$ was examined. The addition of MPG (10 mM) did not alter cardiac function or substrate utilization rates (glucose and palmitate) in normal hearts. We also did not find MPG to alter $MVO_{2\ unloaded}$ in normal

hearts perfused with high fat, nor in *db/db* hearts. The lack of effect may be due to inadequate length of exposure of the anti-oxidant, the possibility that FA-induced ROS production does not increase MVO_2, or the possibility that the changes in O_2 consumption are too small for measurement in the experimental model used in this thesis.

ii) FA–induced changes in metabolic pathways. An excess FA supply to the heart may result in changes of metabolic pathways that have been suggested to be associated with excess ATP utilization. As this will increase MVO_2, they are often referred to as oxygen wasting processes. One such process includes the cycling of FA intermediates from TAG and their subsequent ATP-dependent incorporation back into the triglycerides pool. Under normal aerobic conditions, FA-TAG cycling is a physiologic process that can provide a potential source of energy substrate and, most importantly, reduce the accumulation of FA to dangerous levels within the cytosol (114; 115). However, under conditions of stress for the heart such as ischemia, the consumption of ATP for futile turnover may be detrimental to the energy-challenged cell. Myrmel and Larsen (93) suggested that the cycling of FA and TAG may account for up to 30% of the energy consumption within the cell. This has been proposed to occur under conditions of elevated FA, which due to a superfluous energy expenditure, can contribute to cellular damage (93; 115).

During ischemia, the uncoupling of glycolysis from glucose oxidation has been proposed to result in increased H^+ accumulation (31; 81) and altered cardiac ionic homeostasis which in turn may affect cardiac efficiency in this setting. The proposed cardioprotective effect of DCA during ischemia-reperfusion has been attributed to a reduced H^+ ion accumulation and improved cardiac efficiency (139). In the present thesis we did not find DCA to reduce the FA-induced increase in $MVO_{2\ unloaded}$, which is in line with findings from a recent study (42). This may indicate that although reducing the metabolic uncoupling may improve efficiency following ischemia-reperfusion, FA-induced mechanisms may not be of major importance under aerobic conditions.

iii) FA-induced changes in Ca^{2+} handling. In paper 2 we have shown for the first time that high FA increases the oxygen cost for E-C coupling. Several recent reports have also given reason to believe that high levels of FA will influence Ca^{2+} handling and may therefore alter the oxygen cost of E-C coupling; in cardiomyocytes elevated palmitate has been shown to decrease the amplitude and decay rate of Ca^{2+} transients as well as cellular fractional shortening (37; 51). However, as a decrease in Ca^{2+} transient amplitude should predict a decrease in the oxygen cost for E-C coupling, additional mechanisms must explain the FA-induced increase in $MVO_{2\ ECC}$. Interestingly, a recent

study has linked an elevated expression of UCP2 in cardiomyocytes with altered Ca^{2+} handling; due to a reduced mitochondrial Ca^{2+} uptake these myocytes exhibit a lengthened decay of the Ca^{2+} transient and increased occurrence of Ca^{2+} sparks (140), processes which can lead to increased MVO_2.

The cardiometabolic effect of chronic elevation of fatty acid supply to the heart

Elevated circulating lipids in type 2 diabetes will lead to a chronic exposure to elevated lipid supply to the heart. A hallmark of the diabetic heart is therefore altered cardiac substrate utilization with elevated FA oxidation and a concomitant decrease in glucose utilization (2; 10; 25). During the last years it has become known that diabetic hearts also show reduced cardiac efficiency. Regression analysis of the PVA:MVO_2 relationship has repeatedly shown that this decreased efficiency in hearts from type 2 diabetic *db/db* mice is due to increased oxygen cost for work-independent processes (49; 57). In the present thesis, we have confirmed this finding by direct measurement of $MVO_{2\ unloaded}$ and further demonstrated this increase to be due to an increased O_2 cost for basal metabolism as well as for E-C coupling (paper 1 and 2). Altered Ca^{2+} handling in E-C coupling has been described in models of diabetes (11; 111; 118; 152). Reduced amplitude and longer decay rate of the Ca^{2+} transients as well as reduced SR Ca^{2+} content has been reported in cardiomyocytes (11) as well as in isolated perfused whole hearts (102) from *db/db* mice. Interestingly, Belke *et al.* (2004) (11) have also reported that cardiomyocytes from *db/db* hearts demonstrated Ca^{2+} leak from the sarcoplasmic reticulum (SR), a finding later confirmed by Stølen *et al.* (2009) (130). An increased SR Ca^{2+} leak could increase Ca^{2+} cycling and thus contribute to the increased MVO_2 in unloaded *db/db* hearts (paper 1 and 2). In contrast to that seen in normal mice under elevated FA conditions, Fauconnier *et al.* (2007) have reported that a high palmitate concentration prevented the diabetes-induced decrease in Ca^{2+} amplitude and cell shortening in cardiomyocytes from *ob/ob* mice (37). This further raises the question of whether decreased Ca^{2+} amplitude that has been previously measured in perfusate without FA accurately represents *in situ* Ca^{2+} handling.

Several of the mechanisms proposed to contribute to the reduced cardiac efficiency in diabetic hearts, include those previously discussed in association with the acute FA-induced oxygen waste. Again, despite elevated FA oxidation that has been shown in *db/db* hearts the lower P:O ratio for FA oxidation cannot fully explain the increased MVO_2 in these hearts. Elevated ROS production and mitochondrial uncoupling have been suggested to play an important role in reduced cardiac efficiency (18). Although there is inconsistency regarding the gene and protein expression of UCP2 and 3 in hearts from type 2 diabetic models (17; 27; 92) it is important to point out that mRNA expression and/or protein levels do not necessarily predict functional levels of uncoupling proteins

(21). Murray *et al*. (2004) have reported FA levels to be correlated with increased expression of UCP2 and UCP3 in the heart (91) whereas Boudina *et al.* (2007) have reported increased mitochondrial uncoupling in *db/db* hearts independent of changes in UCP expression levels (18). As increased ROS generation is suggested to affect cardiomyocyte function in type 2 diabetes (150) and is moreover associated with high FA supply (37) there is reason to believe that ROS-induced mitochondrial uncoupling may play an important role in the increased MVO_2 in the diabetic heart.

An important finding in the present thesis is that the acute elevation of FA concentration did not increase MVO_2 in mechanically unloaded *db/db* hearts as compared to non-diabetic controls (paper 2), which thereby confirmed previous data obtained in working *db/db* hearts by regression analysis of the work-MVO_2 relationship (57). Fauconnier *et al*. (2007) found that while elevated FA increases ROS production and decreased mitochondrial membrane potential in non-diabetic cardiomyocytes, this was not the case in myocytes from *ob/ob* hearts (37). This may indicate that prolonged exposure to high FA levels leads to adaptation of the myocyte so that FA becomes the preferred substrate and thus cellular Ca^{2+} handling and contraction actually improve following FA exposure. Furthermore, we have observed that although FA-induced increase in MVO_2 was not found to be associated with changes in FA oxidation rates in normal hearts, a switch in myocardial metabolism towards glucose oxidation using DCA (Hafstad, unpublished data) or following the addition of high glucose and insulin (Paper 1) (49) reduces MVO_2 in *db/db* hearts without any detectable effects in normal hearts. Thus, reducing the rate of FA oxidation improved cardiac efficiency in these hearts as compared to non-diabetic controls.

The cardiometabolic effects of exercise

A low aerobic capacity is considered to be an important predictor for the development of cardiovascular disease (69). Although exercise has been reported to be cardioprotective with respect to post-ischemic functional recovery, the effect of prolonged exercise on cardiac efficiency is not clear. Previous reports on the metabolic effects of exercise have been few and inconsistent, which may be related to different aspects of the exercise training used such as variations in mode, duration and intensity. In accordance with this, we found that the cardio-metabolic effects of exercise are demonstrated to be intensity dependent (paper 3) as only high intensity training (HIT) led to an increase in cardiac glucose utilization and a reduction in unloaded MVO_2. The reduction in unloaded MVO_2 was due to a reduced O_2 cost of basal metabolism and not for E-C coupling. Although the mechanisms behind the decreased MVO_2 remain unclear, it cannot be explained singularly by altered substrate utilization (decreased FA oxidation and increased glucose oxidation) and the higher P:O ratio of glucose. Thus we suggest that there are other potential oxygen-sparing

mechanisms that are induced by HIT. Evidence of increased myocardial antioxidant capacity seen by increased mRNA expression of manganese superoxide dismutase and catalase suggests that exercise training reduced ROS generation due to an increased anti-oxidant expression (106), and/or that the presence of anti-oxidants may contribute to reduced UCP uncoupling activity (14) and thereby reduce energy expenditure for this. Although we did not find indications of altered mitochondrial uncoupling in skinned cardiac fibers receiving malate and glutamate as oxidative substrates, we did find HIT to result in increased mitochondrial capacity (Vmax).

Previous reports have demonstrated HIT-induced increases in cardiomyocyte cell shortening, accompanied by an unchanged or reduced Ca^{2+} amplitude and increased myofilament Ca^{2+} sensitivity (64; 65; 147). Based on this, it would have been expected that HIT would reduce energy cost for E-C coupling and/or increase contractile efficiency in isolated hearts. In contrast, our findings in paper 3 have demonstrated an unaltered O_2 cost for E-C coupling. However, altered contractile function in isolated cardiomyocytes may not necessarily be identical to that seen in the whole heart due to differences in the extracellular milieu (substrate availability) as well as differences in contractility of isolated cells versus those that are attached and developing tension in the whole heart. Interestingly, both types of training resulted in exercise-induced hypertrophy however only HIT induced the concurrent shift towards the α-myosin heavy chain (MHC) isoform. It is possible that this energetically more expensive isoform and/or the hypertrophy itself (90) counteracts the anticipated reduction in $MVO_{2\ ECC}$. Pathological hypertrophy is associated with the reactivation of the fetal gene programme (110; 127) however less is known about physiological hypertrophy. In paper 3, HIT induced many of the same transcriptional changes seen in hearts subjected to increased load and hypoxia (increased hypoxia inducible factor 1-α target genes such as *pdk4*, *ldh*, *vegf* and *hk*, and reduced expression of *ppar-α*) (70; 74; 109) suggesting that continuous high workloads can lead to episodes of reduced O_2 tension in the cardiac tissue and can thereby activate certain pathways also associated with pathological hypertrophy. Thus it is possible that pathways commonly associated with a pathological stimulus indeed may lead to very beneficial adaptations in the heart that enables the heart to perform better during stressful conditions.

The cardiometabolic effect of SERCA2 reduction

Pathological conditions causing depressed myocardial contractility are often associated with reduced myocardial SERCA2 expression, altered myocardial metabolism, and reduced energetic state (11; 59; 75; 143). It is well known that a reduction in the capacity of SERCA2 (30; 122) or absence (5), leads to reduced force generation, delayed relaxation and eventually heart failure. In paper 4, myocardial substrate utilization and cardiac efficiency were examined in isolated, perfused hearts from SERCA2 KO mice, where SERCA2 expression was less than 5% of that seen in controls (SERCA FF). Assessment of cardiac function, MVO_2 and efficiency clearly revealed that SERCA2 excision not only reduced work capacity but also altered MVO_2 and cardiac efficiency. *In vivo* examination of these mice in previous studies with the same myocardial SERCA2 levels has shown that *in vivo* cardiac function is surprisingly well preserved despite the reduced Ca^{2+} transient amplitude and decay rate in found in isolated cardiomyocytes (5; 82) possibly due to enhanced sympathetic stimulation and/or Ca^{2+} sensitivity (5). In the present study, however, *ex vivo* assessment of cardiac function in the absence of neuro-hormonal interference showed that hearts from KO mice exhibited a clear reduction in work capacity as well as signs of diastolic dysfunction. Interestingly, extracellular Ca^{2+} had to be increased (5 mM) in order to make the KO hearts produce sufficient external work to be included in experiments. Moreover, we observed increased expression of *Mhc-β* as compared to FF hearts, a sign that fetal gene activation was initiated and a hallmark of heart failure (80; 110; 127).

In addition to altered Ca^{2+} transients that have previously been shown in cardiomyocytes from SERCA2 KO mice, they also exhibit an increased dependence on other mechanisms to maintain Ca^{2+} homeostasis, such as enhanced presence of plasma membrane Ca^{2+}-ATPase (PMCA) and NCX in the sarcolemma (5; 82; 129). Although the energy expenditure for Ca^{2+} uptake by SERCA2 corresponds to approximately 70% of MVO_2 for E-C coupling, it still remains more energetically efficient as compared to other mechanisms of Ca^{2+} handling (136). This notion is further supported by Sakata *et al.* (2007) (116) who have reported an improved O_2 cost of left ventricular contractility following adeno-viral overexpression of SERCA2 and Shimizu *et al.* (2009) (124) who have reported an increased O_2 cost for Ca^{2+} handling in E-C coupling in hypertrophic hearts where SERCA2 was reduced. With this in mind, our findings of reduced $MVO_{2\ unloaded}$ associated with a decreased oxygen cost for E-C coupling in SERCA2 KO hearts was therefore surprising. This suggests that despite the possibility of a greater energetic cost for Ca^{2+} homeostasis by other ion pumps in the absence of SERCA2, it would seem that the reduction of transient amplitude (60; 78; 127) results in overall less energy expenditure for Ca^{2+} handling during E-C coupling.

An important finding in paper 4 was an increased work-dependent MVO_2 (increased slope of the PVA:MVO_2 relationship), representing a reduced contractile efficiency in the KO heart. Thus KO hearts must exhibit decreased efficiency of mitochondrial oxidative phosphorylation and/or the conversion of ATP to cardiac work as seen in cross-bridge cycling (132).

Despite that fact that heart failure is generally associated with increased glucose oxidation and decrease in fatty acid oxidation (60; 78; 127), the reduced cardiac function in KO hearts was not associated with altered myocardial substrate metabolism. As it is well known that decreased energetic state is a strong predictor for heart failure (95) the cardiac dysfunction following SERCA KO may very well be associated by a decreased energetic state induced by the decreased cardiac efficiency.

Concluding remarks

One of the overall objectives of this work was to establish a reproducible and direct method for measurement of the unloaded MVO_2, an important component of cardiac efficiency. Furthermore, the objective was to measure the individual contributions of work-independent processes associated with unloaded MVO_2 such as BM and E-C coupling, which have been seen to vary under different physiological and pathophysiological conditions. The implications of a reduced cardiac efficiency are paramount under conditions of limited O_2 availability such as myocardial ischemia, where altered cardiac energetics can have crucial consequences.

The direct measurement of unloaded MVO_2 in retrogradely perfused hearts was validated by acutely altering the heart perfusate (*e.g.* inotropic stimulation, FA concentrations, and pharmacological interventions influencing cardiac substrate utilization). This approach also allowed us to obtain mechanistic information, *e.g.* by evaluating the consequences of FA load *vs.* FA oxidation on unloaded MVO_2. Application of this method was further used to assess cardiac efficiency in a variety of mouse models such as i) a monogenic model of obesity and type 2 diabetes (*db/db*), ii) moderate- and high intensity trained mice and finally iii) a genetically engineered mouse model of heart failure (SERCA KO).

A main finding in this thesis is that in contrast to that seen in normal hearts, the chronic exposure to high circulating levels of FA in the hearts of *db/db* mice seems to cause adaptation such that the heart exhibits an altered tolerance to acute elevation of FA. Moreover, work from our research group (49; 50) and this thesis have shown that the inhibition of FA oxidation by metabolic intervention (administration of glucose plus insulin) can reduce unloaded MVO_2 in diabetic hearts. Thus, despite the lack of direct evidence for the mechanism(s) for this observation yet, we speculate that the underlying mechanisms behind the increased MVO_2 following acute and chronic FA load are different and that this issue clearly needs more detailed examination.

Other notable findings include the significance of intensity and duration of exercise performed in the determination of the cardiometabolic effects of exercise, including substrate metabolism, hypertrophy, and myocardial gene expression. Furthermore, measurement of cardiac efficiency in the *ex vivo* SERCA2 KO heart resulted in findings different from those expected based on previous *in vivo* studies showing that early contractile dysfunction can, in some cases, be detected earlier in the *ex vivo* perfusion setting. In summary, the evaluation of cardiac efficiency in combination with the direct measurement of unloaded MVO_2 and O_2 cost of E-C coupling and BM in the heart is a tool that allows for the evaluation of O_2 wasting processes that occur in the heart. An understanding

of these processes can be useful in the development of therapeutic strategies to offer myocardial protection following an ischemic insult.

Future investigations

The inability to identify the precise mechanistic basis for altered unloaded MVO_2 under various physiological and pathophysiological conditions, particularly those leading to O_2 wasting (*i.e.* through evidence of an enhanced generation of reactive oxygen species or uncoupling in the mitochondria) represents a limitation in this thesis. These underlying mechanisms need more detailed examination and should be addressed in future work. For example, the effects of FA load on mitochondrial respiration and the potential corresponding changes in mitochondrial uncoupling and ROS production would provide more mechanistic information. In addition, direct measurement of unloaded MVO_2 in isolated heart perfusions could also be further examined in the uncoupling protein (UCP) KO mouse heart perfused with normal and high FA levels and may further illustrate O_2 wasteful processes. To date, there have been no reports of MVO_2 measured in UCP KO mice however as there is evidence for increased mitochondrial stress and reduced tolerance to elevated FA load in these mice (123) there is also reason to believe that unloaded MVO_2 is altered. As recent studies have implicated adenine nucleotide translocator (ANT) as another mediator of FA-induced mitochondrial uncoupling in the heart (15; 34) examination of the effects of an inhibitor of ANT (carboxyatractyloside, CAT) could be a useful tool in the evaluation of O_2 wasteful processes. Other tools such as the quantification of FA breakdown products such as ceramide and diacylglycerol that are believed to contribute to enhanced ROS generation could also shed light on O_2 wasting in hearts exposed to high FA. Finally, it would be further beneficial to investigate FA-TAG cycling under various physiological and pathophysiological conditions such as acute elevation of FA and in *db/db* hearts, as it is repeatedly referred to as a potential contributor to reduced cardiac efficiency, and yet only few studies have measured this in the heart. In conclusion, future investigations of O_2 wasteful processes in the heart should include a focus on the relationship between enhanced ROS generation and mitochondrial uncoupling, measurement of futile cycling (FA-TAG) and evaluation of mitochondrial function under altered physiological and pathophysiological conditions.

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Paper 1

Paper 2

Paper 3

Paper 4

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