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Myocardial Physiology in Individuals Undergoing Heart Surgery

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
Faculty of Medicine
Department of Circulation and Medical Imaging

NTNU – Trondheim
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Fysiologi i hjertevev hos pasienter som gjennomgår åpen hjertekirurgi

Mitokondrier er uunnværlige organeller i alle kroppens celler. De produserer det meste av energien hjertet trenger for å kunne pumpe oksygenrikt blod rundt i kroppen. Forskning tyder på at mitokondriene er involvert i å formidle en beskyttelseseffekt på hjertet ved en teknikk som kalles fjern iskemisk prekondisjonering (remote ischemic preconditioning, RIPC). RIPC innebærer at man utsetter en del av kroppen for korte perioder med oksygenmangel (iskemi), for å utløse en beskyttelseseffekt på organer i forkant av mer langvarig iskemi. Forskning tyder på at RIPC i form av gjentatt kortvarig iskemi i for eksempel en arm, ser ut til å kunne beskytte hjertet mot skade ved lengre iskemiske perioder. Det er flere aspekter ved RIPC som enda ikke er helt kartlagt til tross for nesten 30 år med forskning på feltet. Det er også antydet at mitokondriene er involvert i underliggende sykdomsprosesser ved atrieflimmer (AF). AF er den vanligste hjerterytmeforstyrrelsen av klinisk betydning, og en grundig forståelse av underliggende mekanismer er viktig for å kunne forbedre forebyggende og behandlingsmessige tiltak. MikroRNA er en gruppe små molekyler som regulerer proteinuttrykk ved å hemme og bryte ned mRNA. Studier tyder på at mikroRNA påvirker iskemi-reperfusjonsskade i hjertet og er involvert i AF. Ettersom mikroRNA ble oppdaget for relativt kort tid siden, er det enda mange ubesvarte spørsmål rundt deres funksjoner. Hovedformålene med denne avhandlingen er å undersøke fysiologi i hjertevevet i sammenheng med RIPC og AF ved hjertekirurgi. Biopsier fra atrie og ventrikkel ble innhentet etter samtykke fra pasienter i forbindelse med åpen hjertekirurgi ved Klinikk for thoraxkirurgi, St. Olavs hospital. Mitokondriefunksjon ble undersøkt i hjertevevet etter åpning av cellemembranene. MikroRNA-ekspresjon ble undersøkt med array- og PCR-teknologi. Undersøkelsene tyder på at RIPC bidrar til å bevare mitokondriefunksjonen ved hjertekirurgi, og påvirker mikroRNA-ekspresjon. Mitokondrienes respirasjonskapasitet var høyere i atrievev fra pasienter med AF, sammenlignet med pasienter med sinusrytme. MikroRNA-ekspresjon var forskjellig mellom høyre og venstre atrie, både blant pasienter med sinusrytme og pasienter med AF, mens mitokondriefunksjon var lik i de to atriene i begge pasientgrupper. Hovedkonklusjonen for avhandlingen er at mitokondriefunksjon og mikroRNA ser ut til å være involvert i underliggende prosesser ved AF, samt ved RIPC, og bør undersøkes videre for å kunne utnytte kunnskapen i behandling av pasienter.

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List of papers

The studies in this thesis were carried out at the Department of Circulation and Medical Imaging, Faculty of Medicine, Norwegian University of Science and Technology and the Department of Cardiothoracic Surgery, St. Olavs Hospital, Trondheim University Hospital in the period 2009-2013.

Paper I

Remote Ischemic Preconditioning Preserves Mitochondrial Function and Influences Myocardial MicroRNA Expression in Atrial Myocardium During Coronary Bypass Surgery. Slagsvold KH, Rognmo Ø, Høydal MA, Wisløff U, Wahba A. *Circ Res.* 2014 Feb 28; 114(5):851-859.

Paper II

Remote ischemic preconditioning preserves mitochondrial function and activates pro-survival protein kinase Akt in the left ventricle during cardiac surgery: A randomized trial. Slagsvold KH, Moreira JBN, Rognmo Ø, Høydal MA, Bye A, Wisløff U, Wahba A. In Press: *Int J Cardiol.* Published Online 2014 Oct 14; DOI 10.1016/j.ijcard.2014.09.206

Paper III

Mitochondrial respiration and microRNA expression in right and left atrium of patients with atrial fibrillation. Slagsvold KH, Johnsen AB, Rognmo Ø, Høydal M, Wisløff U, Wahba A. *Physiol Genomics.* 2014 July 15; 46(14):505-511.

Paper IV

Comparison of left versus right atrial myocardium in patients with sinus rhythm or atrial fibrillation – an assessment of mitochondrial function and microRNA expression. Slagsvold KH, Johnsen AB, Rognmo Ø, Høydal M, Wisløff U, Wahba A. *Physiol Rep.* 2014 Aug 28; 2 (8); DOI: 10.14814/phy2.12124

Abbreviations

ACC	Aortic cross-clamping
ACR	Acceptor control ratio
ADP	Adenosine diphosphate
AF	Atrial fibrillation
Akt	Protein kinase Akt (synonymous to protein kinase B)
ANT	Adenine nucleotide translocator
${}^{app}K_m^{(ADP-Cr)}$	Apparent constant of Michaelis for ADP in the absence of creatine
${}^{app}K_m^{(ADP+Cr)}$	Apparent constant of Michaelis for ADP in the presence of creatine
ATP	Adenosine triphosphate
AUC	Area under the curve
AVR	Aortic valve replacement
BSA	Bovine serum albumin
CABG	Coronary artery bypass graft surgery
cDNA	Complementary DNA
CK	Creatine kinase
CK-MB	Creatine kinase-MB
Cp	Crossing point
CPB	Cardiopulmonary bypass
cTnT	Cardiac troponin T
ECG	Electrocardiogram
FAD^+	Flavin adenine dinucleotide
FEP	Fluorinated ethylene propylene
GSK3ß	Glycogen synthase kinase-3ß
H^+	Proton
HIF-1α	Hypoxia-inducible factor 1-alpha
IPC	Ischemic preconditioning
K_{ATP}-channel	ATP-sensitive potassium channel
LA	Left atrium
LNA	Locked nucleic acids
MCU	Mitochondrial calcium uniporter

miRNA	MicroRNA
mitoK_{ATP}-channel	Mitochondrial ATP-sensitive potassium channel
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA
MtCK	Mitochondrial creatine kinase
mtDNA	Mitochondrial DNA
MVR	Mitral valve replacement
NAD^+	Nicotinamide adenine dinucleotide
NCX	Na^+/Ca^{2+}-exchanger
NHE	Na^+/H^+-exchanger
NT-pro-BNP	N-terminal pro-brain natriuretic peptide
P	Inorganic phosphate
PCr	Phosphocreatine
PCR	Polymerase chain reaction
PDK	Phosphoinositide-dependent kinase
PI3K	Phosphoinositide 3-kinase
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein kinase B (synonymous to Akt)
POAF	Postoperative atrial fibrillation
post ACC	After aortic cross-clamping
pre ACC	Before aortic cross-clamping
pre-miRNA	MicroRNA precursor
pri-miRNA	Primary microRNA
qPCR	real-time PCR
RA	Right atrium
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay buffer
RIPC	Remote ischemic preconditioning
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RT-qPCR	Reverse transcription real-time polymerase chain reaction
RyR	Ryanodine receptor
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
VDAC	Voltage gated anion channel

Summary

Mitochondria are essential organelles in all eukaryote cells. They produce the vast majority of all the energy the heart needs to continuously pump oxygenated blood throughout the body. The myocardium is entirely dependent on well-functioning mitochondria. There are indications that altered mitochondrial function is implicated in mediation of cardioprotection from remote ischemic preconditioning (RIPC), as well as in atrial fibrillation (AF). RIPC involves exposing a part of the body to repeated brief periods of ischemia to evoke a protective effect in another organ such as the heart. Although ischemic preconditioning has been a focus of research for nearly three decades, there are still several aspects that remain unexplained. AF is the most prevalent cardiac arrhythmia of clinical relevance, and a thorough understanding of the underlying pathophysiology is important to improve therapeutic and preventive strategies. MicroRNA (miRNA) are a group of small molecules that regulate protein expression at a post-transcriptional level through inhibition and degradation of mRNA. There is emerging evidence that miRNA may have a role in cardiac ischemia-reperfusion injury, as well as in AF. However, due to their relatively recent discovery, there are still many unanswered questions. The main aims of this thesis are to investigate human myocardial physiology during cardiac surgery in association with RIPC and AF. Atrial and ventricular biopsies were obtained upon consent from patients undergoing open heart surgery at Clinic of cardiothoracic surgery, St. Olav's hospital. Mitochondrial function was assessed in situ after tissue permeabilization by saponin. MiRNA expression was investigated through array and PCR technology. We found that RIPC preserves myocardial mitochondrial respiration in heart surgery, and affects microRNA expression. Mitochondrial respiratory capacity was elevated in patients with AF as compared to patients with normal sinus rhythm. The expression of microRNA differed between the right vs. left atrium of patients with sinus rhythm and AF, whereas mitochondrial function was similar in the right and left atrium of both groups. The main conclusion of our study is that there are indications that mitochondrial function and microRNA are involved in AF pathophysiology, as well as in RIPC, and should be investigated further for future clinical application.

1 Introduction

The original research question that initiated the work behind this thesis was how mitochondrial function is affected in atrial myocardium of patients with atrial fibrillation (AF). The investigation into mitochondrial function in patients with AF involved assessment of myocardial biopsies harvested early in cardiac surgery, and the question of how cardiac surgery itself may affect mitochondrial function came up. Moreover, the puzzling phenomenon of remote ischemic preconditioning (RIPC) as a potential cardioprotective means in heart surgery presented itself as an intriguing, as well as a largely unanswered area of medical research. These interests were integrated, and the ambition to investigate whether mitochondria are affected by, or perhaps effectuates, potential alterations induced by RIPC became a natural focus of the subsequent research project.

Mitochondrial function in the human myocardium is a recurrent theme of this thesis. Because mitochondria are involved in several central aspects of cell function, this thesis also aims to elucidate related topics to provide an outline of the bigger picture in which myocardial mitochondria play their role. With this in mind, this thesis will first aim to discuss cardiac bioenergetics and mitochondria, followed by an exploration into the topics of AF, cardiac ischemia-reperfusion injury, prosurvival kinase Akt, microRNA, and finally, the phenomenon of RIPC, to provide a background for further discussion on the studies included in this dissertation.

1.1 Cardiac bioenergetics and mitochondrial function

Mitochondria constitute the main energy factories of all eukaryotic cells. Each mitochondrion has two membranes where the inner membrane surrounds the matrix, and the outer membrane encircles the intermembrane space.[1] Although several types of mitochondria can produce energy without oxygen, all mitochondria of mammalian cells require a continuous supply of oxygen for energy production through the process of oxidative phosphorylation.[2] Oxidative phosphorylation is the reason why we need to breathe air to survive. Production of adenosine triphosphate (ATP) from glucose is 15 times greater under aerobic conditions with oxidative phosphorylation, as compared to anaerobic glycolysis.[3] The myocardium is a highly energy demanding tissue, and

because 90% of the ATP needed to keep up with this demand is obtained from oxidative phosphorylation, optimal mitochondrial function is imperative.[4] In addition to its pivotal role in energy production, mitochondria are essential in the maintenance of homeostatic electrolyte levels, pH, reactive oxygen species (ROS),[5, 6 7] and ultimately play key roles in regulation of cell survival and cell death.[8] Due to their central tasks in cellular function, proper mitochondrial function is a prerequisite for normal cell function.[1] Consequently, it is not surprising that intricate involvement of mitochondria is suspected in the pathophysiology of AF[9] and ischemia-reperfusion injury,[10] as well as in cardioprotection from ischemic preconditioning,[6] areas that are investigated in the work presented here.

Although mitochondria are responsible for the majority of cardiac energy production, they are not the sole components in cardiac metabolism. To elucidate the context of mitochondria as part of the larger system that fuels the heart, cardiac bioenergetics will be discussed in the following section, prior to an elaboration upon mitochondrial function.

1.1.1 Myocardial bioenergetics

Cardiac energy consumption has been estimated to an average of 30 kg ATP per day, 300 mg ATP per heart beat and approximately 1 mM ATP per second.[4, 11] The heart has a very limited storage capacity for ATP, which entails a continuous requirement for complete renewal of the entire pool of ATP and phosphocreatine (PCr) every 20 seconds to meet cardiac energy demand.[4, 11] Because the vast majority of cardiac ATP is produced through oxidative phosphorylation in the mitochondria, there is a very close correlation between myocardial oxygen consumption and workload of the heart.[4] Although a variety of substrates may serve as fuel for cardiac energy consumption, including carbohydrates, ketone bodies, lactate and amino acid, fatty acids are the fuel of choice in a healthy heart.[4, 12] The heart has the ability to adapt to different metabolic sources according to different pathological and physiological situations. For instance, it has been demonstrated that physical exercise increases cardiac capacity to oxidize fatty acids through oxidative phosphorylation, whereas hypertension decreases the capacity of fatty acid oxidation.[13] Mitochondrial ATP production is pushed towards 90% of

maximum mitochondrial respiratory rate when the mammalian heart is strained to near maximum workloads, indicating that there is a limited capacity for ATP-production that only marginally meets maximal energy consumption.[14] It has been indicated that the production of NADH through intermediary metabolism constitutes the limiting factor in this process.[14] In 1962, Messer et al. found that an average of approximately 70% oxygen was extracted in the myocardium at rest from both patients with coronary insufficiency and control patients.[15] Whereas the level of oxygen extraction remained unaltered during exercise in control patients, myocardial oxygen extraction increased significantly in patients with clinical coronary disease.[15] These results, along with later studies of myocardial oxygen saturation, suggest that oxygen supply does not impose a limiting factor of oxidative phosphorylation during increased cardiac workloads in healthy hearts.[15, 16] Studies in dogs demonstrated that myocardial oxygen extraction could not be increased above 84%, and straining the heart beyond this point of maximal myocardial oxygen extraction lead to contractile dysfunction and cardiac arrhythmias.[17] While oxidation of fatty acids yields a much higher amount of ATP as compared to oxidation of carbohydrates, it involves a larger amount of oxygen consumption.[12] Still, it appears that the heart retains a preference for fatty acids over carbohydrates to fuel the residual capacity of oxidative phosphorylation both during ischemia and upon reperfusion.[18, 19]

The Frank-Starling law of the heart describes how cardiac output is determined by the amount of blood that enters the heart, or stated differently, that cardiac stroke volume increases in response to increased end-diastolic ventricular volume.[20, 21] The metabolic foundation of the Frank-Starling law was published by Starling and Visscher in 1927, and states that any increase in cardiac workload is answered with a corresponding increase in cardiac oxygen consumption.[22] Whereas the rate of oxygen consumption increases substantially with cardiac workload, intracellular concentrations of ATP, adenosine diphosphate (ADP) and PCr are maintained at a stable level.[23] The linear relationship between cardiac workload and oxygen consumption without detectable effects on ATP and PCr, implies that myocardial oxygen consumption represents an appropriate indicator for cardiac energy consumption.[24]

1.1.2 *The creatine kinase-phosphocreatine energy transfer system*

The large intracellular myocardial pool of PCr is administered by the various isoenzymes of creatine kinases (CK).[24] The PCr-CK system functions as an energy buffer, and has an important role in distributing energy reserves where they are needed throughout the cell.

CK is responsible for a reversible reaction where ATP and creatine is converted to PCr and ADP, or conversely; where ATP and creatine are generated from PCr and ADP. CK enables intracellular compartmentalization of energy by releasing ATP in energy-consuming sites, and storing energy as PCr in energy-production sites.[24] Mitochondrial creatine kinase (MtCK) is present in the intermembrane space of the mitochondria where it utilizes ATP produced in the mitochondrial matrix, and creatine imported from the cytosol, to produce PCr. PCr is subsequently transported out into the cytosol where CK will make the energy available for ATPases.[24] Since ATP is produced inside the mitochondrial matrix, ATP must be transported across the inner mitochondrial membrane by adenine nucleotide translocator (ANT), meanwhile ADP is translocated into the matrix during the same process. The reduced permeability of the voltage gated anion channel (VDAC) located in the outer mitochondrial membrane regulates the flux of metabolites out of the mitochondria, thus ensuring separation of energy pools between the mitochondria and cytosol.[24-26] Compartmentalization of energy, where the CK-PCr system is a major contributor, allows tight local regulation of energy substrates in specific parts of the cell. Local availability of ADP for transport by ANT is an important factor in the regulation of oxidative phosphorylation, whereas elevated ADP concentrations near ATPases reduce the velocity of the contraction cycle, illustrating the importance of detailed control of concentrations of energy substrates.[24]

Coordinating excitation-contraction coupling with energy supply is paramount for optimal cardiac function.[24] The ATP-sensitive potassium channel (K_{ATP} channel) in the plasma membrane of the cardiomyocyte is key in coordinating cellular metabolism with excitation-contraction coupling by functioning as a metabolic sensor, responsive to the balance of local concentrations of ATP and ADP.[27] During homeostatic conditions when ATP-supply is sufficient the K_{ATP} channel will remain closed, whereas a dwindling energy supply will induce K_{ATP} channel opening, causing K^+ to leave the

cell.[27] The K^+ efflux shortens the action potential, which in turn reduces Ca^{2+} overload through a decrease in Ca^{2+} influx, while reducing ATP consumption.[27] This is closely linked to CK-PCr system as CK ensures rapid removal of ADP during normal conditions along with sufficient ATP concentration, which inhibits K_{ATP} opening, whereas during energy deprivation a reduced rate of ADP-recycling by CK will lead to accumulation of ADP and consequent opening of the K_{ATP} channel.[27] Figure 2 illustrates the localizations of the main ion channels discussed in throughout the text.

1.1.3 Mitochondrial genetics, biogenesis and dynamics

Mitochondria are thought to descend from bacteria, and they contain their own protein synthesis machinery and their own genome (mitochondrial DNA, mtDNA) that is separate from the nuclear genome of the cell.[28, 29] Whereas some of the genes encoding proteins of the mitochondrial respiratory chain remain in the mtDNA, the majority of the genes are located in the cell nucleus and proteins are transferred to the mitochondrion after translation.[29] Each human mitochondrion contains 2-10 copies of the double stranded circular mtDNA molecule, and every single human cell accommodates approximately one thousand mitochondria.[30] MtDNA is nearly exclusively maternally inherited.[31] The lack of a well-functioning repair system, along with close vicinity to the ROS- producing electron transport chain, renders mtDNA susceptible to injury, resulting in a 10 times higher mutational rate as compared to nuclear DNA.[30] Mitochondrial gene expression is mainly controlled by the number of mtDNA copies present, and there are indications that mutations will only lead to impaired function when the portion of mutated mtDNA molecules surpass a certain ratio of the mtDNA copies in a cell.[32] MtDNA mutations have been linked to several subgroups of cardiovascular disease.[30] Mitochondria are not produced de novo; they continually go through cycles of fusion and fission. Fusion allows dilution of damaged enzymes and mtDNA, whereas fission involves asymmetric division of the contents in a way that the majority of the injured content is concentrated in one of the two resulting mitochondria. The newly produced organelle that contains the majority of the undesired material is isolated and disabled from entering into a new fusion cycle; and subsequently removed by autophagy (mitophagy).[3, 29, 33] There has been controversy

regarding the existence of mitochondrial fusion/fission cycles in mature cardiomyocytes, but a recent study demonstrated that this process is essential to the maintenance of both morphological and functional integrity of adult cardiac cells, as illustrated by compromised mitochondrial respiration after disruption of the mitochondrial fusion process in adult mouse hearts.[33] Morphological examinations of myocardium from different species indicate that oxygen consumption correlates closely to the amount of cellular volume occupied by mitochondria, and although human heart cells have a lower mitochondrial content than smaller animals, more than 25% of the volume of a human cardiomyocyte is made up of mitochondria.[34]

1.1.4 Oxidative phosphorylation

An understanding of mitochondrial ATP production as the main source of cellular energy was established during the second half of the twentieth century.[3] The fundamental premise for energy production in all aerobic organisms is based on utilizing the energy obtained from the reaction $2H_2 + O_2 \rightarrow 2H_2O$ as the driving force for production of ATP from ADP and phosphate.[3] The first descriptions of the basic principles of cellular energy production were published in the 1960s by Peter Mitchell, and in 1978 Mitchell was awarded the Nobel Prize for Chemistry for this work, commonly referred to as the Chemiosmotic Hypothesis.[35-37] The Chemiosmotic Hypothesis was subject to substantial controversy for over a decade before it achieved general acceptance.[3, 38] This constitutes the first descriptive account of the electron transport chain, also referred to as the respiratory chain, that is located in the inner mitochondrial membrane of eukaryotic cells. The respiratory chain (figure 1) involves transfer of electrons between protein complexes in a process where oxygen is consumed through its function as the final electron acceptor. Energy is temporarily stored as an electrochemical proton gradient across the inner mitochondrial membrane and subsequently either released in the process of synthesizing ATP, or dissipated as thermal energy through passive proton leakage or through uncoupling proteins.[3]

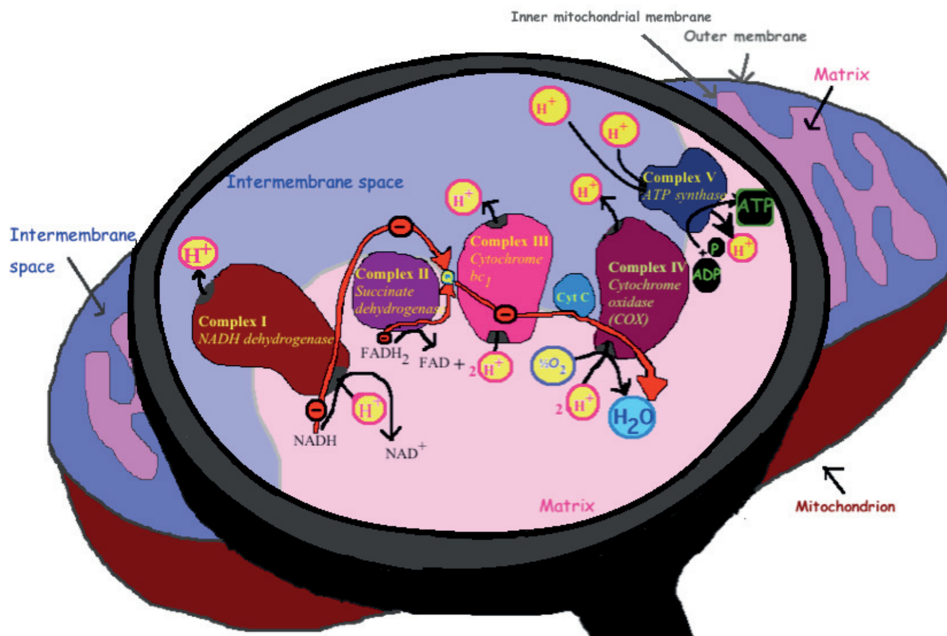

Figure 1. Schematic illustration of the electron transport chain located on the inner membrane of the mitochondria. ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cyt C, cytochrome c; FAD+, flavin adenine dinucleotide; H+, proton; NAD+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide + hydrogen; P, inorganic phosphate.

Electrons are supplied to the respiratory chain through the processes of tricarboxylic acid cycle and ß-oxidation of fatty acids.[3] The primary function of Complex I, III and IV is to utilize energy obtained from electrons passing through redox reactions to pump protons from the mitochondrial matrix out into the intermembrane space, hereby creating the proton gradient that fuels ATP synthesis at Complex V.

Complex I (*NADH dehydrogenase*) is the largest enzyme of the respiratory chain, and transfers electrons to Complex III (*cytochrome bc_1*) via ubiquinone (also known as coenzyme Q_{10}).[39] Complex II (*succinate dehydrogenase*) is the only complex that constitutes both a part of the electron transport chain, as well as part of the tricarboxylic acid cycle.[39] Complex II does not pump protons across the inner mitochondrial membrane, but supplies electrons to the respiratory chain by translocating electrons from succinate to Complex III via ubiquinone, while succinate is oxidated to fumarate in the process.[3, 39] Complex III transfers electrons to Complex IV (*cytochrome oxidase, COX*) via the electron carrier cytochrome c, a reaction that is coupled to the

active pumping of protons across the inner mitochondrial membrane.[39] Complex IV subsequently donates the electrons to O_2 to form H_2O in a process also coupled to pumping H^+ into the intermembrane space.[3] Complex V (*ATP synthase)* uses the energy potential of the proton gradient across the inner mitochondrial membrane to create ATP from phosphate and ADP.[39] In addition to fueling ATP synthesis, the proton gradient is also the driving force that enables sequestering of calcium inside the mitochondria, heat production and transport of protein across the inner mitochondrial membrane.[3]

1.1.5 Mitochondria and generation of reactive oxygen species

Through the process of oxidative ATP-production, mitochondria are also a main source of ROS. This is due to some electrons diverting from their designated path through the respiratory chain, causing interaction between molecular oxygen (O_2) and a single electron, which in turn reduces O_2 into superoxide (O^-_2), a highly reactive ROS-precursor.[40] ROS production leads to molecular damage, some of which is not repaired and consequently may contribute to aging and disease.[41] In addition, it has recently been proposed that ROS have important regulatory roles in many physiological processes through cellular signaling.[40, 42] While significant amounts of ROS may be released as a byproduct during conversion of O_2 into H_2O at Complex I and III, Complex IV is the only oxidase to perform this conversion without the generation of ROS.[3, 41] Whereas mitochondria contribute significantly to ROS production, they also have a substantial capacity to scavenge and remove ROS.[40] Under physiological conditions, it is unlikely that mitochondria emit significant amounts of ROS other than that needed for intracellular signaling because there is a proper balance of ROS production and removal.[40]

1.1.6 The role of mitochondria in cell survival and cell death

When cell death is inevitable, it is less damaging for the surrounding tissue if cell death occurs by apoptosis, rather than through the more injurious process of necrosis.[8] Mitochondria are central both in the process of determining whether the cell will survive at all, and in directing the process into apoptotic or necrotic cell death.[8]

Reduced intracellular availability of ATP may alone trigger apoptosis.[43] However, because apoptosis is an energy-demanding process, ATP-supply must be restored for apoptosis to progress, if not the process will develop into necrosis.[43] It has been demonstrated that if ATP levels are restored within a time frame of three hours after ATP depletion, apoptosis can be reinitiated.[43] Importantly, however, it was found that after a fall in ATP sufficient to initiate apoptosis, cell death was unavoidable despite a subsequent recovery of intracellular ATP.[43]

The mitochondrial permeability transition pore (mPTP) has been pinpointed as a central regulator of cell death in both apoptosis and necrosis.[44] The mPTP remains closed during the ischemic period, but opens upon reperfusion due to increased mitochondrial calcium levels, oxidative stress, ATP-depletion and increased pH in the mitochondrial matrix.[6, 45] Although the mPTP has been a major focus of research in cell death and ischemia-reperfusion injury for many years, its exact structure is not entirely understood.[46] Both VDAC of the outer mitochondrial membrane, and ANT of the inner mitochondrial membrane were previously thought to comprise part of the mPTP, but recent evidence contradict this hypothesis.[46] Opening of the mPTP is irreversible, and allows substances that are normally strictly separated between cytosol and mitochondria to diffuse in and out of the mitochondrion; this causes the mitochondrial membrane potential to collapse, followed by ATP depletion, mitochondrial swelling and ultimately cell death.[46] Cytochrome c release from mitochondria constitutes a signal intricately involved in the regulation of apoptosis by participating in the formation of the caspase-activating complex.[8]

1.2 MicroRNA

MiRNAs are a group of small non-coding RNAs, which influence a wide variety of biological processes by regulating the expression of protein-coding genes. Certain miRNAs exist in great numbers in a single cell, and specific miRNAs are typically present in greater abundance in a particular type of tissue, under specific environmental conditions or at a specific point of development.[47]

Despite their prevalence, miRNAs remained undetected for until as late as 1993 when it was first discovered in the worm C. elegans, commonly referred to as the model

organism in developmental biology because it has been subject of extensive investigation in genetic research.[48] MiRNAs are well-conserved through evolution as illustrated by the fact that over one third of miRNAs in C. elegans have well-recognizable homologs in human miRNA,[49] and miRNAs have been detected in >140 different species including animals, plants and fungi.[50] So far nearly two thousand human miRNAs have been identified, and the number of recognized miRNA sequences is expected to rise substantially.[50, 51]

Due to the relatively short time period since the discovery of miRNA, the exact roles and functions of the majority of miRNAs are still unclear. So far miRNAs have been implicated as influential factors in mitochondrial function,[52, 53] cardiac arrhythmia,[54, 55] ischemia-reperfusion[56-58] and in ischemic preconditioning.[30]

1.2.1 The biogenesis of MicroRNA

MiRNA are initially transcribed in the cell nucleus as long primary transcripts (pri-miRNAs). Pri-miRNAs are subsequently cleaved by the Microprocessor complex containing the RNAse III enzyme Drosha and the double-stranded RNA binding protein Pasha (DGCR8),[59] producing a miRNA precursor that is referred to as pre-miRNA.[47] The pre-miRNA is transferred to the cytosol by exportin-5, where the RNase III endonuclease Dicer cleaves the pre-miRNA into a component which constitutes the mature miRNA, and another fragment referred to as the miRNA* sequence, which is comprised of the opposite arm of the pre-miRNA.[47, 57] The miRNA:miRNA* duplex is loaded into the RNA-induced silencing complex (RISC) where miRNA* is removed and degraded, and the mature miRNA is formed.[47] MiRNAs influence gene expression by directing the RISC to cleave the mRNA if the sequence is complementary, or by repressing translation of the mRNA if it has less degree of complementarity to the miRNA.[47]

1.2.2 MicroRNA and mitochondria

Due to the central role of mitochondria in normal cell function as well as in a variety of human pathologies, significant effort has been directed towards achieving an understanding of the mechanisms behind regulation of mitochondrial function.[53] It has

been established that mitochondria contain unique mitochondrial miRNAs, and that these are cell-specific, meaning that they diverge between different types of cells.[53, 60] Because of the short history of miRNA combined with the fact that the details of how mitochondria influence pathophysiologic processes are still not completely understood, research has so far barely touched the surface of developing an understanding of how miRNA may influence mitochondrial function. It has been demonstrated that miRNA may regulate mitochondrial oxidative phosphorylation, and are involved in mitochondrial processes central to cell survival and cell death.[52, 53, 61]

1.3 Atrial fibrillation with a focus on mitochondria and microRNA

The observation of AF was a subject of investigation in all the studies included in this thesis. AF is the most common cardiac arrhythmia in clinical practice, and incidence is increasing.[7] The incidence is known to increase with age, and the increasing prevalence of AF is partly attributed to an ageing population.[7] AF frequently occurs secondarily to other heart conditions, but may also occur in the absence of concomitant heart disease. The expression “AF begets AF” refers to its common evolvement from paroxysmal to permanent over time, suggesting that the processes underlying AF are progressive in nature.[62, 63] Although the underlying pathophysiology of AF is still not fully understood, the last decades of research have lead to valuable insights into a complex network of interrelated processes of electrical, contractile and structural atrial remodeling, systemic influence through inflammatory processes, hormones and modulation by the autonomous nervous system.[64] Due to the shear complexity of AF pathophysiology, a comprehensive review of the topic is beyond the scope of this thesis; in stead a few of the topics that are closely related to our work will be briefly commented.

1.3.1 Atrial fibrillation and mitochondrial function

In 1982, White et al. measured an increase in atrial blood flow during AF that corresponded to an approximate 2-3 times of baseline, along with a marked increase in atrial oxygen consumption in the atria of dogs.[65] An electron microscopy study of a canine model of sustained AF revealed that both atria were characterized by an increase of mitochondrial quantity and size after 6 weeks of rapid atrial pacing.[66] Investigations

of these canine atria revealed several additional structural alterations, including left atrial dilatation, disarrayed and hypertrophied myocytes, and disruption of the sarcoplasmic reticulum, implicating that structural change of the mitochondria is one of several structural alterations that occurs in association with AF.[66] No structural alterations were observed in the myocardium of the ventricles, suggesting that these effects are limited to the atria only.[66] In a study of sustained AF in goats, induced by biatrial pacing over 9-23 weeks, electron microscopy revealed abnormal mitochondrial morphology in the form of elongated mitochondria with cristae oriented in a longitudinal direction.[67] Accordingly, the mitochondrial structural alterations seen in goats diverge from the alterations found in dogs, which may, as suggest by Ausma et al., be attributed to a difference in species, duration of AF, or mode of pacing.[67] Another marked difference between the two animal models of AF was that, in contrast to the hypertrophied cardiomyocytes seen in the canine atria, the cardiomyocytes of the goats were characterized by atrophy and myolysis.[66, 67] A later investigation of a goat model of AF, this time investigating pacemaker-induced AF after a specified time period varying from 1 to 16 weeks, revealed an increased quantity of mitochondria, but a decrease in the size of each individual mitochondrion as assessed in the right atrium of animals in AF as compared to SR.[68] Despite the structural alterations of the mitochondria, mitochondrial enzyme activities for cytochrome c oxidase, proton-translocating ATPase and NADH-oxidase remained normal in right atrium of goats even after 16 weeks of sustained AF.[68] Elevated protein levels of respiratory Complex I-V have been detected in right atrial tissue from patients with permanent AF, as compared to in patients with SR.[69] Seppet et al. investigated mitochondrial respiration the right atria of patients with SR and AF, and found that AF was associated with increased succinate-dependent respiration and augmented proton leak, suggesting that alterations of mitochondrial oxidative phosphorylation may contribute to AF pathophysiology.[70] Most previous studies investigating AF pathophysiology in human myocardium have been limited to the right atrial appendage,[69, 71, 72] whereas investigation of left atrium has mainly been left to animal studies.[68] While animal models indisputably provide invaluable insights into the pathophysiology of AF, results obtained in experimental models may differ from those obtained in patients with AF. For instance, no increase in fibrosis was observed in either of the animal models

involving goats and dogs discussed previously, whereas increased interstitial fibrosis has been found in right atrial appendages from patients with AF.[66-68, 71] This supports that findings observed in the atria of animal models in AF should be verified in human myocardium before clinical applicability to AF can be established. However, studies of AF in human myocardium are scarce. Investigations of left atrial tissue from patients with AF is particularly interesting due to the limited knowledge regarding the characteristics of the left atrial myocardium in general, and in the setting of AF in particular.

1.3.2 MicroRNA in atrial fibrillation

It has been suggested that miRNA may play important roles in AF, yet research on miRNA and AF is still at a preliminary stage.[73] It can be argued that any miRNA that has a role in Ca^{2+} handling, electrical or structural myocardial remodeling, has the potential to influence processes involved in AF.[54] Several miRNAs have been implicated in cardiac arrhythmia,[54, 55] and in AF.[73] It has been suggested that miRNA may prove to be suitable biomarkers, or as therapeutic targets in the prevention, treatment and management of AF.[74] There is still, however, a long way to go before we have accomplished a thorough understanding of the precise mechanisms behind miRNA modulation in AF, and many questions need answers before miRNA can be exploited clinically.[54]

1.4 Myocardial ischemia-reperfusion injury

Proper cardiac function is entirely dependent on maintaining a complex and highly specific balance of ion concentrations and fluxes. An outline of the main myocardial ion channels discussed in the text is provided in figure 2. Cardiomyocytes are striated muscle cells with a single nucleus, closely connected to one another by intercalated disks that enable electrochemical coupling.[75] A highly specific uneven distribution of electrically charged ions creates the membrane potential across the cell membrane, and closely regulated changes in ionic currents initiate the action potential and ultimately enable cardiac contraction. Maintaining a fine balance of Ca^{2+} is crucial during diastole as well as systole, due to its central role in excitation-contraction coupling. The entry of

a minor amount of Ca^{2+} into the cytosol through a voltage sensitive Ca^{2+} channel (L-type Ca^{2+} channel, LTCC) in the cell membrane triggers the release of substantially larger amounts of Ca^{2+} from the sarcoplasmic reticulum through Ryanodine receptors (RyR) in a process referred to as calcium-induced calcium release.[75] When intracellular Ca^{2+} concentration increases, Ca^{2+} binds to troponin C of the troponin complex and thereby enables contraction during systole through the interaction of actin and myosin cross-bridge.[75] Removal of Ca^{2+} is essential for the relaxation phase (diastole) to commence, and this is achieved mainly by the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and the Na^+/Ca^{2+} exchanger (NCX), whereas a minor amount of Ca^{2+} is removed by the Ca^{2+}-ATPase of the cell membrane and the mitochondrial Ca^{2+}-uniporter.[76]

Due to a lack of oxygen and essential nutrients in situations with insufficient coronary blood supply, the cardiomyocytes are left mainly dependent on glycolysis for energy production. Consequently, the myocardium is faced with challenges of metabolic acidosis, hyperkalemia, Ca^{2+} overload and accumulation of ROS.[77] Although reperfusion is necessary to resolve the situation, reestablishing blood flow is also associated with exacerbated cellular injury through further increase in ROS, rapid restoration of physiological pH and additional increase of intracellular Ca^{2+}.[78] Mitochondria are intricately involved in these processes as they partake in ROS production and constitute targets for the damaging effects of ROS and Ca^{2+} during ischemia and reperfusion.[6]

Lactic acid accumulates and intracellular pH decreases as a consequence of switching from oxidative phosphorylation to glycolysis as the main fuel supply. To counteract the drop in pH, the Na^+/H^+-exchanger (NHE) is activated in an attempt to remove intracellular H^+ along with the Na^+ HCO_3^- cotransporter.[79] When the supply of ATP becomes insufficient, the Na^+/K^+ ATPase of the cell membrane is not able to remove the excess Na^+ that is brought into the cell. This results in elevated levels of intracellular Na^+, thus diminishing the gradient driving the Na^+/Ca^{2+}exchanger (NCX), in turn inhibiting Ca^{2+} efflux and promoting Ca^{2+} entry, with a net result of intracellular Ca^{2+} accumulation.[6, 79]

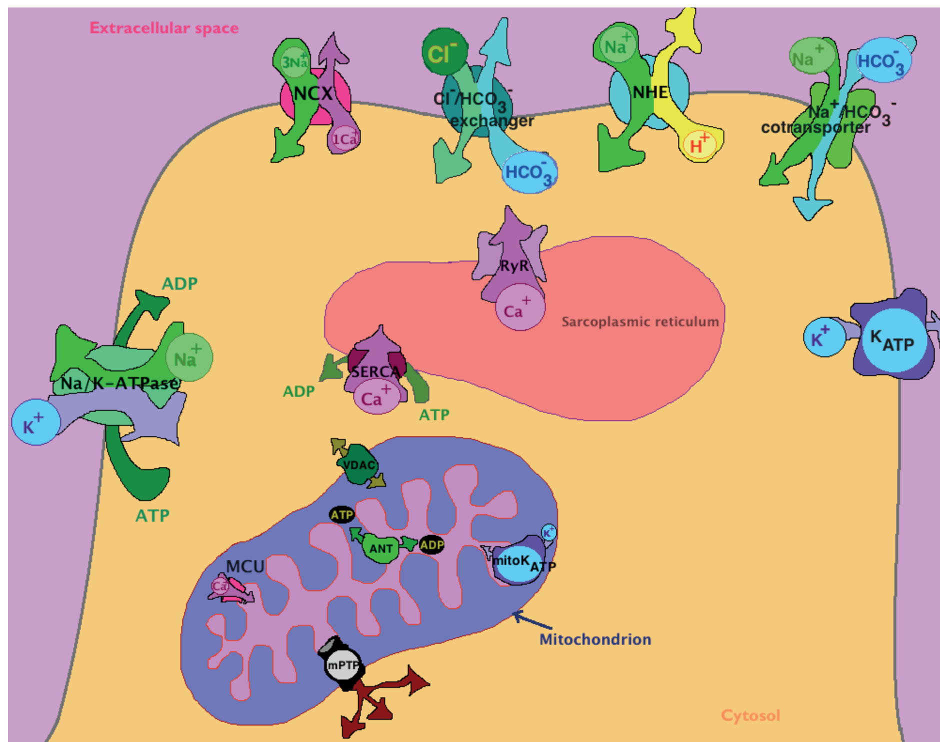

Figure 2. Simplified illustration indicating the locations of the main myocardial ion channels that are mentioned in the text. K_{ATP}-channel, ATP-sensitive K^+-channel (of the cell membrane); MCU, mitochondrial calcium uniporter; mito-K_{ATP}, mitochondrial ATP-sensitive K^+-channel; mPTP, mitochondrial permeability transition pore; NCX, Na^+/Ca^{2+} exchanger; NHE, Na^+/H^+-exchanger; RyR, Ryanodine receptor; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; VDAC, voltage dependent anion channel.

ROS levels increase few seconds after the onset of cardiac ischemia and remains at a stable level for approximately 20 minutes before it increases dramatically.[80] There are indications that the large increase in ROS after prolonged ischemia contributes substantially to the extent of cardiac injury.[6] Although the precise sources of ROS have not yet been entirely clarified, a significant proportion of ROS is derived from the mitochondria.[6]

The mPTP remains closed during ischemia because the low pH efficiently inhibits mPTP opening.[81, 82] Mitochondrial Ca^{2+} overload, oxidative stress, restoration of pH as lactate is washed out, along with a deficiency of ATP, triggers the mPTP to open after the onset of reperfusion.[78] Exactly how the cardioprotective signal is transferred from

the outside of the mitochondrion to the inner mitochondrial membrane is unknown. It has been stipulated that the mitoK_{ATP} channel is involved in mPTP inhibition, but the precise mechanisms have not been discovered.[46] The extent of mPTP opening depends on the duration of the preceding ischemic period.[81] Opening of the mPTP will ultimately lead to cell death by necrosis or apoptosis.[45]

1.5 Akt (protein kinase B)

The protein kinase Akt is another important component in the cellular response to ischemia-reperfusion, and is also linked to mitochondria. Akt is also known as protein kinase B, and is commonly referred to as a prosurvival kinase due to its involvement in processes promoting cell survival. Akt has a central role in several essential cellular processes, including metabolism, cell survival, differentiation and proliferation.[83] Through its complex regulatory processes and functions, Akt appears to be important in a wide spectrum of human disease.[83] Evidence suggests that activation of prosurvival kinase signaling pathways constitutes an important component in cardioprotection from ischemia-reperfusion injury through mobilization of inborn cellular protection against apoptosis.[84, 85]

1.5.1 The PI3K-Akt signaling cascade

Akt is a serine-threonine kinase and a downstream component in phosphoinositide 3-kinase (PI3K) signaling.[83] The PI3K signaling pathway is activated by a variety of receptors, including receptors involved in cellular growth and survival.[86] In its inactive state, signaling protein kinase Akt is located in the cytosol.[83] Activation of PI3K induces production of the second messengers phosphatidylinositol 3,4,5 triphosphate (PIP_3) and phosphatidylinositol 3,4 bisphosphate ($PI(3,4)P_2$), which in turn interacts with Akt and phosphoinositide-dependent kinase (PDK). This interaction causes Akt to translocate to the cell membrane, which is followed by Akt activation through phosphorylation of its primary phosphorylation sites Thr308 and Ser473 by PDK.[86, 87] Activation of Akt causes phosphorylation of numerous substrates, including apoptotic proteins, transcription factors and protein kinase C.[86]

1.5.2 Akt in cell survival

Akt has the ability to inhibit several stages of the apoptotic process.[86] It has been demonstrated that Akt is able to phosphorylate, and thereby inactivate, the pro-apoptotic protein BAD, a member of the BCL-2 family that constitutes central regulators of the common cell-death pathway.[88] Phosphorylation of BAD forces it to remain in the cytosol, precluding it from binding to its dimerization partner BCL-X_L located in the mitochondria, thereby inhibiting apoptosis.[89] It has also been demonstrated that Akt promotes cell survival by preserving the integrity of the outer mitochondrial membrane, hence inhibiting mitochondrial swelling and hyperpolarization, thereby preventing cytochrome c release that otherwise will activate caspase-9 upon release from the mitochondrion.[90] Whereas it is not precisely known how Akt preserves mitochondrial integrity, it has been hypothesized that it may be linked to preserved accessibility to ADP in the mitochondrial matrix.[90]

1.5.3 Akt in cardioprotection

Several experimental studies have demonstrated that Akt is an essential component in mediating cardioprotection from ischemic preconditioning. Inhibition of the Akt signaling pathway inhibits the protective effects of RIPC in mice,[91, 92] and studies of rodent hearts have illustrated that activation of Akt leads to potent reduction in myocardial infarction size as well as improves cardiac function after transient ischemia.[93] A study of coronary ischemia in pigs showed increased activation of the PI3K-Akt cascade after RIPC, whereas inhibition of PI3K and Akt abolished the cardioprotective effect of RIPC, indicating that PI3K and Akt are necessary components for cardioprotection from RIPC.[94]

Although the precise mechanisms by which Akt confers cardioprotection are not thoroughly understood, glycogen synthase kinase-3ß (GSK3ß) has been pinpointed as an important downstream target of Akt in ischemic preconditioning.[95] GSK3ß is a common target in multiple signal pathways, as well as an important regulator of the mPTP, which is a key determinant in cell survival.[95] Inhibition of GSK3ß appears to be the main event in effectuating cardiomyocyte protection through the regulatory effect on mPTP-opening.[95] In addition, evidence indicate that activation of Akt signaling inhibits

the activity of the NHE during intracellular acidosis, and it has been hypothesized that a consequent prolongation of the acidic period after ischemia may contribute to the prevention of mPTP-opening upon reperfusion.[96] Although results from several experimental studies strongly indicate that Akt has a central role in ischemic preconditioning, this has not previously been demonstrated in human myocardium. Further knowledge of the effects of Akt in a clinical setting may yield useful information for further improvement of cardioprotective strategies in cardiac surgery.

1.6 Remote ischemic preconditioning

RIPC involves exposing a tissue to brief, non-lethal periods of ischemia to increase resilience against subsequent ischemic challenge distal to the application site.[97] The first studies on the effects of ischemic preconditioning were published in 1986.[98, 99] Reimer et al. investigated the effect of brief episodes of ischemia on myocardium of canine hearts, and hypothesized that although a single limited time period of ischemia only induce reversible injury to the myocardium, repetitive episodes of ischemia would cause cumulative metabolic derangements.[98] Surprisingly, despite a substantial reduction in ATP after the first 10 min period of ischemia, they found that repetition of intermittent ischemia in total 10 min x 4 did not significantly worsen ATP depletion, and that several intermittent ischemic periods had substantially less impact on ATP depletion as compared to an equivalent time period of 40 minutes continuous myocardial ischemia.[98] The same research group proceeded with investigations of intermittent cardiac ischemia, and another report was published by Murry et al. the same year, where they preconditioned dogs by occluding the circumflex coronary artery intermittently 5 min x 4 with 5 min reperfusion intervals, followed by 40 min sustained occlusion.[99] The preconditioned dogs sustained a myocardial infarction that was only 25% of the size of the infarction of the control group that underwent a single 40 min period of occlusion.[99] The phenomenon that intermittent brief ischemia appear to induce protection from subsequent ischemic insult elicited a great deal of research aiming to understand its underlying mechanisms, as well as towards finding ways to utilize the technique for benefit in the clinic.[100] In 1993, Przyklenk et al. demonstrated that preconditioning by intermittent occlusion of the circumflex coronary artery not only protected the

myocardium directly supplied by this vessel, but in addition ameliorated ischemic injury in other regions of the heart, in this case tissue supplied by the left anterior descending coronary artery.[101] It was later demonstrated an ability to evoke protection even in remote organs, a phenomenon now referred to as RIPC.[102, 103] In recent studies, RIPC is commonly performed by applying intermittent ischemia to an arm, or in some cases a leg, with the intent to trigger cardioprotection.[104-106]

RIPC has apparent practical advantages over applying intermittent ischemia directly to the heart, and has been a major area of research since it was first discovered.[100, 107] It has become evident that preconditioning induces two phases of cardioprotection; the first period of protection starts shortly after the preconditioning stimulus and lasts for approximately two hours, whereas the "second window" of protection emerges approximately 24 hours after preconditioning, and lasts until approximately 72 hours after the stimulus has been applied.[108] In addition, a phenomenon referred to as ischemic postconditioning, which involves the application of intermittent ischemia upon the onset of reperfusion, is able to induce similar beneficial effects as those observed with preconditioning.[108]

1.6.1 Mitochondria and cardioprotection from ischemia-reperfusion injury

Results from a number of studies indicate that mitochondria are important modulators of ischemia-reperfusion injury,[5, 6] and it has been demonstrated that cardiomyocyte survival after ischemia-reperfusion strongly correlates with the fraction of depolarized mitochondria.[95] Multiple hypotheses have been proposed regarding the precise mechanism by which mitochondria are involved in cardioprotection after RIPC. Here different mechanisms of how mitochondria are suggested to confer cardioprotection in ischemia and reperfusion will be discussed.

A main hypothesis suggests that ischemic preconditioning generates a subset of signaling substances such as adenosine, bradykinin and opioids that activate intracellular signaling cascades through G-protein coupled receptors, where many of the end-effectors ultimately converge on the mitochondria.[46] It is further stipulated that these pathways lead to mitochondrial ROS production, which in turn cause activation of downstream mediators such as protein kinase C, Akt and Erk1/2 that forwards the

cardioprotective signal to the ultimate effectors, of which the precise identity remains unknown.[46]

The decline in O_2 during ischemia halts the flow of electrons through the electron transport chain, causing mitochondrial ATP synthesis to be blocked. The respiratory chain is unable to maintain the proton gradient across the inner mitochondrial membrane, and the function of the ATPase (Complex V) is reversed; instead of conducting its main task of producing ATP, it switches to ATP hydrolysis while pumping protons out of the mitochondrial matrix in an attempt to restore the membrane potential.[109] The reversed function of the ATPase contributes significantly to ATP consumption during ischemia, thus causing further ATP depletion.[109] It has been indicated that selective inhibition of ATP hydrolysis by Complex V may conserve ATP during ischemia and confer cardioprotection, whereas nonselective inhibition of normal Complex V function will prevent recovery of ATP-levels upon reperfusion when the ATPase resumes ATP synthesis.[109] Although some studies have given promising results from studies of selective ATPase inhibition in cardioprotection, other studies have not, rendering the importance of ATPase in cardioprotection unclear.[109, 110]

There is evidence of an essential role for mPTP in cardioprotection from ischemic preconditioning.[95] It has been demonstrated that ischemic preconditioning inhibits mPTP opening in the myocardium of rats.[111] Moreover, whereas pharmacological inhibition of mPTP opening by Cyclosporin A decreased infarction size in a similar manner to ischemic preconditioning, pharmacologically induced mPTP opening was associated with increased infarction size after ischemia-reperfusion.[111] A connection has also been demonstrated between the mitochondrial ATP-sensitive potassium ($mitoK_{ATP}$) channel and mPTP, as indicated by a reduction in calcium-induced mPTP opening in rat cardiac mitochondria after exposure to the $mitoK_{ATP}$-channel opener diazoxide.[111]

1.6.2 MicroRNA in remote ischemic preconditioning

Several miRNAs have been defined as modulators of ischemia-reperfusion injury,[112] and studies of animals have revealed that miRNAs are implicated in the processes of ischemic preconditioning both assessed directly in the myocardium and in plasma.[58]

Multiple authors have promoted continued research into miRNA in the setting of ischemia-reperfusion injury and ischemic preconditioning, and it has been argued that features that characterize miRNAs such as stability, affinity and specificity make them promising as potential future agents in medical therapy.[58, 112, 113] The role of miRNAs in human myocardium during CABG surgery and in RIPC remains largely unexplored, and further investigations into the role of miRNA in RIPC have been called for with the aim of finding a mediator of cardiac conditioning for clinical use.[57]

1.6.3 Remote ischemic preconditioning in cardiac surgery

Through the last decade of research on RIPC, substantial effort has been put forward aiming to establish whether RIPC can provide better protection of the heart through cardiac surgery.[106, 114] About 4000 patients undergo open heart surgery in Norway every year, of which approximately 50% undergo CABG surgery due to ischemic coronary disease.[115] Conventional "on-pump" cardiac surgery involves cardiopulmonary bypass (CPB) where a heart-lung machine provides oxygenated blood supply for the body while bypassing the heart and lungs, a process also referred to as extracorporeal circulation (ECC). Cardiac arrest is induced peroperatively by the administration of cold cardioplegia, and the coronary circulation is excluded from the remaining circulation by aortic cross-clamping (ACC). Ever since the first cardiac surgery with a heart-lung machine took place in 1953, there has been continuous research aiming to improve cardioprotective strategies during open heart surgery.[116]

Cardiac surgery represents a particularly convenient circumstance for the application of RIPC because it entails that cardiac ischemia is a planned event, presenting an ideal situation for the implementation of prophylactic measures against ischemia-reperfusion injury. Several researchers have proposed that RIPC may constitute a harmless and efficient method of protecting the heart during cardiac surgery.[117] The first investigation to demonstrate an effect of RIPC in cardiac surgery was published in 2006 by Cheung et al.[104] They found that preconditioning by intermittent preoperative lower limb ischemia in children undergoing repair of congenital heart defects was associated with reduced postoperative release of Troponin I and inflammatory markers, reduced the requirement for inotrope medication and

lowered airway resistance.[104] Subsequently, a number of trials have been published investigating RIPC in cardiac surgery, several demonstrating a reduction of postoperative cardiac marker release,[105, 114] and even improved long-term prognosis, including reduced mortality of patients undergoing elective CABG surgery.[118] However, results have been inconsistent and there are also studies that have been unable to confirm a beneficial effect of RIPC.[119]

1.6.4 Associations of mitochondria, Akt and microRNA in remote ischemic preconditioning

Despite substantial effort, researchers have not yet been successful in bringing RIPC into clinical practice. This has been attributed to a shortcoming in the understanding of the mechanisms behind cardioprotection from RIPC.[107] Although there are still several aspects of RIPC that are not fully understood, RIPC has been found to improve maintenance of cardiac ATP, reduce production and increase removal of ROS, and promote preservation of physiological pH and calcium balance; all factors susceptible to mitochondrial influence.[5, 6] Both direct ischemic preconditioning and RIPC have been demonstrated to preserve mitochondrial respiration in rat skeletal muscle[120] and there is strong evidence that mitochondrial protection plays an important role in protecting the heart.[121-123] A majority of current knowledge of RIPC suggests that cardioprotective signaling pathways converge on mitochondria, induce mitochondrial ROS production and consequently activate mediators in the form of pro-survival kinases such as Akt and protein kinase C, which in turn may confer cardioprotection by inhibiting mPTP-opening.[46] It has been demonstrated that Akt may delay the correction of acidosis during reperfusion, another way of potentially contributing to inhibition of mPTP opening.[96] A recent study of RIPC in porcine hearts exposed to ischemia-reperfusion provides convincing evidence of that the PI3K-Akt pathway has an essential role in conveying the cardioprotective effect in RIPC.[94] Moreover, studies have indicated that diabetes may interfere with the effect of ischemic preconditioning due to alterations in the PI3K-Akt pathway.[124] Several miRNAs have been implicated as modulators of cardiac ischemia-reperfusion injury, and although research on miRNA in RIPC still is at a preliminary stage there are indications that miRNAs may play a role in the mechanism

underlying cardioprotection from ischemic preconditioning.[57] Moreover, it has been hypothesized that manipulating miRNAs through miRNA agonists and antagonists may have potential for facilitating protection against ischemia-reperfusion injury in the future.[57]

1.6.5 Remote ischemic preconditioning and postoperative atrial fibrillation

Postoperative atrial fibrillation (POAF) is one of the most common complications after open heart surgery, with a commonly reported incidence around 25-35% for patients undergoing isolated CABG surgery.[125-127] Consequences of POAF are prolonged postoperative in-hospital stay, increased mortality and increased risk of cerebrovascular accidents.[125-127] Onset of POAF is most frequent within 5 days after surgery,[126] and may be symptomatic or asymptomatic. Incidence is higher in patients with advanced age, hypertension and impaired renal function, and increased duration of aortic cross-clamping has been identified as a peroperative risk factor for POAF.[127] Treatment approaches are highly variable between clinics, but most commonly involves betablockers, calcium channel blockers, antiarrhythmic drugs (most frequently amiodarone) and electroconversion.[127] A variety of prophylactic strategies have been suggested, and betablockers, calcium antagonists, amiodarone, biatrial overdrive pacing, correction of electrolyte disturbance and hypoxia are recommended options.[126]

Oxidative stress and inflammation have been implicated as underlying processes in the pathophysiology of POAF.[126] Moreover, POAF may be associated with alterations of mitochondrial function during ischemia-reperfusion.[128] An experimental study demonstrated that ischemic preconditioning may reduce the incidence of ventricular fibrillation upon reperfusion.[129] It is however not known whether RIPC has an effect on POAF in patients undergoing cardiac surgery.

2 Aims

The overall aim of this thesis was to investigate myocardial physiology in specific clinical settings of patients undergoing cardiac surgery. Assessments of mitochondrial function and miRNA expression were common aims for all the papers included in this thesis.

Aims of Paper I:

The primary aim of Paper I was to investigate the effects of RIPC on mitochondrial function in right atrial myocardium of patients undergoing CABG surgery. We also aimed to assess potential alterations of right atrial miRNA expression of patients undergoing CABG surgery with or without preoperative application of RIPC. As a secondary aim, we investigated the incidence of POAF in the two patient groups.

Aims of Paper II:

Paper II aimed to investigate effects of RIPC on mitochondrial function in left ventricular myocardium of patients undergoing CABG surgery, and to assess whether RIPC would activate myocardial Akt. Additionally, we aimed to examine potential alterations of left ventricular miRNA expression through CABG surgery with or without RIPC.

Aims of Paper III:

The primary aim of Paper III was to assess atrial mitochondrial respiration of patients with AF as compared to patients with normal sinus rhythm. A secondary aim was to assess atrial miRNA expression in patients with AF as compared to patients without any history of AF.

Aims of Paper IV:

Paper IV aim to compare the right and left atria with respect to mitochondrial respiratory function and miRNA, both within patients with normal sinus rhythm, as well as in patients with AF.

3 Methodological considerations

3.1 Patients

Investigations conducted in this thesis conform to the principles outlined in the Declaration of Helsinki. The Regional Committee for Medical Research Ethics of Central Norway approved the studies prior to their initiation. Written informed consent was obtained from all patients prior to enrollment. According to the Norwegian Health Research Act, all patients have the right to seek compensation from the Norwegian System of Compensation to Patients NPE in case of injury or complications arising due to participation in the research project. Regulations specified in the Patient Injury Act apply in case of any injury that were to occur in relation to the research projects, and attend to patient's rights in the event of injury in relation to public health services in general.

Participants for Paper I and II were recruited amongst patients referred for primary isolated CABG surgery at the Clinic of Cardiothoracic Surgery of St. Olav's Hospital. Patients with severe hepatic, renal or pulmonary disease, and peripheral vascular disease affecting the upper limbs were excluded. Patient characteristics were similar in the two groups including preoperative assessment scores, age, gender distribution, body-mass index, creatine clearance and preoperative evaluation of left ventricular function. Despite randomization there was a difference in the use of calcium blocking agents, with a higher percentage using calcium blockers in the control group. No evidence could be obtained from the literature that this difference between the groups would influence mitochondrial function or affect outcomes of RIPC vs. control, although it cannot be entirely excluded.

Participants for Paper III and IV were recruited amongst patients with or without a history of AF referred to the Clinic of Cardiothoracic Surgery of St. Olav's Hospital for isolated CABG surgery, mitral valve replacement (MVR) surgery, aortic valve replacement (AVR) surgery, or combined CABG/AVR surgery at St. Olav's Hospital, Trondheim University Hospital, Norway. Due to the high prevalence of comorbidity in

patients with AF, obtaining comparable patients in the experimental groups are a challenge. The patient groups were similar in AF vs. sinus rhythm for most parameters, except for a higher proportion of patients with mitral valve disease in patients with AF. There was also a non-significant tendency of left atrial dilatation in the AF group as compared to sinus rhythm. Since mitral valve disease is a predisposing factor for left atrial dilatation, which in turn is associated with increased frequency of AF, it is a particularly difficult to obtain similar patient groups with regards to mitral valve disease, and one may not exclude the possibility that this may influence the results. The possibility that comorbidities may influence results is inevitably present when investigating patient material, whereas investigations of lone AF (AF without any apparent comorbidity) is virtually exclusively possible in animal studies, and assessments in goat and dog models have demonstrated changes in experimental models of lone AF.[66, 67]

3.2 Clinical procedures

Standard procedures of our department were followed for all patients participating in the studies included in the thesis (Paper I-IV). Premedication in the form of acetaminophen and morphine-scopolamine was administered 1-3 hours before surgery. Anesthesia consisted of intravenously administered thiopental, fentanyl, propofol and cisatracurium, and isoflurane was administered during pulmonary ventilation before and after CPB. CPB was conducted with a membrane oxygenator at mild systemic hypothermia of 32-34°C. Heparin was administered to achieve an activated clotting time above 480 seconds. Protamine was administered to reverse the effect of heparin after CPB. Distal coronary anastomoses were constructed under ACC. Cold crystalloid or blood cardioplegia was administered for myocardial protection immediately after application of ACC and repeated approximately every twenty minutes. Heart rhythm was monitored by ECG telemetry.

3.2.1 Anesthetics and remote ischemic preconditioning

The potential interference of anesthetic regiments is a concern when investigating the effects of RIPC in a clinical setting (Paper I-II). Whereas there are reports that common

anesthetic agents, including isoflurane,[130] propofol[131] and opioids,[132] may induce preconditioning, the significance of the potential interference of anesthesia with the effects of RIPC remain a debated issue due to diverging conclusions between studies. RIPC has been demonstrated to have an effect on cardiac enzyme release in cardiac surgery both with[131] and without isoflurane.[105] Yet other studies found no such effect of RIPC either with[133, 134] or without isoflurane,[119] and another study interestingly demonstrated an effect of RIPC when combined with isoflurane, but not with propofol.[131] Although one cannot exclude that anesthetic regimens may influence results by potentially concealing RIPC effects, this should not affect between-group differences in our results as all patients received equivalent treatment.

3.3 Interventional procedures

For Paper I and II, an internet-based database provided by the Unit for Applied Clinical Research of St. Olav's Hospital was used to randomize patients to either RIPC or control. A blood pressure cuff was placed around the upper arm prior to anesthesia in all patients. RIPC involved intermittent limb ischemia after induction of anesthesia by inflating the blood pressure cuff to 200 mmHg for 3 x 5 minutes, interrupted reperfusion intervals lasting 5 minutes. For patients allocated to the control group, the blood pressure cuff remained deflated for an equivalent time period. Patients, surgeon, personnel in postoperative intensive care and laboratory personnel were blinded to which group the patients were randomized.

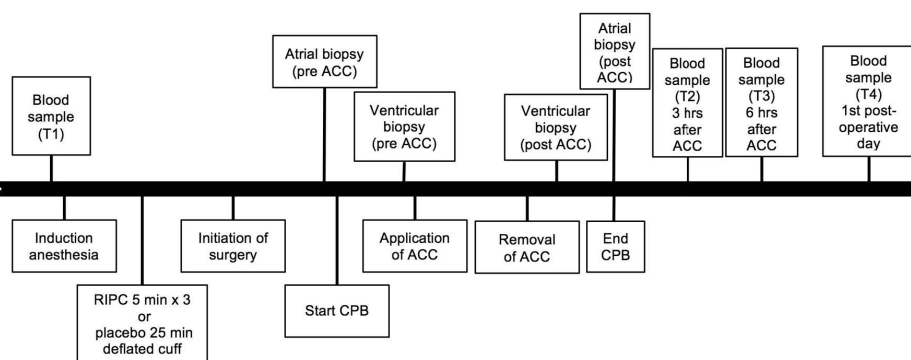

Figure 3. Sequence of sampling and procedures in Paper I and II.

3.4 Biopsies

Right atrial biopsies were obtained with surgical scissors upon venous cannulation for Paper I, and during cannulation and decannulation in Paper III-IV. Left atrial biopsies were obtained during CPB with either surgical scissors or a disposable surgical stapler for Paper III-IV. Left ventricular biopsies were obtained with a disposable automatic 16G biopsy needle (BioPince® Full Core Biopsy Instrument) during CPB for Paper II. Figure 3 (above) illustrates sequence of sampling in Paper I-II.

3.5 Tissue handling

For Paper I-II, biopsies were divided into three parts: one sample was immersed into a mitochondria-preserving storage solution and kept on ice for mitochondrial analyses, one was put on RNAlater® (Ambion ®) for RNA-analyses and one sample immediately snap-frozen in liquid nitrogen for protein analyses. Both the sample on RNAlater® and the snap-frozen sample were thereafter kept at -80 °C until further analyses.

For Paper III-IV, one part of each atrial sample was immediately transferred to a preservation solution and used for mitochondrial respiratory assessment, and another was immediately snap-frozen in liquid nitrogen and kept at -80 °C for miRNA analyses.

3.6 Blood samples

Blood samples for the study discussed in Paper I and II were obtained at six different time points pre- and postoperatively from an arterial catheter (preoperatively before and after RIPC/control, 15 minutes after ACC, 3 hours after ACC, 6 hours after ACC and upon the first postoperative day). Only samples from four different time points were used in the final papers, as two of the samples were timed very close to one another and provided little additional value. One may have suggested that blood samples taken at a later time point e.g. 48 and/or 72 hours postoperatively could have provided additional information with regards to postoperative cardiac marker release. However, the largest difference of cTnT release between RIPC and control in previous studies has been found within the first 24 hours after heart surgery,[105] and there are also studies measuring cardiac markers 48-72 hours postoperatively where no difference in cardiac marker release was observed.[133, 134] The original intent of the blood samples that were

taken preoperatively immediately after RIPC or control (Paper I-II), as well as those obtained 15 min after ACC, was to be able to detect circulating RIPC-associated signaling substances peroperatively. The samples were intended for miRNA analyses, but interference of heparin precluded these samples from being analyzed.

No additional blood samples were obtained for research purposes in Paper III-IV; laboratory values were obtained from routine preoperative samples registered in the patient medical records.

3.7 Laboratory analyses

All blood sample analyses for biochemical markers were performed at the laboratory of St. Olav's University Hospital according to standard routines of the hospital (Paper I-IV). The hospital laboratory follows manufacturer's instruction for each respective analysis conducted.

3.8 Assessment of mitochondrial respiration

Measurement of mitochondrial respiration for all the papers was performed in situ according to well-established procedures previously described and reviewed by others.[135-139] An advantage of measuring mitochondrial respiration in situ is that the mitochondria remain in their natural intracellular location with a preserved cytoskeleton, in contrast to methods based on isolated mitochondria where the mitochondria are removed from their habitual environment.[139, 140] Although our measurements were made at an unphysiological temperature of 22 °C, measurements at this temperature has been thoroughly tested and validated by others.[136] Because all measurements were performed at the same temperature, this should not interfere with group differences as all samples were treated equally. The preparation process involves exposing the tissue to saponin. Saponin forms a complex with cholesterol in the cell membrane and makes holes in the membrane, leaving the plasma membrane permeabilized. The membranes of the intracellular organelles such as the mitochondria contain much less cholesterol compared to the cell membrane, leaving these membranes intact throughout saponin treatment despite permeabilization of the plasma membrane. An advantage of the permeabilization process is that it enables manipulation of the

intracellular environment through exchange of soluble molecules between the cytosol and the experimental incubation solution in which the sample is submerged.[139] Any alteration in the experimental incubation solution is rapidly transferred to the intact mitochondria, sarcoplasmic reticulum and other organelles, and alterations in mitochondrial respiration can be assessed through measurement of changes in oxygen consumption in the solution. While enabling manipulation of the intracellular milieu and leaving intracellular organelles intact, permeabilization also represents a disadvantage of the method; because as all solutes are removed, factors in the cytosol that normally affect mitochondrial are also removed.[139] The cytosolic factors such as pH, metabolites and electrolyte concentrations may fluctuate rapidly and have significant impact on mitochondrial function under normal conditions. Consequently, the in situ method is limited in the way that mitochondrial function is measured in a standardized setting with solutes as specified for the experiment. Another limitation of the in situ method is that it measures oxygen consumption per weight of tissue without yielding any information regarding the number of mitochondria in the sample. Consequently, the in situ method of assessing mitochondrial function does not distinguish whether a difference in mitochondrial respiration is due to qualitative or quantitative differences in the mitochondrial population being assessed. An alternative to measuring mitochondrial respiration in situ is by isolating the mitochondria before respiration assessment, which allows for exact quantification of the number of mitochondria being assessed, i.e. eliminating the quantitative factor, and allowing focus on qualitative differences in mitochondrial respiration only. However, because the intracellular organelles are preserved through the in situ method, one can argue that it is the method that allows assessment of mitochondria closest to their physiological environment.[139] Moreover, it is the only method suitable for assessment of the degree of coupling between energy production in the mitochondria and transfer of energy substrates to energy-consuming sites of the cell.[139] Another argument that was crucial in favor of selecting the in situ method is that is requires only a few milligrams of tissue, and this argument was an apparent advantage to us when working in a clinical setting with myocardial biopsies.

Detailed description of the method used for assessment of mitochondrial respiration is provided in the respective papers (Paper I-IV). In brief, connective tissue

was removed and the dense tissue was transformed into a fine net through fine dissection under a microscope (figure 4). Proper dissection of the tissue is an important factor in facilitating oxygen diffusion into the mitochondria, to prevent that a limitation in mitochondrial oxygen supply will influence the respiration measurements. To ensure equivalent sample preparation for all experiments, dissection of all the tissue samples studied in this thesis was performed by the same person.

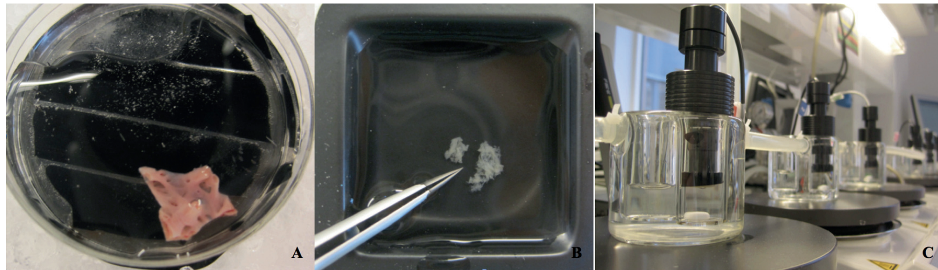

Figure 4. Images from our lab depicting steps involved in the mitochondrial assessment protocol; A) atrial myocardial biopsy prior to dissection; (B) atrial myocardial tissue after dissection; C) water-jacketed respiration chambers with Clark-type microcathode oxygen electrodes used for respiration experiments.

Respiration rates were assessed at 22°C in respiration solution utilizing a Clark-type microcathode oxygen electrode (Strathkelvin Instruments, Glasgow, UK) (Figure 4). A fluorinated ethylene propylene (FEP) membrane was used due to their high degree of permeability for fast response to alteration of oxygen concentration. Basal respiration rate (V_0) was assessed in the presence of glutamate and malate (substrates for Complex I in the electron transport chain) without adenosine diphosphate (ADP). A subsaturating amount of ADP was added (V_{ADP}) prior to the addition of creatine ($V_{creatine}$) followed by addition of saturating amount of ADP. Assessment of the respiration rate of Complexes I through IV with glutamate and malate as substrates is defined as the maximal respiration rate (V_{max}). Succinate was added to supplement electrons to Complex II via FAD^+ ($V_{succinate}$). Amytal was added to block Complex I and allow assessment of respiration form Complex II, III and IV (V_{amytal}).[136] Ascorbate is an artificial electron donor, which supplies electrons to cytochrome c via the redox shuttle N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). TMPD must be added after ascorbate to avoid uncontrollable autoxidation, as ascorbate maintains TMPD in a reduced state. In turn, cytochrome c transfers electrons to Complex IV, thereby enabling assessment of

Complex IV function, without dependence on electron transport from Complex I-III ($V_{ascorbateTMPD}$). In Paper III and IV there was a final supplement consisting of azide, which is an electron transport inhibitor blocking Complex IV function. Inhibition of Complex IV prevents the cell from consuming oxygen, thus causing cellular asphyxia as the cell is unable to produce energy aerobically.[3] Figure 5 illustrates the mitochondrial respiratory chain with the substrates we used in the protocol applied for respiratory assessment.

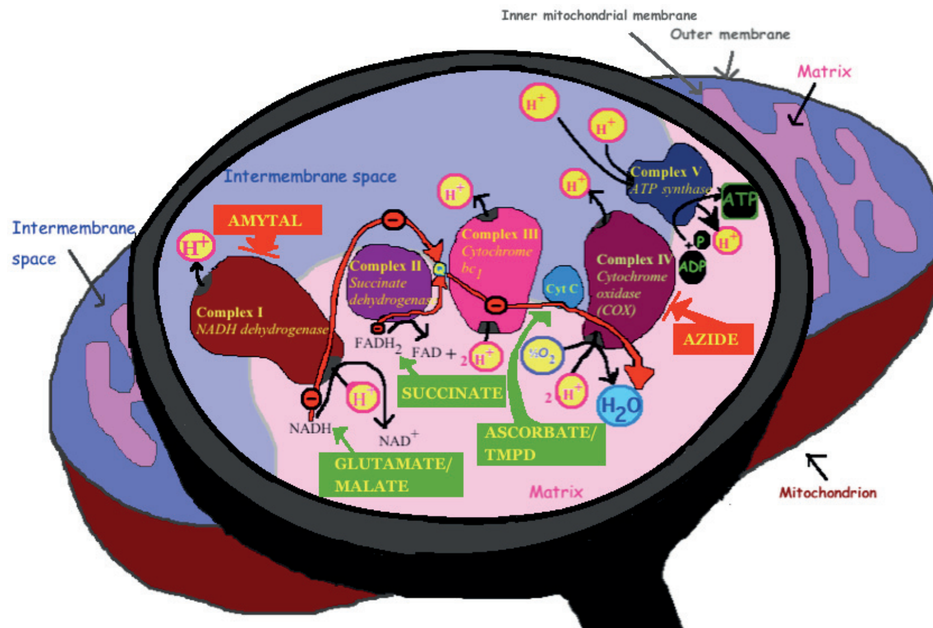

Figure 5. Schematic illustration of oxidative phosphorylation in the mitochondria with indications upon which complexes of electron transport chain the supplements used in our protocol exert their function (more elaborated description in the text). ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cyt C, cytochrome c; FAD+, flavin adenine dinucleotide; H+, proton; NAD+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide + hydrogen; P, inorganic phosphate; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

3.9 MicroRNA expression

Achieving high specificity and sensitivity in the assessment miRNA expression is challenging for several reasons, including the small size of the miRNA molecules and a substantial variation in melting temperature of the nucleic acid duplexes between different miRNA.[141] Moreover, the target sequence is present in the pri-miRNA, the

pre-miRNA, as well as in the mature miRNA, and in some cases as little as a single nucleotide may distinguish one miRNA from another.[141] In addition, there is no mutual sequence that characterizes miRNAs to promote selective isolation of the miRNA population.[141] For these reasons, it is essential to optimize all steps involved in the processes involved in miRNA analyses to obtain accurate results.

Prior to the initiation of miRNA analyses, total RNA must be extracted from the sample of interest. Importantly, RNA quality must be verified before further analyses may proceed because the quality of the isolated RNA is a deciding factor in the accuracy of miRNA expression profiling; both with regards to array and PCR technology.[142] In the studies included in this thesis (Paper I-IV), isolated RNA from every sample was extensively assessed with regards to RNA integrity, content of small RNA and RNA concentration before inclusion in the data analyses. A requirement of RNA integrity number (RIN) above seven was applied to all experiments, as recommended by previous methodological assessments of both miRNA array and miRNA real-time quantitative PCR (RT-qPCR).[143] It has been demonstrated that poor quality of total RNA increase the rate of false positives in miRNA-array, and limits the ability of miRNA-array and miRNA-qPCR analyses to accurately detect biological differences.[143]

3.9.1 MicroRNA array

MiRNA array involves whole-genome screening to provide a qualitative and quantitative assessment of miRNA profiles. MiRNA array technology is used for rapid, high-throughput analysis that allows assessment of a substantial number of miRNAs simultaneously.[142] There are multiple steps involved in miRNA array; miRNA probes designed to pair with (hybridize with) specific miRNAs are fixated onto glass slides, and this unit constitutes the miRNA microarray.[142]

The miRNAs that have been isolated from the samples under investigation is labeled with fluorescent dye, and subsequently they are hybridized with the miRNA microarray. During the hybridization process, the fluorescence-labeled miRNAs from the sample bind to the matching probes on the microarray slide. The fluorescent dye identifies the specific miRNAs that are present in the sample, and this information is

attained through analysis of the pattern of fluorescence emission on the microarray slide.

Despite recent improvements in miRNA array technology, there are several challenges associated with assessment of miRNA expression.[144] MiRNA are especially prone to measurement errors in array technology due to a relatively small number of total miRNA in comparison to total RNA, as well as the low expression of most miRNAs.[144] In addition, considerable variation has been demonstrated in comparing miRNA array results between different platforms and laboratories, a feature that makes comparison of miRNA-array data from different laboratories a significant challenge.[144] For these reasons, whereas we performed miRNA as an initial investigative approach in our studies, miRNA RT-qPCR was performed to verify all miRNA results in our studies (Paper I-IV).

3.9.2 MicroRNA real-time PCR

Real-time PCR (qPCR) is the most common method for validating results from miRNA array.[141] The first step of qPCR is reverse transcription of mature miRNA into complementary DNA (cDNA). The method used in this thesis (Paper I-IV) involves marking all miRNAs with a common sequence (poly-A tail), and subsequent universal reverse transcription of all present miRNAs into cDNA. Universal transcription allows miRNAs to be analyzed from small samples.[141] The next step consists of amplifying the cDNA template. The cDNA is marked with a fluorescent dye, and the rate at which the intensity of the fluorescence increases is proportional to the amount of cDNA present in the sample. The intensity of the fluorescence is monitored throughout the entire PCR process (which is why it is referred to as "real-time"), and the cycle number where fluorescence first can be detected is referred to as the crossing point (Cp). This means that lower Cp values indicate a larger amount of the target miRNA was present in the sample from the start. Normalization of the results obtained from RT-qPCR is necessary to correct for variation between samples.[141] Identifying appropriate reference genes (normalizers) must be specified for each individual experiment, and is performed by identifying miRNAs that are present in similar amounts in all samples.[141] Our miRNA-data is given as normalized Cp (dCp) values, which is calculated by subtracting the Cp

of a specific miRNA from the average Cp of all the normalizer miRNAs (dCp = average Cp (normalizers) – assay Cp). Consequently, a higher dCp indicates that the specific miRNA is present in larger amounts in the sample. Moreover, the process of reverse transcription was performed in 3 replicates for each sample to limit interference due to technical variation.

3.9.3 Locked Nucleic Acids in microRNA technology

Locked nucleic acid (LNA)-modified oligonucleotide probes were applied in all miRNA array and RT-qPCR analyses performed in this thesis (Paper I-IV). LNA is a synthetic RNA nucleotide characterized by a modified ribose backbone and a structure that closely resembles that of naturally occurring nucleic acids.[145] The major advantage of LNA is its ability of high-affinity hybridization with nucleic acids to complementary RNA.[145] Application of LNA-modified capture probes improves the thermostability during the hybridization process and increases the sensitivity and accuracy of the miRNA array and RT-qPCR.[141, 146] Moreover, these features reduce the amount of RNA required for the analyses, which is a major advantage in clinical samples.[146]

3.10 Protein electrophoresis and western blotting

Western blotting is a qualitative and semi-quantitative method used for detecting the presence of, as well as evaluating the expression levels, of proteins in a specific sample.[147-149] Appropriate sample handling, as well as careful attention to accurate performance of each specific step is a prerequisite for ensuring a high specificity and sensitivity.[148]

In our studies, tissue handling was standardized to prevent variability in protein degradation and expression, and all samples were immediately frozen at -80 °C and prepared for proteins analyses on the same day to ensure identical processing. The samples must first be broken down to release the proteins, making them available for analyses. In Paper II, this was performed mechanically and biochemically by subjecting the tissue samples to mechanical homogenization as well as exposure to radioimmunoprecipitation assay (RIPA) buffer, which is a method that has been demonstrated to yield solid data for western blot analysis.[147] The total protein content in

the resulting lysate (the liquid containing the lysed cells) was used for normalization of the results from protein expression measurements. The protein is subjected to gel electrophoresis, sorting proteins dependent on their size, charge and conformation. The pattern generated by the protein distribution in the gel is thereafter transferred to a membrane characterized by its ability to bind protein effectively (the process of loading of protein onto a membrane is referred to as blotting).[150] Staining with specific dyes (such as Ponceau S, which was used in our experiments (Paper II)) is used to ensure that protein was transferred effectively and uniformly from the gel to the membrane. After blotting, the membrane must be prevented from binding antibody other places than onto the protein of interest, and this is achieved by adding a protein such as bovine serum albumin (BSA) in a process called blocking. The proteins deposited on the membrane are subsequently labeled with antibodies that bind to the protein of interest (primary antibodies). It is essential that the primary antibody binds effectively end exclusively to the protein of interest, and these features must be verified before the experiment is initiated. Unbound primary antibody must be removed by washing the membrane, before incubation with fluorescent dye-labeled secondary antibodies. The fluorescent dye-labeled secondary antibodies bind to the primary antibodies, enabling quantification of protein expression through image analysis with a fluorescence scanner. A housekeeping protein, i.e. a protein that is assumed to be ubiquitously present in the tissue under investigation (in this thesis glyceraldehyde 3-phosphate dehydrogenase) is used as an internal reference to ensure that equal amounts of protein was loaded into the electrophoresis gel. An equivalent amount of protein loading for each sample is paramount to the accuracy of results because expression levels of specific target proteins are given as relative densities between samples, and this semi-quantitative assessment of protein levels has been described as a limitation of western blotting.[149]

3.11 Statistical analysis

For Paper I-II, sample size was calculated based upon postoperative release of cardiac markers after CABG surgery in a previous clinical trial investigating the effects of RIPC.[105] We estimated that a sample size of 27 patients per group was required for 80% statistical power. For Paper III-IV, sample size was calculated from previous

investigations of mitochondrial respiration on myocardial tissue, as measured in our lab at NTNU. Statistics were performed by the use of SPSS 19.0 and 21.0 for Mac (IBM SPSS Statistics, Chicago, Illinois). Unpaired Student's t-test was used for between-group comparison for continuous variables, and paired Student's t-test was applied for within-group comparisons. A two-tailed p-value <0.05 was considered significant. For categorical variables Pearson's chi squared test and the Fisher's exact test were used. Hochberg-Bonferroni-correction was applied to analyses of miRNA expression. Graphics were produced by the use of GraphPad Prism 5 (GraphPad Software, Inc., San Diego, California).

4 Results and discussion

4.1 Remote ischemic preconditioning preserves myocardial mitochondrial respiration

In Paper I and II, we found that maximal mitochondrial respiratory capacity is preserved through CABG surgery in both the left ventricle and the right atrium of patients exposed to RIPC preoperatively. In contrast, maximal mitochondrial respiratory capacity was reduced by an average of -28% ($p<0.05$) towards the end of surgery in the right atrium (Paper I), and an average of -15% ($p<0.05$) in the left ventricle (Paper II) compared to patients in the control group.

Preservation of mitochondrial function is a prerequisite for preventing myocardial injury and cell death. Through their vital role as the cell's main energy supply, mitochondria are intricately involved in potential consequences of ischemia-reperfusion including ATP-depletion, decline in pH, intracellular calcium overload and accumulation of ROS. Moreover, potential recovery after ischemia-reperfusion is entirely dependent on the resumption of ATP-production to fuel cellular repair. Consequently, the maintenance of well-functioning mitochondria is essential in both prevention and recovery from ischemia-reperfusion injury.[5, 6] Our results indicate that mitochondria of the right atrium and left ventricle in patients exposed to RIPC are able to maintain normal respiratory rates for Complex I (with malate and glutamate as substrates), Complex II (assessed with succinate as substrate for Complex II and amytal as inhibitor of Complex I) and for Complex IV (with ascorbate and TMPD feeding electrons to Complex IV through cytochrome c).

In contrast, a general impairment of right atrial and left ventricular mitochondrial function was observed in control patients undergoing CABG surgery (Paper I-II). A previous study on mitochondrial function in chronically ischemic human left ventricular tissue implicated Complex II as the site of defect, causing reduced mitochondrial respiratory capacity after ischemia,[151] whereas a general impairment of all respiratory complexes have been demonstrated after ischemia-reperfusion in skeletal muscle of rats.[152] Other authors have demonstrated specific defects and protection of mitochondrial Complex I and II by ischemic preconditioning in the setting of acute

ischemia in skeletal muscle of rats.[120] The inconsistency of the precise localization of the respiratory alterations may be due to differences in species or tissue, the method used for mitochondrial assessment, or it could be attributed to differences in exposure prior to mitochondrial assessment, such as duration and severity of the preceding ischemia and reperfusion.

Because the mitochondria produce over 90% of the energy required by the heart,[4] a 28% and 15% reduction in atrial and ventricular mitochondrial capacity respectively, may impact the ability to meet cardiac energy demand during a challenge of ischemia, as well as in the aftermath when energy is needed for repair processes and resumption of normal cell function. One may hypothesize that insufficient ATP-supply may impact myocardial function, as suggested by the observation that reduced availability of ATP may be linked to cardiac failure.[153] It has previously been reported that ischemic preconditioning applied directly to the heart conserve myocardial ATP levels in cardiac surgery.[154, 155] Both remote and direct ischemic preconditioning of rats resulted in protection from mitochondrial respiratory deficiency in skeletal muscle after ACC.[152] Preservation of myocardial ATP levels through cardiac surgery have been demonstrated with direct ischemic preconditioning both in the setting of intermittent cross-clamp fibrillation[154] as well as with cold crystalloid cardioplegia.[155] Studies of cell cultures demonstrated that a reduction of ATP content by $^1/_3$ caused irreversible cell death, even when ATP levels were restored after only a transient fall in ATP-concentration.[43] It may be hypothesized that maintained ATP-supply through improved mitochondrial function constitute part of the explanation behind cardioprotection by RIPC.

Although our results indicate that RIPC may influence mitochondrial function, our investigations do not reveal the mechanisms underlying these effects in further detail. In light of previous studies, there are several potential ways by which mitochondria may be involved in cardioprotection from RIPC. One may stipulate that altered mitochondrial handling of ROS is implicated as part of the effects of RIPC. Although it has been demonstrated that a certain level of ROS is necessary to evoke a preconditioning effect, further increase in ROS production produces tissue damage.[80] Results from a study performed in guinea pigs indicate that ischemic preconditioning causes a delayed and reduced rise in ROS accumulation during ischemia.[80] It has also been established that ischemic preconditioning leads to improved ROS removal.[156]

There are several indications that a phenomenon termed uncoupling contributes to the effects from RIPC. Uncoupling refers to an alteration in the ratio between the rate of electron transport and ATP-production in the mitochondria, where mitochondrial ATP production in essence is rendered less efficient. During uncoupling, protons are allowed to pass through the inner mitochondrial membrane and back into the mitochondrial matrix without passing through Complex V ATP synthase, in other words, without contributing to energy production. There are indications that partial uncoupling may represent part of the innate defense system against cardiac ischemia-reperfusion injury, and this has been proposed as one of the mechanisms behind protection by ischemic preconditioning.[157] The process of uncoupling appears to be mediated through proteins of the inner mitochondrial membrane (uncoupling proteins), which enable proton transport across the inner mitochondrial membrane.[158] The protons that enter the mitochondrial matrix without passing through ATP synthase, contributes to heat production in stead of ATP synthesis, and is associated with reduced ROS production.[157, 159, 160] Moreover, uncoupling has been linked to decreased mitochondrial Ca^{2+} overload provoked by oxidative stress in myocardium.[161] Whereas assessment of mitochondrial membrane potentials and establishing the degree of uncoupling regrettably was not performed in the studies included in this thesis, assessment of these parameters may provide important supplemental information in future studies.

Assessment of mitochondrial respiration in situ does not enable us to measure directly whether the respiration was better preserved in RIPC patients as compared to control due to maintained function of the mitochondria, or to a potential compensatory increase in the number of mitochondria. However, the duration of a mitochondrial fusion/fission cycle of adult mice cardiomyocytes has been estimated to last for 16 days,[33] and the samples in both Paper I and II were obtained within ~35-70 and 70-90 minutes respectively between pre- and post samples. This indicates that although the number of mitochondria was not assessed in our study, the preservation of mitochondrial respiration rates are likely to be due to the functional status of the mitochondria.

4.2 Remote ischemic preconditioning induces early phosphorylation of Akt

No differences were observed between RIPC and control, or between pre ACC and post ACC regarding total protein expression of Akt, p70S6 kinase, GSK3β and ribosomal protein S6 (Paper II). However, phosphorylated Akt at ser473 was 43% higher in RIPC vs. control pre ACC, indicating that RIPC induced phosphorylation of Akt before the onset of global myocardial ischemia. The phosphorylation level of Akt in RIPC was maintained at reperfusion. Within RIPC, phosphorylation of Akt substrates increased by 27% from pre to post ACC, and the phosphorylation level was higher in RIPC as compared to control in post ACC samples. There was no significant increase in phosphorylation of Akt substrates in the control group from pre to post ACC.

Studies have demonstrated that activation of Akt is necessary to confer protection from ischemic preconditioning, and there is increasing evidence that the cardioprotective role of Akt is closely linked to its interaction with the mitochondria.[162] Opening of the mPTP in the mitochondria constitutes the key mediator of cell death, and it has been demonstrated that Akt may confer cardioprotection by inhibiting mPTP opening upon activation.[90, 163] Our results indicate that RIPC induced early activation of Akt before the onset of ACC, allowing time for phosphorylation of Akt targets during the period of global myocardial ischemia. In contrast, we found that there was no significant increase in the level of phosphorylation of Akt targets within the control group post ACC, despite significant increased level in phosphorylation of Akt, indicating that there is a delay between Akt phosphorylation and the time when Akt phosphorylates its targets. After activation by phosphorylation, Akt counteracts cell death by stabilizing the mitochondrial membrane, inhibiting activation of caspases, and by directly inhibiting proapoptotic proteins.[88, 95] Akt-mediated phosphorylation of BAD is an important step in promoting cell survival as this inactivates its proapoptotic functions, thereby blocking the initial events leading to cell death.[88]

Phosphorylation levels of GSK3β were similar in the control and RIPC groups both pre and post ACC. Whereas inhibition of GSK3ß has been proposed as the main end effector in mediation of cardioprotection by inhibiting mPTP opening,[95] later studies have demonstrated that GSK3ß have distinct roles during the different phases of ischemia and reperfusion.[164] A study in mice showed that GSK3ß-activation by

dephosphorylation during ischemia, and inhibition by phosphorylation during reperfusion are both important for cardioprotection.[164] As our study did not reveal any alterations in the phosphorylation state of GSK3ß in the left ventricle during cardiac surgery in either RIPC or control, it is not clear whether RIPC influences GSK3ß phosphorylation at all, or whether these alterations are only detectable at a later time point than the relatively brief period of reperfusion that was allowed in our study.

A five-fold increase in phosphorylated ribosomal protein S6 was observed from pre to post ACC both in control and RIPC, without differences between groups. Ribosomal protein S6 is a target substrate of p70S6 kinase, and is involved in regulating global protein synthesis during a wide variety of physiological conditions.[165]

The findings of early Akt phosphorylation in RIPC in our material indicate that Akt may constitute a key event in mediating cardioprotection during ischemia-reperfusion injury in heart surgery, and suggests that this aspect should be further studied as a potential target for pharmacological therapy in a clinical setting.

4.3 Remote ischemic preconditioning affects peroperative microRNA expression

In Paper I, we found that miRNA-133a and miRNA-133b were upregulated in atrial samples obtained post ACC compared to pre ACC in both groups, suggesting that expression of these miRNAs were not influenced by RIPC. Due to extensive similarity in sequence between miRNA-133a and miRNA-133b, these miRNAs likely have similar biological functions.[166] There are indications that miRNA-133a is involved in a rapid response after cardiac ischemia-reperfusion injury, and may serve as a marker for acute coronary syndrome[167] as well as an indicator of the extent of tissue injury in myocardial infarction.[168] Our results agree with these investigations, as they demonstrate an increased level of miRNA-133a in atrial samples harvested only approximately 25 minutes after onset of reperfusion, suggesting that miRNA-133a may be induced as part of an acute reaction to ischemia-reperfusion.

Another important observation presented in Paper I, was the increase in atrial expression level of miRNA-1 from pre ACC to post ACC within the control group, but not within RIPC, suggesting that RIPC interfered with upregulation of miRNA-1. In light of the increased frequency of POAF in the control group (Paper I), it is particularly

interesting to note that overexpression of miRNA-1 in rat cardiomyocytes has been shown to induce a significantly increased frequency of spontaneous Ca^{2+} release from the sarcoplasmic reticulum during diastole due to hyperactivity of the RyR receptors, a feature that is associated with arrhythmogenesis.[169] Investigations of a rat model of myocardial infarction demonstrated that miRNA-1 expression increased significantly in myocardium directly affected by the ischemic insult.[170] Moreover, administration of the ß-receptor agonist isoproterenol increased miRNA-1 expression in the absence of ischemia, and conversely, administration of the ß-blocker propranolol reduced miRNA-1 expression in ischemic myocardium, while at the same time reducing both the infarction size and the propensity for post-ischemic arrhythmias within the first 12 hours of myocardial infarction.[170] Moreover, ischemic preconditioning has been shown to reduce the level of miRNA-1 in the myocardium of rats, and this reduction of miRNA-1 expression was associated with a reduced extent of tissue injury during myocardial infarction.[171, 172]

Atrial expression of miRNA-338-3p post ACC was higher in the RIPC group as compared to in control patients, a difference not present in pre ACC samples. There has been substantially less research upon miRNA-338-3p as compared to miRNA-1 and miRNA-133a, and consequently there is limited information available on miRNA-338-3p. In particular, there is no known role for miRNA-338 in the heart. In neuronal cell culture, miRNA-338 has been found to regulate oxidative phosphorylation through local control of mitochondrial mRNAs involved in protein synthesis for the respiratory chain.[61, 173] Further studies of miRNA-338-3p must be performed before it can be established whether miRNA-338-3p may have a link to our findings regarding mitochondrial function.

In contrast to the alterations we observed in atrial miRNA expression (Paper I), no significant alterations of miRNA expression were detected in ventricular tissue (Paper II). The exact time frame for changes of miRNA expression has not been established, but previous experimental studies of miRNA in ischemic preconditioning have allowed a greater time interval between samples; generally not less than 2 hours, and the highest expressional differences have been observed after approximately 6 hours.[174] The lack of alterations in miRNA expression could be due to the relatively short time-interval between the pre ACC and post ACC samples in our study (~35-70

minutes), which was shorter than the interval between atrial samples (~70-90 minutes between samples) (figure 3). Sampling at a later time-point would have been ideal; nonetheless there are obvious practical obstacles that precluded later sampling in our study. With regards to the short time perspective of sampling, our results do not exclude the possibility that RIPC may affect ventricular miRNA expression.

4.4 Remote ischemic preconditioning and postoperative atrial fibrillation

A large difference in POAF was observed in our trial on RIPC (Paper I), where 50% of the patients in the control group developed POAF versus only 14% in RIPC. Incidence of POAF may vary as widely as 5-70% after cardiac surgery,[125, 126, 175] although the incidence is most commonly around 25-35% for patients undergoing isolated CABG surgery.[125-127] Risk factors associated with POAF include older age, chronic obstructive pulmonary disease and duration of CPB or ACC.[125, 176] As the patient groups in our material were similar in these respects, a difference in the frequency of known risk factors does not appear to explain the diverging incidence of POAF in our study. Whereas we observed a lower frequency of POAF amongst RIPC patients, the incidence in the control group was higher than expected. Due to the limited number of patients included in our study, these results must be interpreted with caution and no definitive conclusions can be drawn with regards to an association between RIPC and POAF based on our study. Although a potential association between RIPC and arrhythmia has been noted in a previous study where a significant reduction in reperfusion tachyarrhythmia was observed after RIPC in rats, there has been limited focus on RIPC and cardiac arrhythmia.[102] In this respect it is worth noting that a larger multi-center trial has been commenced to investigate the effects of ischemic conditioning on POAF.[177]

Results from several studies have revealed that new-onset AF after CABG surgery is associated with a substantial increase in the likelihood of developing AF, as well as an increased mortality in the long term after CABG surgery.[178, 179] Although it is not understood whether the occurrence of POAF itself constitutes a risk factor, or whether the incidence of POAF is simply higher in patients with a more serious underlying health conditioning,[180] it is important to understand the mechanisms

underlying the occurrence of POAF in cardiac surgery in order to optimize prophylactic and therapeutic strategies.

A possible connection between the incidence of POAF and mitochondrial function, as well as with the expression profile of miRNA warrants further exploration. As discussed previously, miRNA-1 has been associated with increased propensity of cardiac arrhythmia, and further investigation of a potential link between miRNA-1 and POAF in patients undergoing cardiac surgery is warranted.[169] A recent study found that impaired mitochondrial function after simulated ischemia-reperfusion injury of right atrial myocardium was associated with the development of POAF.[128] Myocardial oxidative stress has been indicated as an important contributory factor in POAF, and it has previously been reported that patients suffering from new-onset POAF have significantly elevated levels of oxidative stress on both a systemic and a myocardial level compared to patients that maintain sinus rhythm.[181] Due to mitochondrial involvement in arrhythmogenesis through their modulation of ROS and calcium homeostasis,[9] one may hypothesize that preservation of mitochondrial function may reduce the risk of POAF through moderation of ROS production and better-balanced calcium homeostasis. This notion is supported by animal studies where antioxidant therapy reduce the incidence of AF the following 1-2 days after rapid atrial pacing to promote electrical remodeling.[182, 183] In addition, supplements of the antioxidant ascorbate has been associated with a reduction in POAF in patients undergoing CABG.[183]

4.5 Effects of remote ischemic preconditioning on cardiac marker release

In our study of RIPC, no significant reduction in cardiac markers was found after RIPC as compared to control (Paper I-II). A number of clinical studies investigating the effects of RIPC in cardiac surgery have focused on the release of cardiac markers, yielding conflicting results.[105, 106, 119] Levels of troponins and CK-MB in peripheral blood are used as indicators for myocardial necrosis, and measurements following coronary surgery yield highly variable results. Postoperative levels of cardiac markers are influenced by a variety of intraoperative factors, and even the process of cardiopulmonary bypass itself has been shown to induce release of cardiac markers.[184,

[185] Moreover, a study with myocardial perfusion imaging demonstrated that remote ischemic conditioning as an addition to primary angioplasty reduced the extent of cardiac tissue injury in patients undergoing myocardial infarction, without any measurable reduction in serum troponin T.[186] These findings lead us to hypothesize that mitochondrial respiration may represent a more sensitive measure of cardiac ischemia-reperfusion than serum levels of CK-MB or cTnT for assessment of the effect of RIPC in heart surgery.

4.6 Diabetes mellitus in remote ischemic preconditioning

We observed no difference in postoperative release of cTnT or CK-MB between RIPC and control groups when investigating the entire study population in Paper I and II (n=60). However, due to conflicting evidence regarding the effect of RIPC in diabetic patients,[124] a subanalysis was performed of non-diabetic patients only (n=47). Interestingly, when the diabetic patients were excluded, a significantly reduction in the area under the curve (AUC) was observed for both cTnT and CK-MB in patients subjected to RIPC as compared to control patients (figure 6).

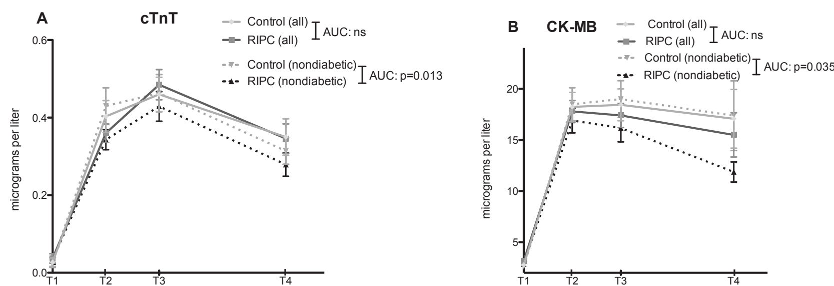

Figure 6. Levels of (A) cTnT and (B) CK-MB in all patients within RIPC (n=30) and all patients in the control group (n=30), as well as non-diabetic patients within RIPC (n=23) vs. non-diabetic patients in the control group (n=24). Values are given as mean±SE (error bars). T1, preoperative; T2, 3 hours after ACC; T3, 6 hours after ACC; T4, 1 day postoperative; AUC, area under the curve.

Our data indicate that diabetic patients may gain less benefit from RIPC than non-diabetic patients. This conclusion is supported by results from several previous studies.[124, 187] An investigation of the effect of preconditioning in the form of prodromal

angina prior to acute myocardial infarction revealed that non-diabetic patients appeared to benefit from preconditioning as evaluated by a lower peak CK-value and lower in-hospital mortality, compared to non-diabetic patients without prodromal angina, whereas no such amelioration was seen in association with prodromal angina in the diabetic population.[187] Still, there are indications that diabetes does not abolish the effect of ischemic conditioning entirely, but rather that the preconditioning stimulus must be augmented to overcome a threshold for induction of cardioprotection.[124] Interestingly, there are also indications that diabetes may ameliorate cardiac injury from ischemia-reperfusion, as demonstrated by smaller myocardial infarct size in diabetic animals, as compared to non-diabetic individuals.[124]

The reduced effect of ischemic preconditioning in association with diabetes has been partly attributed to limited phosphorylation of Akt, or rather that the preconditioning stimulus must be increased as compared to in non-diabetics, in order to achieve a phosphorylation level of Akt that is sufficient to mediate cardioprotection.[124] Our study included an insufficient number of diabetic patients to allow assessment of the diabetic subcohort by itself, but it is interesting to note that an increased tendency of Akt phosphorylation was observed when investigating the non-diabetic population separately (Paper II). In the subanalysis of non-diabetic patients, phosphorylation levels of Akt were higher after RIPC compared with control before ACC, and was further augmented at reperfusion. Further investigation of the role of diabetes in ischemic preconditioning is an area that merits further investigation.

4.7 Mitochondrial function in atrial fibrillation vs. sinus rhythm

Maximal mitochondrial respiratory capacity, as measured in both right and left atrial myocardium, was increased in AF patients as compared to patients with normal sinus rhythm (SR) (Paper III). Most previous human studies investigating underlying mechanisms of AF have included the right atrial appendage only,[68, 69, 71, 72] whereas investigations of the left atrial appendage have mainly been reserved for animal studies.[68] Our findings suggest that the myocardium of both atria are subject to alterations in AF, and this conclusion is in agreement with assessment of atrial remodeling in animal experimental models of AF.[66, 68]

Previous reports have indicated that atrial oxygen consumption and coronary blood flow increase substantially during AF, findings that suggest an increased myocardial energy demand in AF.[188] An elevated maximal mitochondrial respiratory capacity in the atria of patients with AF may be a functional response serving to ensure adequate energy under conditions of increased energetic demand in AF.[68, 188] A study of myocardial PCr levels in a goat model of AF indicate an increased energy consumption relative to energy production in the early stage of permanent AF, whereas the energy balance is restored after 8-16 weeks.[68] One could hypothesize that a restoration of energy balance could be explained by an increase in mitochondrial respiratory capacity, as indicated by our measurements in patients with AF. Because the population studied in Paper III includes patients with both paroxysmal and permanent AF, we are not able to distinguish whether features of mitochondrial respiration differ between these subgroups. Further distinction of myocardial features between paroxysmal and permanent AF, as well as between different durations of AF, will be an important subject for clarification in future investigation of patients with AF.

Whereas the mitochondrial measurements in our study do not enable distinction between qualitative or quantitative alterations of the mitochondria as the cause of the elevated respirator capacity of atrial myocardium in AF, previous studies of both atria in a canine model of AF, as well as measurements of atrial mitochondrial complexes in the right atrium of patients with AF, indicate an increased numbers of mitochondria.[66, 69] Proceeding with investigations of the mitochondrial quantity in atria of patients with AF as compared to patients with SR, would be beneficial for clarification of this issue and should be pursued in future research.

AF has been associated with elevated myocardial levels of oxidative stress,[71] and mitochondrial respiratory complexes have been indicated as main contributors to atrial ROS production in AF.[69] Although reduction and prevention of ROS accumulation has been implicated as an important aspect in the prevention and treatment of AF, the clinical effect of antioxidant supplements have been discouraging.[189, 190] In stead, a recent proposal suggests that a more promising strategy may be to target ROS production in mitochondria specifically for the management of AF.[189] Whereas results indicate that mitochondrial function is altered in both atria of patients with AF as compared to SR, assessments of mitochondrial metabolism in

association with ROS may provide valuable additional information into AF pathophysiology.

4.8 MicroRNA-expression in atrial fibrillation vs. sinus rhythm

As discussed in Paper III, our assessment of miRNA expression in AF indicate that several miRNAs have been upregulated in both or one of the atria in AF as compared to SR. In contrast, we only detected a single miRNA, miRNA-208a, that was downregulated in AF as compared to SR. Due to the limited knowledge of the specific functions for each miRNA in AF, in particular in humans, only a few of the more relevant findings will be discussed here.

Downregulation of miRNA-208a is a particularly interesting observation, due to experimental evidence suggesting an important role of miRNA-208a as a central regulator of the cardiac conduction system.[191] We found that miRNA-208a was downregulated in both the right and the left atrium in patients with AF as compared to patients with SR. MiRNA-208a deletion was associated with an incidence of AF in 80% of the mice investigated.[191] In contrast, overexpression of miRNA-208a was associated with significantly prolonged PR intervals on ECG, consistent with first degree AV-block in the same study of mice.[191] These results suggest that miRNA-208a may have an important role in cardiac arrhythmia and should be examined in human atrial tissue as a potential modulator of AF.

Amongst our findings, miRNA-18a, -19a and -19b have also been linked to arrhythmia. MiRNA-18a, -19a and -19b were upregulated in the left atrium of patients with AF as compared to SR (Paper III). These miRNAs are considered part of the miRNA-17-92 cluster, which recently was demonstrated to cause dilated, hypertrophic cardiomyopathy in mice in a dose-dependent manner, promoting arrhythmogenesis and a significant degree of premature mortality.[192] Overexpression of miRNA-17-92 was associated with spontaneous atrial and ventricular arrhythmias, a feature attributed to a miRNA-mediated repression of Connexin 43.[192]

MiRNA -106b (upregulated in both atria of AF patients), miRNA-15b (upregulated in right atrial tissue of AF patients) and miRNA-25 (upregulated in the left atrium of AF patients) have been related to mitochondria in experimental studies.

Overexpression of miRNA-106b was associated with mitochondrial swelling, reduced mitochondrial membrane potential and increased mitochondrial ROS production in cultured myocytes from mice, despite unaltered ATP-levels.[193] Overexpression of miRNA-15b has also been shown to reduce mitochondrial membrane potential, as well as to disturb mitochondrial integrity in mice cardiomyocytes, whereas anti-miRNA-15b reversed several of the unfavorable effects associated with the subsequent cardiac dysfunction.[194] Increased miRNA-25 has been demonstrated to reduce mitochondrial calcium uptake through reduced expression of the mitochondrial calcium uniporter, preventing lethal Ca^{2+} accumulation in cell in cancer cells.[195]

Due to the early phase of current miRNA research, these are only indications of potential roles for miRNAs in cell function based on current literature, strictly serving as indicators for potential future directions of miRNA research.

4.9 Myocardial differences of the left atrium as compared to the right atrium in patients with or without atrial fibrillation

Electrophysiological mapping has indicated that myocardial areas in both the right and the left atrium may be responsible for activating atrial premature complexes in patients with AF,[196] and there are indications that AF-associated electrical remodeling affects the left atrium differently than the right atrium.[197] Biatrial alterations on the cellular level have been demonstrated in animal models of AF,[66, 68] but investigations in humans have mainly been performed in right atrial tissue.[68, 69, 71, 72] In Paper IV, we compare findings of mitochondrial respiration and miRNA expression in the left versus the right atrium of patients with SR, as well as in patients with AF.

4.9.1 Interatrial comparison of mitochondrial respiratory capacity

We found similar mitochondrial respiratory rates in the left atrium as compared to the right atrium regarding all mitochondrial parameters assessed. This pertained to patients with SR, as well as for patients with AF (Paper IV). To our knowledge, biatrial mitochondrial respiration rates have not previously been assessed in patients with AF. However, a study of patients with heart failure also revealed similar respiration rates between the two atria.[198] In contrast, increased mitochondrial respiratory capacity was

measured in the left ventricle as compared to the atria of the patients with heart failure, a feature that was explained by an increased number of mitochondria in ventricular myocardium.[198] These results may be attributed to an increased workload of the ventricles, also under normal conditions, which in turn may be related to a difference of pressure under normal working conditions of the particular cardiac chamber.[199] The similar respiratory rates measured in the left and right atrium in our study suggest that the physiological pressure difference between the two atria are not associated with a detectable difference in mitochondrial respiratory capacity. Whereas there was no statistically significant difference in incidence of left atrial dilatation in the AF patients in our patient groups, one may not exclude the possibility that a difference of mitochondrial respiratory capacity may occur in groups of patients characterized by significant left atrial dilatation due to an increased frequency of elevated left atrial pressure in this patient group.[200]

4.9.2 Interatrial comparison of microRNA expression

We found a number of interatrial differences of miRNA expression that were identified in both patient groups, whereas other interatrial differences in miRNA expression were found in patients with SR only, or within AF patients only (Paper IV).

Interatrial differences found only in AF patients included increased expression level of miRNA-1 and -208a in the left compared to the right atrium. In light of the potential role of overexpression of miRNA-1,[169] as well as both overexpression and underexpression of miRNA-208a[191] in cardiac arrhythmia discussed previously, these miRNAs are of particular interest for further research in atrial myocardium. Further discussion of interatrial differences of miRNAs has been provided in Paper IV.

5 Limitations

As in any research involving human subjects (Paper I-IV), patient heterogeneity must be taken into account in interpretation of results, and in particular because all research subjects were recruited from a single treatment center, findings must be verified through further studies to establish generalizability of results.

A significant amount of research remains to be done before we have achieved a comprehensive understanding of the specific functions of the various miRNAs in human myocardium. Our findings describe characteristics of mitochondrial respiration, miRNA, RIPC and AF, without providing evidence for a causal relationship. Causal relations must be established through further research.

Whether AF-related cellular alterations of the atrial appendage prevail throughout the atrium has not been assessed in human, thus it has not been ascertained that tissue from the atrial appendage is representative to the remaining atrial myocardium of patients. The atrial appendage is selected for investigation, as it constitutes the most accessible location for sampling human myocardial tissue. However, an investigation of structural changes in a goat model of experimental AF indicates that alterations in the atrial appendages also applies to other localizations of atrial myocardium in general.[67]

The investigation of myocardial alterations in AF included patients with both paroxysmal and permanent AF, and information regarding the duration of AF was not available. It cannot be excluded that these different subcategories of AF may present differences in myocardial physiology. Consequently, studies of carefully selected patients according to specific clinical parameters, both with regards to clinical features of AF, as well as comorbidities and medication regimens, would provide valuable additional information in future investigations of AF.

Direct measurement of myocardial levels of ROS and ATP, as well as investigations of mitochondrial morphology would have added valuable information in the investigations of AF and RIPC, but was precluded due to limited tissue availability.

6 Conclusions

The main results of the studies presented in this thesis may be summarized as follows:

- Myocardial respiratory capacity was significantly reduced in both atrial and ventricular myocardium through routine CABG surgery using cold crystalloid or blood cardioplegia for cardioprotection.
- RIPC preserved mitochondrial respiratory capacity in the myocardium of both the right atrium and the left ventricle through CABG surgery.
- Significantly increased phosphorylation levels of prosurvival kinase Akt were detected in left ventricular myocardium of patients after exposure to RIPC before ACC as compared to control patients. No significant phosphorylation of Akt was detected between pre and post ACC in control patients, nor was any significant phosphorylation of Akt targets detected in these patients. These results indicate that RIPC induce early activation of Akt. This may in turn cause Akt to trigger prosurvival pathways that could be important for protection during subsequent episodes of cardiac ischemia-reperfusion.
- A significant effect of RIPC on mitochondrial function and Akt phosphorylation was observed despite the use of propofol and isoflurane anesthesia.
- The effects of RIPC on mitochondrial function and Akt were detected despite a lack of difference in postoperative cardiac marker release. It remains to be established whether the effects on mitochondrial respiration have clinical significance.
- There was a significant reduction in the incidence of POAF in patients subjected to RIPC as compared to control patients. However, due to the limited sample size in our study, these results are not conclusive and the effect of RIPC on cardiac arrhythmias must be investigated in larger patient cohorts.
- MiRNA expression was significantly altered through CABG surgery in the right atrium of patients from both control and RIPC groups. In addition, miRNA-1 expression was upregulated post ACC as compared to pre ACC in the control group only. No significant alterations of miRNA expression were observed in ventricular tissue of either patient group.
- AF was associated with increased maximal myocardial oxygen consumption in the right and left atrium compared to patients with normal SR.
- Mitochondrial respiration was similar in the left versus the right atrium of patients with AF as well as in patients with SR.

- MiRNA expression differed in atrial tissue from patients with AF as compared to patients with SR, as well as between the right versus the left atrium in both patient groups.

7 Implications and future perspectives

Through the work involved in this thesis, it has become clear that the role of mitochondrial function in human myocardium is an area where many questions remain unanswered, and continued work in the field of mitochondrial assessment is warranted. A potential link between the activation of prosurvival kinase Akt and mitochondrial function in myocardium should be explored, because this may provide a more precise understanding of the mechanisms behind RIPC. In addition, potential uncoupling of mitochondrial respiration in the myocardium should be assessed in association with RIPC in cardiac surgery. Continued investigation into the effect of RIPC on diabetic versus non-diabetic patients may help us achieve a further understanding of the mechanisms behind RIPC in general. The potential effect of exercise as a form of preconditioning may be another area worth investigating. More detailed descriptions of mitochondrial alterations in AF should be established, and patients for future studies should be selected carefully according to clinical characteristic, including specific features of AF, to provide more precise information of associations and differences in myocardial alterations. In addition, a potential link between POAF after cardiac surgery and myocardial mitochondrial function may provide valuable insight into potential approaches to prevent postoperative arrhythmias. An important next step in research on miRNA is to acquire a thorough understanding of the functions of individual miRNAs in cardiac conditions. Furthering research into miRNAs towards both prophylactic and therapeutic aspects of cardiovascular pathophysiology appears to be an area worthy of further exploration. Influencing miRNA for the benefit of both translating beneficial effects of RIPC as well as in the treatment and prevention of AF may have potential, and there are also several other fields of cardiovascular medicine where these areas may prove relevant.

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Paper I

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Paper II

Remote Ischemic Preconditioning Preserves Mitochondrial Function and Activates Pro-survival Protein Kinase Akt in the Left Ventricle During Cardiac Surgery: A Randomized Trial

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ABSTRACT

***Background**:* Understanding the intracellular mechanisms induced by remote ischemic preconditioning (RIPC) in the human left ventricle opens new possibilities for development of pharmacological cardioprotection against ischemia and reperfusion injury. In this study we investigated the effects of RIPC on mitochondrial function, activation of pro-survival protein kinase Akt and microRNA expression in left ventricular biopsies from patients undergoing coronary artery bypass surgery (CABG).

Methods: Sixty patients were randomized to control (n=30) or RIPC (n=30). A blood pressure cuff was applied to the arm of all patients preoperatively. The cuff remained deflated in control group, whereas RIPC was performed by three cycles of cuff inflation to 200mmHg for 5 min, separated by 5 min deflation intervals. Left ventricular biopsies were obtained before and 15 min after aortic clamping. The primary outcome was mitochondrial respiration measured in situ. Secondary outcomes were activation of protein kinase Akt, assessed by western immunobloting, and expression of microRNAs assessed by array and real-time polymerase chain reaction.

***Results**:* Mitochondrial respiration was preserved during surgery in patients receiving RIPC (+0.2 μmol O_2/min/g, p=0.69), and reduced by 15% in controls (-1.5μmol O_2/min/g, p=0.02). Furthermore, RIPC activated protein kinase Akt before aortic clamping (difference from control +43.3%, p=0.04), followed by increased phosphorylation of Akt substrates at reperfusion (+26.8%, p<0.01). No differences were observed in microRNA expression.

***Conclusions**:* RIPC preserves mitochondrial function and activates pro-survival protein kinase Akt in left ventricle of patients undergoing CABG. Modulation of mitochondrial function and Akt activation should be further explored as cardioprotective drug targets.

Clinical Trial Registration: http://www.clinicaltrials.gov, unique identifier: NCT01308138

ABBREVIATIONS

RIPC	remote ischemic preconditioning
CABG	coronary artery bypass graft
CPB	cardiopulmonary bypass
ACC	aortic cross-clamping
cTnT	cardiac troponin T
CK-MB	creatine kinase-MB
NT-pro-BNP	N-terminal pro-brain natriuretic peptide
ADP	adenosine diphosphate
ACR	acceptor control ratio
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
AUC	area under the curve
ATP	adenosine triphosphate
qRT-PCR	quantitative reverse-transcription polymerase chain reaction

1. INTRODUCTION

Remote ischemic preconditioning (RIPC) has emerged as a promising strategy to reduce myocardial reperfusion injury after cardiac surgery.[1] RIPC involves exposing a tissue to brief, non-harmful periods of ischemia to induce protection against subsequent ischemic challenge distal to the application site. The cardioprotective effects of RIPC have been extensively investigated in animal models, revealing potential molecular targets for pharmacological cardioprotection. Previous studies have shown that regulation of mitochondrial function and activation of pro-survival protein kinase Akt are involved in the cardioprotection induced by RIPC,[2, 3] and that these cellular mechanisms interact.[4]

Conserved mitochondrial function is required for optimal cardiac function, since it directly influences physiological processes that are essential for cardiomyocyte survival and proper contractile activity, including maintenance of energy substrates (ATP), pH control and scavenging of reactive oxygen species.[5] Previous studies have shown that both local ischemic preconditioning and RIPC prevent impairment of mitochondrial respiration induced by ischemia in rat skeletal muscle,[6] and that maintaining an optimal mitochondrial function plays an important role in protecting the heart against ischemia.[3, 7] Moreover, mitochondrial damage has been unequivocally demonstrated as a trigger of apoptotic cardiomyocyte death.[8, 9] Preclinical studies showed that local ischemic preconditioning and RIPC reduce ischemic cardiac damage by blocking apoptosis through activation of pro-survival protein kinase Akt.[10] Accordingly, inhibition of Akt signaling completely blocks the effects of RIPC in a porcine model,[11] while targeted activation of Akt renders potent cardioprotection in vivo.[12]

Experimental studies also reported a causal involvement of microRNAs (e.g. microRNAs 199a and 320)[13, 14] in ischemia-reperfusion injury and mitochondrial physiology.[15] However, the effects of RIPC on left ventricular mitochondrial function and microRNA expression have never been explored in humans.

Despite robust preclinical evidence, intracellular mechanisms induced by RIPC in the

human left ventricle are nearly unexplored. Therefore, we investigated the effects of RIPC on left ventricular mitochondrial function, microRNA expression and activation of protein kinase Akt in patients undergoing coronary artery bypass graft (CABG) surgery.

2. METHODS

2.1. Study Design and Participants

This single-center, randomized, prospective, double-blinded study included sixty patients admitted for urgent or elective first-time on-pump CABG surgery at St. Olav's Hospital, Trondheim University Hospital, Norway. The study was conducted in 2011. Exclusion criteria were severe hepatic, renal or pulmonary disease, and peripheral vascular disease of the upper limbs. A database provided by the Unit for Applied Clinical Research at St. Olav's Hospital was used for randomization. Randomization was accomplished immediately before performing the procedure. Patients, surgeons and personnel (both in postoperative intensive care as well as in the laboratory) were blinded to group allocations until after completion of data collection and analyses. This investigation conforms to the principles outlined in the Declaration of Helsinki and was approved by the Regional Committee for Medical Research Ethics of Norway (REK 2010/461-9). Written informed consent was obtained from all patients prior to inclusion. The study is registered at www.clinicaltrials.gov, identification number NCT01308138.

Premedication in the form of acetaminophen and morphine-scopolamine was administered 1-3 hours before surgery. Intravenous thiopental, fentanyl, propofol and cisatracurium were used for anesthesia, supplemented by the volatile anesthetic isoflurane, which was administered during pulmonary ventilation before and after cardiopulmonary bypass (CPB). CPB was conducted with a membrane oxygenator at mild hypothermia of 34 °C. Cold crystalloid or blood cardioplegia with standard St. Thomas' solution (Martindale Pharmaceuticals, United Kingdom) was given every ~20 minutes. Distal coronary anastomoses were constructed under aortic cross-clamping (ACC). All perioperative procedures were

performed according to standard routines of the department, including presurgical preparations, anesthetics, drug administration, surgical technique and postoperative care.

2.2. Intervention

All patients included in the study had a blood-pressure cuff applied to the upper arm before induction of anesthesia. For patients randomized to RIPC (n=30), the cuff was inflated to 200 mmHg for 3 cycles of 5 min ischemia and 5 min reperfusion, after the induction of anesthesia. The cuff remained deflated for an equivalent period in control patients (n=30).

2.3. Biochemical markers

Blood samples were collected preoperatively (T1), 3 hours after removal of ACC (T2), 6 hours after removal of ACC (T3), as well as on the first postoperative day (T4). Analyses of circulating biochemical markers (creatinine; CRP, C-reactive protein; cTnT, cardiac troponin T; CK-MB, creatine kinase-MB; and pro-BNP, N-terminal pro-brain natriuretic peptide) were performed at the laboratory of St. Olav's Hospital according to standard procedures.

2.4. Ventricular biopsies

Left ventricular samples were obtained by use of a disposable automatic 16G biopsy needle (BioPince™ Full Core Biopsy Instrument) during CPB at two separate intraoperative time-points; the first before application of ACC (pre ACC) and the second ~15 minutes after removal of ACC (post ACC). One part of the biopsy was added to ice-cold storage solution for mitochondrial analyses, one part was immersed in RNAlater® (Ambion®) and one part was immediately snap-frozen in liquid nitrogen for protein analyses. The sample in RNAlater and the snap-frozen sample were maintained frozen at -80 °C until further processing.

2.5. Mitochondrial respiration in situ

Mitochondrial respiration was measured *in situ,* as described.[16, 17] The tissue was continuously kept at 4 °C during preparatory procedures. The myocardium was kept in storage solution from harvest until membrane permeabilization with 50 µg/ml saponin for 30 minutes, followed by a rinse cycle of 10 minutes in pure storage solution and an additional 10 minutes in respiration solution. The storage solution contained (in milimol/L) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 6.56 $MgCl_2$ (1 milimol/L free Mg^{2+}), 20 taurine, 0.5 dithiothreitol (DTT), 20 imidazole, 50 potassium-methanesulfonate (CH_3KO_3S), 5.7 Na_2ATP, 15 phosphocreatine (PCr) (pH 7.1 at 22°C). The respiration solution contained (in milimol/L) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 1.38 $MgCl_2$ (1 milimol/L free Mg^{2+}), 20 taurine, 0.5 dithiothreitol (DTT), 20 imidazole (pH 7.1 at 22°C), 90 potassium-methanesulfonate (CH_3KO_3S), 10 sodium-methanesulfonate (CH_3SO_3Na), 3 K_2HPO_4, 10 glutamate, 4 malate, and 2 mg/ml bovine serum albumin. Assessment of mitochondrial function was performed in 3 ml of respiration solution at 22 °C with a fluorinated ethylene propylene membrane on a Clark-type microcathode oxygen electrode (Strathkelvin Instruments, UK). Measurement of basal respiration rate (V_0) with glutamate and malate as substrates for respiratory Complex I was followed by addition of subsaturating amount (0.1 milimol/L) of adenosine diphosphate (ADP) (measuring V_{ADP}) and subsequently 20 milimol/L creatine (measuring $V_{creatine}$). Supplement of a saturating amount of ADP (2 milimol/L) allowed assessment of respiration rates involving the entire respiratory chain including Complex I through IV with glutamate and malate as substrates, recording maximal respiration rate (V_{max}). Complex II substrate succinate (10 milimol/L) was added to assess $V_{succinate}$, after which supplement of Complex I inhibitor amytal (1milimol/L amobarbital) allowed assessment of Complex II (V_{amytal}). Lastly, ascorbate (0.5 milimol/L) and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD, 0.5 milimol/L) was added to induce respiration of Complex IV ($V_{ascorbateTMPD}$). Respiratory rates are given as micromoles O_2 per minute per gram dry weight of left ventricular tissue (µmol O_2/min/g dw). The acceptor control ratio (ACR) was calculated

from the ratio of V_{max}/V_0 to quantify the degree of coupling between oxidation and phosphorylation. Mitochondrial sensitivity to ADP was estimated by the ratio of V_{ADP}/V_{max}. Excess respiration of the cytochrome oxidase complex was quantified by the ratio V_{amytal}/V_{max}. Effect of creatine is given as percent increase in respiration rate after the addition of creatine (↑RR Cr). The apparent constant of Michaelis for ADP was estimated in the absence (${}^{app}K_m^{(ADP-Cr)}$) and presence of creatine (${}^{app}K_m^{(ADP+Cr)}$).[16]

2.6. MicroRNA expression

Random selection of pre ACC and post ACC ventricular samples from 10 patients in RIPC and 10 patients in control group were used for microRNA analyses. Samples were transferred on dry ice transportation-medium from St. Olav's Hospital, Trondheim University Hospital, Norway to Exiqon Services, Denmark (< 24 hours), where microRNA analyses were performed. RNA samples were reverse transcribed into complementary DNA in triplicates. Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, California) was used to evaluate RNA quality. Samples were labeled for microRNA array with miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ (Exiqon, Denmark), and hybridization was performed on the miRCURY LNA™ microRNA Array (6th gen) (Exiqon, Denmark) including capture probes for all human miRs registered in the miRBASE 16.0. miRCURY LNA™ Universal RT miRNA PCR (Exiqon, Denmark) human pick and mix panel was used for microRNA quantitative reverse-transcription polymerase chain reaction (qRT-PCR). qRT-PCR was performed for microRNA-1, -125b-1*, -129*, -133a, -133b, -185, -191, -199a-3p, -199a-5p, -208b, -21, -210, -23a, -299-5p, -320a, -320b, -338-3p, -423-3p, -494, -525-5p, -630, -92a, and miR-943. Average values from three out of four preselected normalization assays were applied for normalization.

2.7. Western immunobloting

Biopsies were kept stored at -80 °C and untouched until all samples were collected. All biopsies were prepared for analysis on the same day to guarantee identical handling procedures. Samples were homogenized in ice-cold RIPA buffer (150 milimol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 milimol/L Tris, pH 8.0; Sigma-Aldrich, Germany) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Germany). Lysates were centrifuged (12000g, 4°C, 15 min) to remove pelleted debris. Total protein concentration in the lysates was measured in triplicates by the BCA method (Thermo Scientific, Norway). Total protein yield did not differ between control and RIPC groups. Sample loading buffer supplemented with reducing agent was added to the lysates and samples were heated at 95°C for 10 minutes in a heating block for protein denaturation. Ten micrograms of total proteins were loaded into polyacrylamide Bis-Tris gels (4-20% or 10% acrylamide, Bolt Precast Gels, Life Technologies, Norway) and subjected to electrophoresis at 165V. Gels were removed from cassettes and proteins were blotted to nitrocellulose membranes by dry transfer (P0 protocol with iBlot system, Life Technologies, Norway). Effective and even transfer was verified by Ponceau staining (Sigma-Aldrich, Norway) of the membrane (5 minutes at room temperature). Membranes were blocked for 1 hour at room temperature (5% BSA in PBS plus 0.05% Tween-20), followed by incubation with primary antibody (1:1000 dilution in blocking buffer, overnight at 4°C). Primary antibodies against total Akt, total P70-S6k, total GSK3β, total ribosomal S6 protein, phosphorylated Akt (serine 473), phosphorylated targets of Akt, phosphorylated GSK3-beta (serine 9) and phosphorylated ribosomal S6 protein (Serine 240/244) were used. Information regarding antibodies is available in Supplemental Table 1. All membranes were also incubated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody, which was adopted as loading control. We tested all antibodies in advance to verify specificity (absence of unspecific binding). Membranes were washed three times with PBS without BSA (5 min each, room temperature) and incubated for one hour with fluorescent-

labeled secondary antibodies (anti-Rabbit at 1:20000 or anti-Mouse at 1:10000) (LICOR Biosciences, UK). Membranes were washed again and imaged by fluorescence scanner (Odyssey system, LI-COR Biosciences, UK). Signals were objectively quantified. Images were analyzed without any modification from the original scanning, and sample pictures were converted to grey-scale for illustration purposes in the manuscript. Phosphorylated protein expression was normalized to total protein expression. Mean values of control group before ACC were arbitrarily set as 100% and data are presented as relative percentage difference from the control group (pre ACC).

2.8. Statistical analysis

Sample size was estimated from previous assessment of the effect of RIPC on troponin release after CABG, requiring a minimum of 27 patients per group to maintain a statistical power of 80%.[1] SPSS 19.0 for Mac (IBM SPSS Statistics, Chicago, Illinois) was applied for statistical calculations. For continuous variables, unpaired or paired students t-test was used to assess between-group and within-group (pre ACC vs. post ACC) differences, respectively. For categorical variables Pearson's chi squared test and Fisher's exact test were used. A two-tailed p-value <0.05 was considered significant. Bonferroni correction was applied to microRNA analyses. GraphPad Prism 5 (GraphPad Software Inc., USA) were used for graphical presentation of data.

3. RESULTS

A diagram of the inclusion process is presented in Figure 2. Sixty patients were included in the study and there was no 30-day mortality. RIPC and control groups were comparable with respect to anesthetics, intraoperative parameters and patient characteristics. Patient characteristics, previous medication and operative parameters are provided in Table 1. There was no significant difference in length of stay in postoperative intensive care.

3.1. Biochemical markers

Laboratory values were similar between groups pre- and postoperatively, including creatinine and C-reactive protein. No differences were observed in postoperative levels of cTnT, CK-MB or pro-BNP, for peak values or area under the curve (AUC) (Figure 3).

Studies have suggested that the effects of ischemic preconditioning might be hindered by diabetes.[18, 19] For this reason we conducted a subanalysis with data from nondiabetic patients only and observed that AUC (24h) for cTnT and CK-MB were significantly lower in RIPC (n=23 nondiabetic patients) than control (n=24 nondiabetic patients) group (Supplemental Figure 1). Patient characteristics, medication and intraoperative parameters of the nondiabetic subgroups are presented in Supplemental Table 2.

3.2. Mitochondrial respiration

All mitochondrial respiratory rates within the control group were reduced post ACC compared to pre ACC, including a significant reduction of V_{max} by an average of -15% (pre ACC 10.2±3.9 vs. post ACC 8.7±2.5 µmol O_2/min/g, p=0.02, Figure 4A). On the other hand, all respiration rates within RIPC were preserved throughout surgery, including V_{max} (pre ACC 9.8±3.9 vs. post ACC 10.0±3.0 µmol O_2/min/g, p=0.69, Figure 4B). The protective effect of RIPC on mitochondrial function was maintained when the subset of nondiabetic patients were analyzed (Supplemental Figure 2).

No significant alterations were observed throughout surgery regarding ACR, ↑RR Cr, ${}^{app}K_m^{(ADP-Cr)}$, ${}^{app}K_m^{(ADP+Cr)}$, V_{ADP}/V_{max} or V_{amytal}/V_{max} in either group (Table 2).

3.3. MicroRNA expression

No significant differences were observed in left ventricular microRNA expression from pre ACC vs. post ACC within RIPC or control, or between RIPC vs. control.

3.4. Protein kinase Akt activation

Protein kinase Akt is activated by phosphorylation. Therefore, we assessed both total (non-phosphorylated) and phosphorylated forms of Akt. We also assessed phosphorylation of Akt-specific targets, as well as downstream players of Akt signaling that have been altered by RIPC in animal models.

When total protein expression of Akt, P70-S6k, GSK3β and ribosomal S6 protein was analyzed, no differences were observed between groups or between pre ACC and post ACC biopsies (Figure 5, panels A-D). Abundance of phosphorylated Akt at serine-473 was 43.3% greater (95% CI for mean difference, +1.7 to +84.9%; $p<0.05$) in RIPC than control (Figure 5E), demonstrating early Akt activation by RIPC before the onset of ischemia (pre ACC). Phosphorylation status of Akt in RIPC group was maintained at reperfusion. Phosphorylation of Akt substrates increased 26.8% from PRE- to post ACC only in RIPC group (95% CI, +10.5 to +43.2%; $p<0.01$) and was higher in RIPC than control at reperfusion (post ACC; $p<0.05$) (Figure 5F). Phosphorylation of GSK3β did not differ between groups before ACC or at reperfusion (Figure 5G). Increased phosphorylation of ribosomal S6 protein (target of P70-S6k) was observed from pre- to post ACC in both groups, without differences between RIPC and control (Figure 5H).

In the subanalysis of nondiabetic patients, the phosphorylation level of Akt was higher after RIPC compared with control before ACC, and was further augmented at reperfusion (Supplemental Figure 3). In this sub-analysis we also observed a trend towards more abundant ($p=0.07$) levels of phosphorylated GSK3β in RIPC than control group post ACC (Supplemental Figure 3).

4. DISCUSSION

This is the first study to demonstrate that RIPC preserves mitochondrial respiration in the left ventricle of patients undergoing cardiac surgery. Maintaining adequate mitochondrial respiratory capacity is fundamental for limiting the extent of damage caused by cardiac ischemia-reperfusion.[5, 20] As mitochondria contribute to approximately 90% of the ~30 kg ATP needed for optimal cardiac function on an average day,[21, 22] a 15% reduction of maximal mitochondrial respiratory capacity poses a substantial energetic disadvantage to the left ventricle, both during and after cardiac ischemia. Our results add support to previous studies demonstrating that ischemic preconditioning applied directly to the heart conserved myocardial ATP levels in patients during cardiac surgery.[23]

Our data indicate that RIPC protected several complexes in the electron transport chain of left ventricular mitochondria, as we observed preserved respiratory capacity in Complex I, II and IV after RIPC. Research on mitochondrial function in human myocardium is sparse, but a recent study demonstrated a more specific defect in Complex II of the respiratory chain in human ventricular tissue exposed to chronic ischemia.[24] A progressive defect in respiratory Complex I has been detected in human myocardium from patients at early stages of heart failure.[25] Studies of mitochondrial respiration in rodent skeletal muscle have implicated both a specific ischemia-induced dysfunction and subsequent protection by ischemic preconditioning localized to complexes I and II in the aftermath of acute ischemia,[6] whereas others observed a general malfunction of all respiratory complexes after ischemia-reperfusion.[26] The inconsistency in the precise localization of the respiratory alterations might be due to differences in species, tissue or severity of the ischemic challenge, which complicates a direct comparison with our results. RIPC preserved mitochondrial capacity in all steps of the protocol used in our study, indicating that the protection was not limited to specific mitochondrial complexes.

ACR remained similar from pre ACC to post ACC in samples from both groups, indicating that coupling between oxidation and phosphorylation was not altered by cardiac

ischemia-reperfusion, despite the reduced respiration rates observed in control samples after ACC. This result indicates that the reduced oxygen respiration observed in control group post ACC resulted in lower amounts of ATP produced by mitochondria. Furthermore, values for ${}^{app}K_m^{(ADP+Cr)}$ and ${}^{app}K_m^{(ADP-Cr)}$ did not change significantly throughout surgery in either group indicating that the sensitivity to ADP was maintained at a stable level, and that mitochondrial creatine-kinase system was not affected by cardiac ischemia-reperfusion during surgery.

Increasing evidence demonstrates that energy metabolism and cell survival are not isolated phenomena, but interlaced mechanisms with reciprocate feedback,[27] where mitochondria play a crucial role. Besides producing ATP for cardiac work, mitochondria are pivotal regulators of apoptotic signaling in cardiac myocytes. Dysfunctional mitochondria release cytochrome C to the cytosol, thereby activating apoptotic proteins (caspases) and causing myocardial necrosis in humans.[9] Such mechanism is blocked by RIPC in animal models through activation of protein kinase Akt.[10] Our study demonstrates that RIPC led to increased Akt phosphorylation before ACC when compared to control group, suggesting an early activation of the kinase. Given this observation, we also used an antibody that recognizes proteins phosphorylated at motifs specifically targeted by Akt. Phosphorylation of Akt targets increased between pre and post ACC within the RIPC group only, reaching values significantly higher than the control group post ACC. These observations suggest a delay between the time Akt is phosphorylated and the time that the kinase effectively acts (i.e. phosphorylates its targets). This phenomenon is unexplored in the context of ischemia-reperfusion, but might be important because phosphorylation of Akt is the trigger for kinase action, while not the final event to stop cell death. After activation by phosphorylation, Akt directly inhibits apoptotic proteins, thereby blocking the signaling leading to cell death.[28] Therefore, activation of Akt before ischemia might benefit myocardial cell survival during cardiac surgery.

The lack of alterations in microRNA expression contradicts a previously suggested association with mitochondrial function.[15] However, unchanged microRNA expression may be

explained by the short interval between collection of pre and post ACC biopsies (35-70 min) in our study.[29] There were obvious obstacles impeding collection of left ventricular biopsies postoperatively, and therefore we cannot exclude the possibility that microRNAs play a role in cardiac responses to ischemia-reperfusion or RIPC at later time-points.

In this study we did not perform gain- or loss-of-function interventions to verify whether mitochondrial preservation and Akt activation are causes or consequences of RIPC-mediated cardioprotection in humans. Interventional disruption of mitochondrial function or pro-survival signaling is not, and will probably never be, an option in human studies, therefore, we must rely on experimental reports to extrapolate our results. In this regard, several studies demonstrated that cardiac ischemic preconditioning is abolished by blockage of mitochondrial ATP-dependent potassium channels due to collapse of mitochondrial membrane potential and oxidative phosphorylation (reviewed by Sato and Marban).[30] In addition, a clinical study demonstrated that inhibition of mitochondrial transition pore opening by intravenous cyclosporine A (2.5mg/kg body weight) reduces ischemic damage of the human heart.[31] Similarly, cardiac protection by RIPC is completely abolished in pigs pretreated with an inhibitor of Akt signaling,[11] while transgenic activation of Akt protects the mitochondria,[4] inhibits apoptosis and reduces ischemic damage after myocardial infarction in rodents.[12] Therefore, overwhelming evidence supports the rationale that pharmacological activation of Akt is an attractive strategy to preserve mitochondrial function and reduce ischemic cardiac damage. However, a selective Akt agonist has not been described in the literature, but should be pursued.

Our data suggest that diabetic patients have reduced benefit of RIPC, as we observed significantly lower AUC for cTnT and CK-MB only in non-diabetic patients (vs. controls) receiving RIPC (Supplemental Figure 1). This is in line with a previous study, reporting that diabetic patients do not benefit from the preconditioning effect of prodromal angina 24h prior myocardial infarction.[19] Interestingly, experimental evidence suggested that diabetes negatively interferes with preconditioning due to a reduced ability of diabetic hearts to enhance activation

of Akt upon preconditioning.[32]

Most studies on RIPC adopted postoperative circulating levels of cardiac biomarkers as the only endpoint to infer cardioprotection.[1, 33, 34] This created a precipitated sense that similar levels of cardiac biomarkers between control and RIPC mean that the intervention promoted no cardioprotection at all. Such assumption led studies to conclude that RIPC was ineffective[33] or even “disappointing”. [35] On the other hand, a recent RIPC clinical trial reported enhanced myocardial salvage after 30 days and improved long-term outcomes in RIPC group, even though no differences were observed in postoperative troponin release.[36, 37] Therefore, circulating “cardiac biomarkers” should not be the only indexes to assess cardioprotection, especially when ventricular biopsies permit assessment of biological phenomena in myocardial tissue.

Interfering effects of anesthetics such as isoflurane,[38] opioids[39] and propofol[34] are frequently debated when it comes to assessing RIPC effects on serum cardiac markers, but a consensus on to what extent they interfere with RIPC has not been reached. RIPC has been demonstrated to reduce postoperative levels of circulating cardiac markers both with[34] and without isoflurane,[1] while other studies found no such effect with[40] or without isoflurane,[35] or with propofol.[34] In our study, the anesthetic regimen included isoflurane, propofol and fentanyl for all patients. As the anesthetic protocol was equivalent for both groups, this would not affect the differences observed between control and RIPC.

4.1. Limitations

Despite randomization, the control group included a statistically higher number of patients taking calcium antagonists. We could however not find any report indicating that this should affect the influence of RIPC on mitochondrial respiration or Akt activation. Additionally, mitochondrial respiration and Akt activation did not differ between patients with and without calcium antagonists preoperatively.

Due to sample availability, we were unable to perform experiments that required a different sample preparation. For instance, assessment of apoptosis by gold-standard TUNEL assays (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) could have provided additional insight on a relationship between mitochondrial function, Akt activation and cardiomyocyte death.

5. CONCLUSION

RIPC preserved mitochondrial function and activated the anti-apoptotic protein kinase Akt in the left ventricle of patients undergoing CABG surgery. Our results indicate that RIPC induces alterations in the left ventricular myocardium and provides a novel aspect in the investigation of RIPC in the clinical setting. Further investigations focusing on modulation of mitochondrial function and activation of Akt may provide valuable knowledge in optimizing cardioprotection against ischemia-reperfusion.

6. AUTHORS CONTRIBUTION

Katrine Slagsvold and Jose Moreira take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation. Study concept and design: Slagsvold, Rognmo, Høydal, Wisløff and Wahba. Acquisition of data: Slagsvold and Moreira. Analysis and interpretation of data: Slagsvold, Moreira, Rognmo, Høydal, Bye, Wisløff and Wahba. Drafting of the manuscript: Slagsvold and Moreira. Critical revision of the manuscript for important intellectual content: Slagsvold, Moreira, Rognmo, Høydal, Bye, Wisløff and Wahba. Statistical analysis: Slagsvold and Moreira. Obtained funding: Slagsvold, Wahba and Wisløff. Study supervision: Wahba and Wisløff.

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8. DISCLOSURES

None

9. REFERENCES

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10. TABLES

Table 1. Clinical data

	Control (n=30)	RIPC (n=30)	p-value
Baseline characteristics			
Gender, men/women, n	23/7	27/3	0.17
Age, years	68 ± 8	64 ± 9	0.09
Body mass index, kg/m^2	28 ± 4	29 ± 8	0.48
ASA score	3.6 ± 0.6	3.4 ± 0.5	0.23
Euroscore II, %	1.31 ± 0.9	1.35 ± 1.2	0.90
Urgency, elective/urgent*, n	13/17	15/15	0.61
Left ventricular ejection fraction, %	53 ± 7	52 ±8	0.65
Creatinine clearance, mL/min	90 ± 25	102 ± 41	0.19
Risk factors and comorbidities			
Chronic obstructive pulmonary disease	2	3	>0.99
Current smoker	8	8	>0.99
History of smoking	21	20	0.78
Diabetes Mellitus	6	7	0.75
History of atrial fibrillation	2	1	>0.99
Hypertension	15	12	0.44
Peripheral arterial disease	1	1	>0.99
Previous cerebral insult	0	3	0.24
Previous myocardial infarction	14	19	0.19
Previous PCI	5	5	>0.99
Unstable angina < 3 days preoperative	3	0	0.24

Pharmacotherapy			
ACE-inhibitor or ARB	11	14	0.43
Aspirin	29	28	>0.99
Beta blocker	24	25	0.74
Calcium channel blocker	9	1	0.01
Clopidogrel	11	13	0.60
Dipyridamole	0	2	0.49
Diuretics	4	7	0.32
Glibenclamide	1	0	>0.99
Insulin	1	2	>0.99
Lipid-lowering agent	27	25	0.71
Metformin	4	3	>0.99
Organic nitrates	6	3	0.47
Sulfonylurea	1	1	>0.99
Warfarin	2	1	>0.99
Intraoperative data			
Aortic cross-clamping, min	42± 11	41 ± 11	0.80
Cardiopulmonary bypass, min	76 ± 23	71 ± 14	0.31
Cardioplegia, mL	1000 ± 284	911 ± 257	0.21
Distal coronary graft anastomoses, n	3.3 ± 0.7	3.5 ± 1.0	0.31

Data are presented as mean ±SD or number. ASA-score, preoperative physical assessment score developed by the American Society of Anesthesiology; PCI, percutaneous coronary intervention; ACE, angiotensin converting enzyme; ARB, angiotensin-II-receptor blocker. *as defined according to Euroscore II (2011).

Table 2. Mitochondrial respiratory parameters

	Control (N=30)			RIPC (N=30)		
	pre ACC	post ACC	p-value	pre ACC	post ACC	p-value
V_{amytal}/ V_{max}	1.02 ± 0.4	0.98 ± 0.2	0.72	0.96 ± 0.2	0.89 ± 0.3	0.35
V_{ADP}/ V_{max}	0.46 ± 0.2	0.37 ± 0.1	0.06	0.42 ± 0.2	0.35 ± 0.2	0.11
ACR	4.07 ± 2.0	4.96 ± 2.7	0.16	4.80 ± 3.0	5.70 ± 3.4	0.21
↑RR Cr, %	72 ± 58	85 ± 36	0.21	94 ± 59	101 ± 53	0.62
${}^{app}K_m^{(ADP+Cr)}$, μM	57 ± 42	70 ± 56	0.41	44 ± 36	60 ± 56	0.19
${}^{app}K_m^{(ADP-Cr)}$, μM	151 ± 95	198 ± 121	0.14	174 ± 92	222 ± 128	0.11

V_{amytal}/V_{max}, ratio quantifying excess respiration of the cytochrome oxidase Complex; V_{ADP}/V_{max}, ADP sensitivity ratio; ACR, acceptor control ratio; ↑RR Cr, percent increase in respiration rate after addition of creatine; ${}^{app}K_m^{(ADP+Cr)}$, apparent Michaelis-Menten constant for ADP in the presence of creatine; ${}^{app}K_m^{(ADP-Cr)}$ apparent Michaelis-Menten constant for ADP in the absence of creatine.

11. FIGURES CAPTIONS

Figure 1. Timeline illustrating sequence of procedures and sampling.

Figure 2. CONSORT diagram of inclusion process.

Figure 3. Biochemical markers. Circulating (A) cTnT, (B) CK-MB and (B) NT-proBNP in control (n=30) vs. RIPC (n=30) group. Values are given as mean ± standard error (SEM). T1, preoperative; T2, 3h after ACC; T3, 6h after ACC; T4, 1-day postoperative.

Figure 4. Mitochondrial respiration rates pre vs. post ACC within (A) control group (n=30) and (B) RIPC (n=30). V_0, basal respiration; V_{ADP}, subsaturating ADP (0.1 milimol/L); $V_{creatine}$, 20 milimol/L creatine; V_{max}, maximal respiration (2 milimol/L ADP); $V_{succinate}$, 10 milimol/L succinate (Complex-II substrate); V_{amytal}, 1 milimol/L amytal inhibits Complex-I; $V_{ascorbateTMPD}$, 0.5 milimol/L ascorbate and 0.5 milimol/L TMPD stimulate Complex-IV. Data are displayed as mean ± SEM. *p<0.05.

Figure 5. Protein expression and phosphorylation in control group (n=24) and RIPC (n=24). Non-phosphorylated abundance and phosphorylated levels pre ACC and post ACC are show. Original representative bands are presented. Data are presented as mean ± SEM . *p<0.05 vs. Control-PRE; #p<0.05 vs. RIPC-PRE; &p<0.05 vs. Control-POST.

12. FIGURES

FIGURE 1

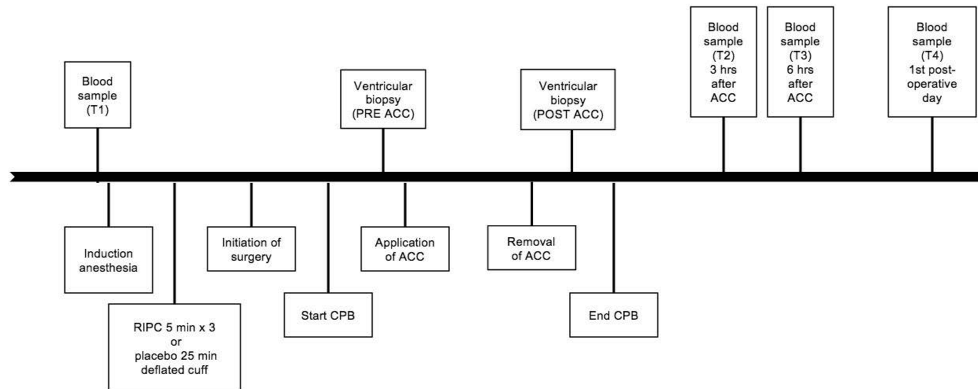


FIGURE 2

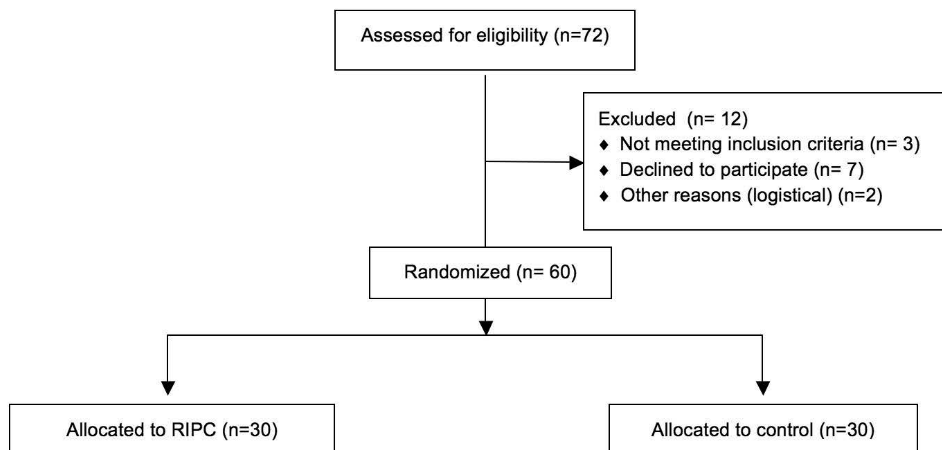


FIGURE 3

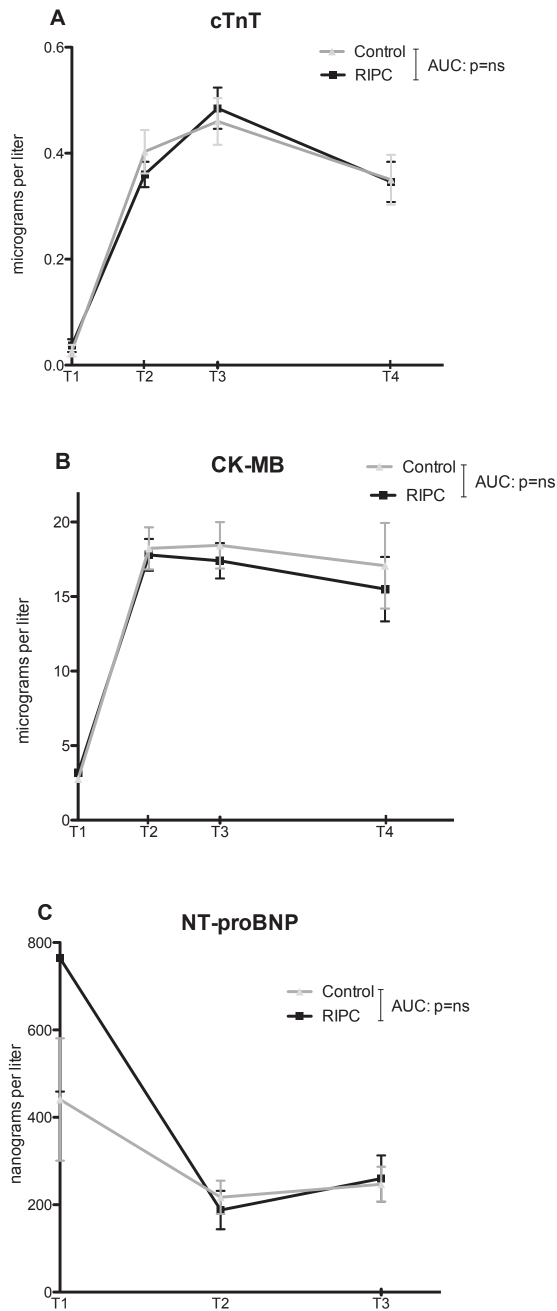


FIGURE 4

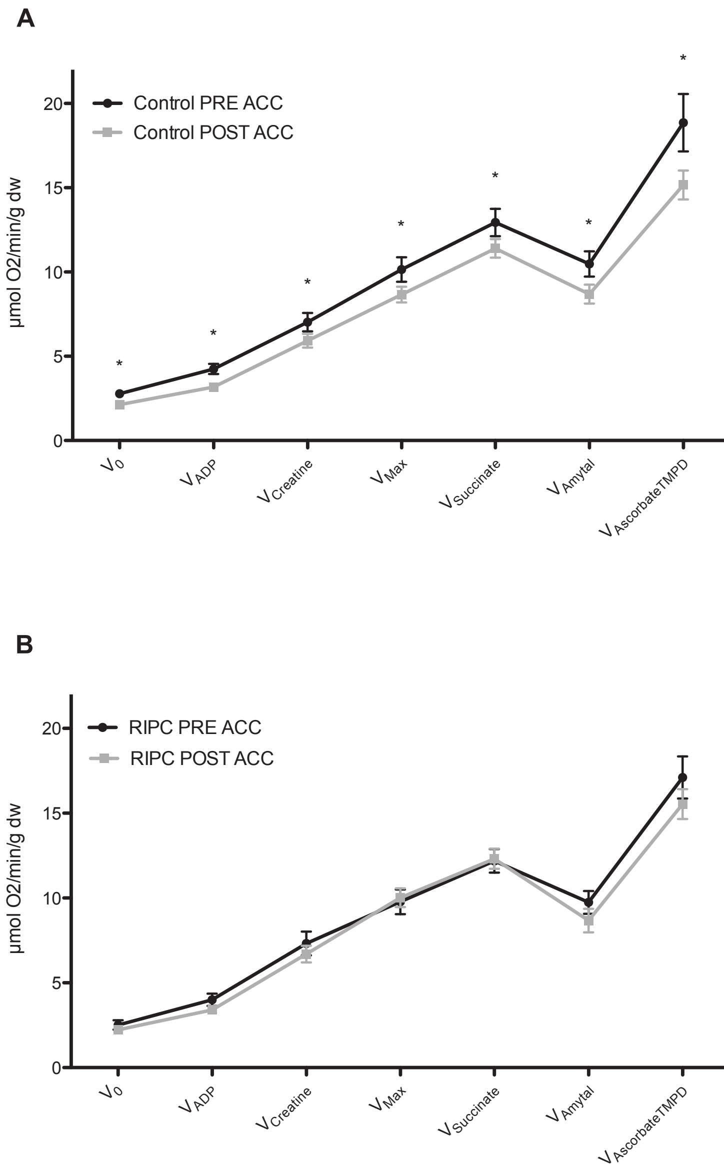
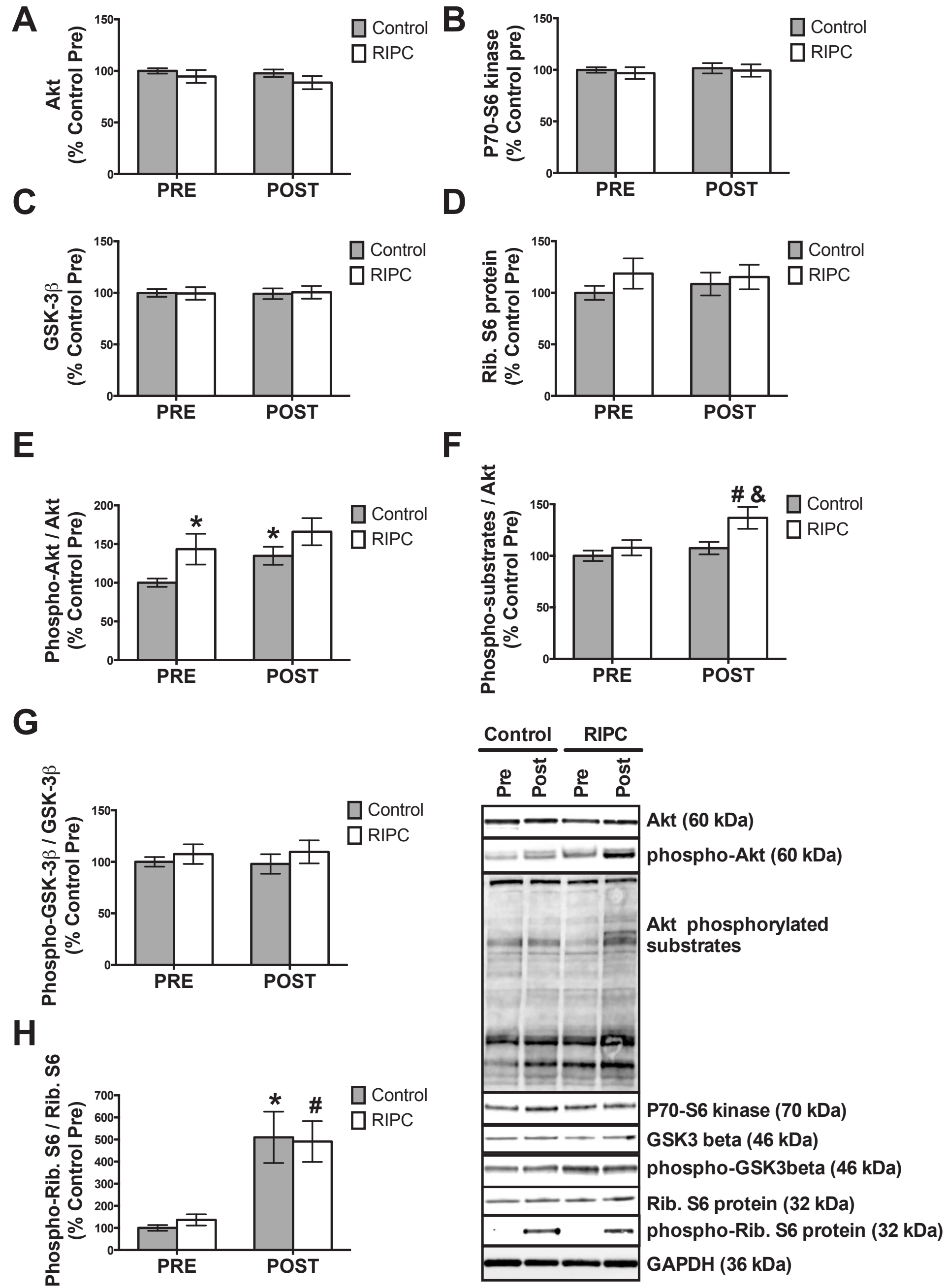


FIGURE 5



SUPPLEMENTAL ONLINE MATERIAL

SUPPLEMENTAL TABLES

Supplemental Table 1. List of all antibodies used in the study for western immunobloting analysis.

Antibody	Supplier	Product	Secondary
Akt (total)	Cell Signaling	4685	Rabbit
P70-S6 kinase (total)	Cell Signaling	2708	Rabbit
GSK3β (total)	Cell Signaling	12456	Rabbit
Ribosomal S6 protein (total)	Cell Signaling	2317	Mouse
Phosphorylated Akt (ser 473)	Cell Signaling	4060	Rabbit
Phosphorylated substrates of Akt	Cell Signaling	10001	Rabbit
Phosphorylated GSK3β (ser 9)	Cell Signaling	5558	Rabbit
Phospho-Rib. S6 protein (ser 240/244)	Cell Signaling	5364	Rabbit
GAPDH	Pierce	MA5-15738	Mouse
Secondary anti-rabbit	LICOR Biosciences	926-68021	-
Secondary anti-mouse	LICOR Biosciences	926-32210	-

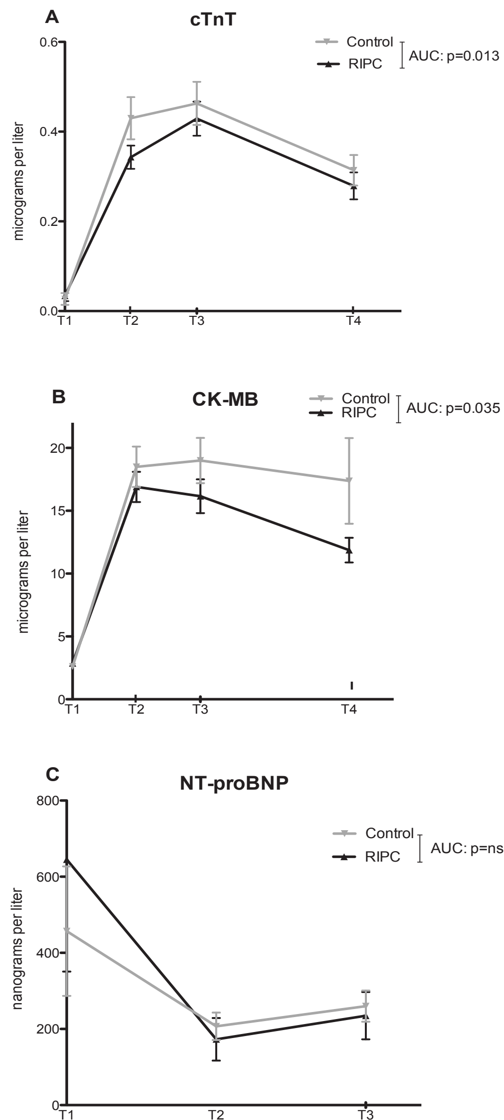
Supplemental table 2. Clinical data of the subcohort of nondiabetic patients

	Control (n=24)	RIPC (n=23)	p-value
Baseline characteristics			
Gender, men/women, n	18/6	20/3	0.46
Age, yrs	68 ± 9	63 ± 10	0.11
Body mass index, kg/m^2	27 ± 4	27 ± 4	0.83
ASA score	3.5 ± 0.6	3.5 ± 0.5	0.70
Euroscore II, %	1.24 ± 0.6	1.31 ± 1.2	0.81
Urgency, elective/urgent*, n	10/14	10/13	0.90
Left ventricular ejection fraction, %	53 ± 7	53 ± 7	0.65
Creatinine clearance, mL/min	90 ± 22	104 ± 43	0.16
Risk factors and comorbidities			
Chronic obstructive pulmonary disease	2	3	0.67
Current smoker	8	8	0.92
History of smoking	19	17	0.67
History of atrial fibrillation	1	0	>0.99
Hypertension	9	9	0.91
Peripheral arterial disease	1	1	>0.99
Previous cerebral insult	0	3	0.11
Previous myocardial infarction	10	15	0.11
Previous PCI	2	5	0.19
Unstable angina < 3 days preoperative	3	0	0.23
Pharmacotherapy			

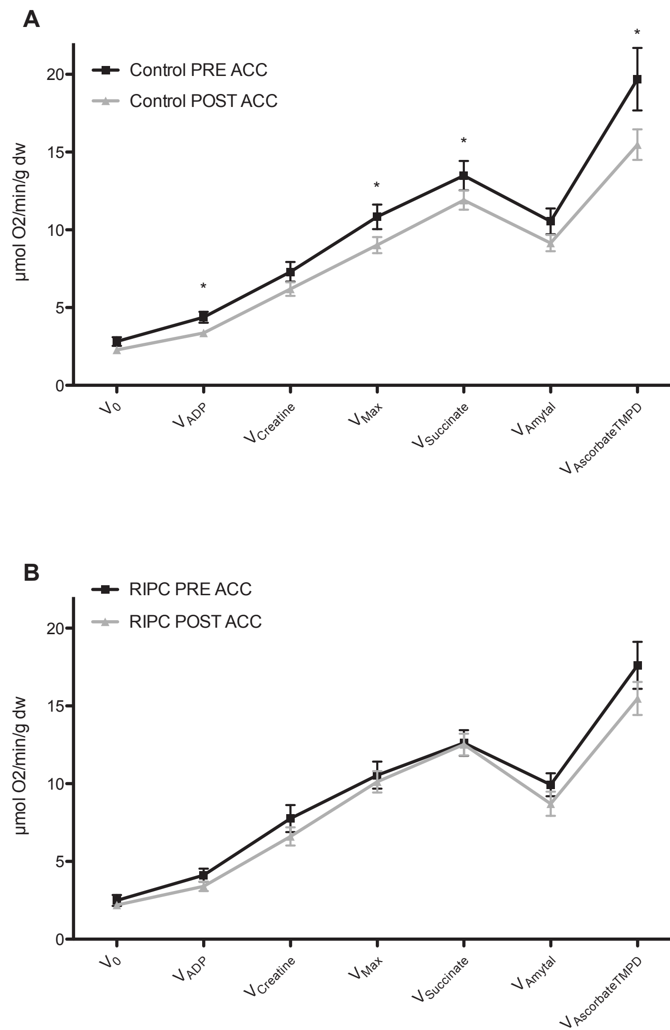
ACE-inhibitor or ARB	7	11	0.19
Aspirin	23	22	>0.99
Beta blocker	19	20	0.70
Calcium channel blocker	7	1	0.05
Clopidogrel	99	11	0.60
Dipyridamole	0	2	0.23
Diuretics	4	5	0.72
Antidiabetic agent	0	0	>0.99
Lipid-lowering agent	21	18	0.46
Organic nitrates	5	2	0.42
Warfarin	1	0	>0.99
Intraoperative data			
Aortic cross-clamping, min	44± 11	41 ± 11	0.38
Cardiopulmonary bypass, min	75 ± 16	68 ± 13	0.09
Cardioplegia, mL	985 ± 237	901 ± 244	0.24
Distal coronary graft anastomoses, n	3.3 ± 0.7	3.5 ± 0.8	0.31

Data are presented as mean ± SD or number. ACE, angiotensin converting enzyme; ARB, angiotensin-II-receptor blocker; ASA-score, preoperative physical assessment score developed by the American Society of Anesthesiology; PCI, percutaneous coronary intervention; RIPC, remote ischemic preconditioning. *As defined in Eurscore II (2011).

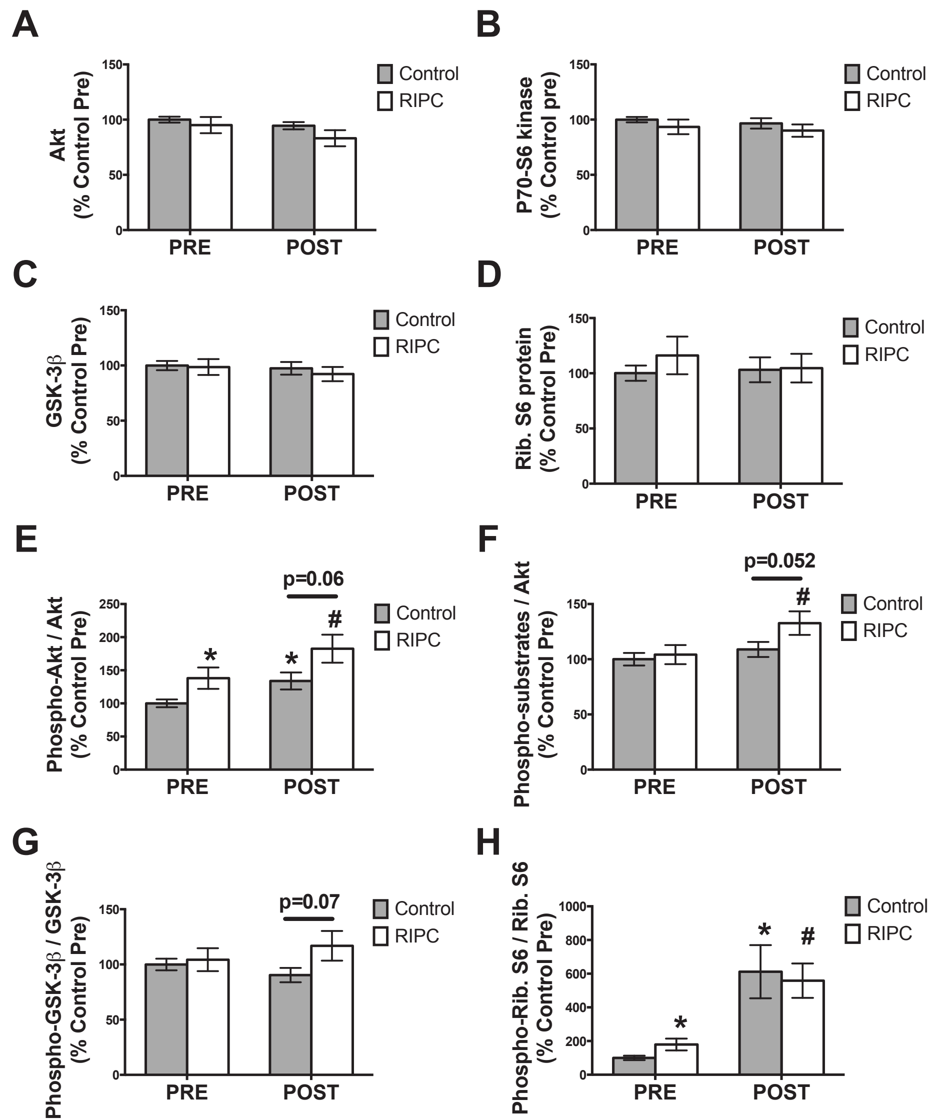
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS



Supplemental Figure 1. Biochemical markers in the subcohort of nondiabetic patients. Circulating (A) cTnT, (B) CK-MB and (B) NT-proBNP in control (n=24) vs. RIPC (n=23) group. Values are given as mean ± standard error (SE). T1, preoperative; T2, 3 hours after ACC; T3, 6 hours after ACC; T4, 1 day postoperative.



Supplemental Figure 2. Mitochondrial respiration rates in nondiabetic patients, PRE vs. POST-ACC (A) within control group (n=24) and (B) RIPC (n=23). V_0, basal respiration; V_{ADP}, subsaturating ADP (0.1mM); $V_{creatine}$, 20mM creatine; V_{max}, maximal respiration (2mM ADP); $V_{succinate}$, 10mM succinate (complex-II substrate); V_{amytal}, 1mM amytal inhibits complex-I; $V_{ascorbateTMPD}$, 0.5mM ascorbate and 0.5mM TMPD stimulate complex-IV. Data are displayed as mean ± SE.*$p<0.05$.



Supplemental Figure 3. Protein expression and phosphorylation in nondiabetic patients within control group (n=19) and RIPC (n=19). Total (non-phosphorylated) abundance and phosphorylated protein levels in controls and RIPC before (PRE) and after (POST) ACC. Representative western blotting images are presented. Data are presented as mean ± standard error. *p<0.05 vs. Control-PRE; #p<0.05 vs. RIPC-PRE.

Paper III

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Paper IV

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ORIGINAL RESEARCH

Comparison of left versus right atrial myocardium in patients with sinus rhythm or atrial fibrillation – an assessment of mitochondrial function and microRNA expression

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Keywords
Arrhythmia, atrium, microRNA, mitochondria.

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Clinical Trial Registration–URL: at http://www.clinicaltrials.gov. Unique identifier: NCT01493128.

Abstract

Several of the cellular alterations involved in atrial fibrillation (AF) may be linked to mitochondrial function and altered microRNA (miR) expression. A majority of studies on human myocardium involve right atrial (RA) tissue only. There are indications that AF may affect the two atria differentially. This study aimed to compare interatrial differences in mitochondrial respiration and miR expression in the RA versus left atrium (LA) within patients with sinus rhythm (SR) and AF. Thirty-seven patients with AF (n = 21) or SR (n = 16), undergoing coronary artery bypass surgery and/or heart valve surgery, were included. Myocardial biopsies were obtained from RA and LA appendages. Mitochondrial respiration was assessed in situ in permeabilized myocardium. MiR array and real-time quantitative polymerase chain reaction were performed to evaluate miR expression. Mitochondrial respiratory rates were similar in RA versus LA. Expression of miR-100, -10b, -133a, -133b, -146a, -155, -199a-5p, -208b, and -30b were different between the atria in both SR and AF patients. In contrast, differential expression was observed between RA versus LA for miR-93 in patients with SR only, and for miR-1, -125b, -142-5p, -208a, and -92b within AF patients only. These results indicate that mitochondrial respiratory capacity is similar in the RA and LA of patients with SR and AF. Differences in miR expressional profiles are observed between the RA versus LA in both SR and AF, and several interatrial differences in miR expression diverge between SR and AF. These findings may contribute to the understanding of how AF pathophysiology may affect the two atria differently.

Introduction

Atrial fibrillation (AF) is prevalent in the increasingly aging population, and is associated with serious complications (Go et al. 2001; Fuster et al. 2011). The underlying pathophysiology of AF is still not fully understood (Mathew et al. 2009). There are indications that AF may affect the two atria differentially, and because the human right atrial (RA) appendage is more accessible for sampling than the left atrial (LA) appendage, investigations of the human LA are particularly scarce (Caballero et al. 2010). Since the majority of studies investigating human

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tissue are restricted to the RA only, there is limited knowledge regarding interatrial differences in AF and sinus rhythm (SR) in human myocardium.

Despite the central role of mitochondrial respiration in cardiomyocyte function, research on mitochondrial function in AF is limited. Many of the pathophysiological processes implicated in AF are linked to mitochondrial function, including calcium homeostasis (Mihm et al. 2001; Neef et al. 2010; Reilly et al. 2011), formation of reactive oxygen species (ROS) (Mihm et al. 2001), and alterations of oxygen consumption (White et al. 1982). Findings of altered levels of phosphocreatine (Ausma et al. 2000), electron transport chain proteins (Reilly et al. 2011), and differences in mitochondrial distribution (Morillo et al. 1995) further implicate that mitochondria have a role in AF.

MicroRNA (miR) regulates gene expression through inhibition or degradation of mRNA and has been implicated as an important influential element in mitochondrial respiration (Li et al. 2012; Sripada et al. 2012) and cardiac arrhythmias (Luo et al. 2010; Wang et al. 2011), including AF (Shi et al. 2013; Santulli et al. 2014).

We recently presented a report where we compared biatrial mitochondrial function and miR expression between patients with AF versus patients with SR, and found that AF was associated with altered miR expression and elevated maximal mitochondrial respiratory rate as compared to SR (Slagsvold et al. 2014). In this paper, we assess interatrial differences in mitochondrial respiration and miR expression within each of these two patient groups.

Methods

Patients

A total of 37 patients with either SR (n = 16) or a history of AF (n = 21) scheduled for elective isolated coronary artery bypass grafting (CABG) surgery, mitral valve replacement (MVR) surgery, aortic valve replacement (AVR) surgery, or combined CABG/AVR surgery at St. Olav's Hospital, Trondheim University Hospital, Norway were included. Patients with either paroxysmal AF (pAF) (n = 11) or chronic AF (cAF) (n = 10) were included in the AF group. Blinding of patient characteristics was ensured until completion of data collection and processing. The study conformed to the principles outlined in the Declaration of Helsinki, and was approved by the Regional Committee for Medical Research Ethics of Norway. Written informed consent was obtained from patients prior to inclusion.

Study design

Standard pre- and peroperative procedures of the Department of Cardiothoracic Surgery, St. Olav's Hospital were followed. Thiopental, fentanyl, propofol, and cisatracurium were administered intravenously for anesthetics. Isoflurane was given during pulmonary ventilation, but not during cardiopulmonary bypass (CPB). CPB was performed at mild hypothermia of 32–34°C. Standard St. Thomas (Martindale Pharmaceuticals, Essex, United Kingdom) crystalloid or blood cardioplegia was administered for myocardial protection. Cardiac rhythm was monitored pre- and peroperatively by electrocardiography.

Tissue samples

Biopsies from the RA appendage were obtained during RA cannulation, and biopsies from the LA appendage during CPB. Each biopsy was immediately divided into two; one piece was promptly submerged in an ice-cold preservation solution for assessment of mitochondrial function, and the other snap-frozen in liquid nitrogen and kept on −80°C for miR analyses.

Mitochondrial respiration in situ

Mitochondrial respiration rates were measured *in situ* according to methods thoroughly described by other authors (Veksler et al. 1987; Saks et al. 1998; Zoll et al. 2002; N'Guessan et al. 2004; Kuznetsov et al. 2008). The myocardium was separated from connective tissue under a microscope, and myocardial cell membranes permeabilized by 50 μg/mL saponin for 30 min at 4°C while leaving mitochondrial membranes intact. This was followed by a > 10 min rinsing cycle in pure storage solution (2.77 mmol/L CaK_2EGTA, 7.23 mmol/L K_2EGTA (100 nmol/L free Ca^{2+}), 6.56 mmol/L $MgCl_2$ (1 mmol/L free Mg^{2+}), 20 mmol/L taurine, 0.5 mmol/L dithiothreitol (DTT), and 20 mmol/L imidazole 50 mmol/L potassium-methanesulfonate (CH_3KO_3S), 5.7 mmol/L Na_2ATP, 15 mmol/L phosphocreatine (PCr) (pH 7.1 at 22°C)), and subsequent 10 min rinsing in respiration solution (2.77 mmol/L CaK_2EGTA, 7.23 mmol/L K_2EGTA (100 nmol/L free Ca^{2+}), 1.38 mmol/L $MgCl_2$ (1 mmol/L free Mg^{2+}), 20 mmol/L taurine, 0.5 mmol/L dithiothreitol (DTT), and 20 mmol/L imidazole (pH 7.1 at 22°C), 90 mmol/L potassium-methanesulfonate (CH_3KO_3S), 10 mmol/L sodium-methanesulfonate (CH_3SO_3Na), 3 mmol/L K_2HPO_4, 10 mmol/L glutamate, 4 mmol/L malate, and 2 mg/mL bovine serum albumin). Mitochondrial respiratory assessment of the permeabilized atrial myocardium was performed by use of a Clark-type microcathode oxygen electrode (Strathkelvin Instruments, Glasgow, UK) covered by a fluorinated

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ethylene propylene (FEP) membrane, with tissue submerged in 3 mL respiration solution maintained at 22°C. Assessment of respiration in the presence of respiratory complex I substrates glutamate and malate, without adenosine diphosphate (ADP) (basal respiratory rate, V_0), was followed by assessment of respiration in the presence of subsaturating 0.1 mmol/L ADP (V_{ADP}). A volume of 20 mmol/L creatine was supplemented prior to measurement of $V_{creatine}$, and subsequently the addition of a saturating 2 mmol/L ADP allowed assessment of maximal respiration rate (V_{max}) with glutamate and malate as substrates for complex I through IV of the electron transport chain. $V_{succinate}$ was measured in the presence of complex II - substrate succinate (10 mmol/L) and subsequent complex I inhibition by amytal (1 mmol/L) preceded assessment of complex II (V_{amytal}). Ascorbate (0.5 mmol/L) and N,N, N',N'-tetramethyl-p-phenylenediamine (TMPD, 0.5 mmol /L) was added to induce complex IV activity ($V_{ascorbateTMPD}$) and before completion irreversible inhibition of complex IV by azide (4 mmol/L) allowed assessment of V_{azide}. Respiratory rates are given as micromoles O_2 per minute per gram dry weight myocardial tissue (μmol O_2/min/g dw). Acceptor control ratio (ACR), as calculated by the ratio V_{max}/V_0, estimates degree of coupling between oxidation and phosphorylation. The ratio of V_{ADP}/V_{max} quantifies mitochondrial sensitivity to ADP. Percent increase in respiratory rate after addition of creatine is given (↑RR Cr). The ratio V_{amytal}/V_{max} estimates excess respiration of the cytochrome oxidase complex. Estimation of the apparent constant of Michaelis for ADP was calculated in the absence (${}^{app}K_m^{(ADP-Cr)}$) and presence of creatine (${}^{app}K_m^{(ADP+Cr)}$) (N'Guessan et al. 2004).

MicroRNA expression

RNA isolation, miR array and real-time quantitative polymerase chain reaction (qRT-PCR) were performed by Exiqon Services, Vedbaek, Denmark. RNA quality was ascertained by use of Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA) prior to labeling for miR array by use of Exiqon's miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ and subsequent hybridization on the miRCURY LNA™ microRNA Array 6th gen (Exiqon, Denmark). Capture probes targeting all human miRs registered in miRBASE 16.0 were applied. Hybridization was performed with a Tecan HS4800™ hybridization station (Tecan, Austria). Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc.) was used to scan the array slides, followed by image analysis with ImaGene® 9 (miRCURY LNA™ microRNA Array Analysis Software, Exiqon, Denmark). Background correction of quantified signals was performed prior to normalization by applying the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. miR RT-qPCR was performed with miRCURY LNA™ Universal RT miRNA PCR pick and mix custom panel, and LightCycler® 480 Real-Time PCR System (Roche, USA) was used for amplification. PCR was performed for miR-1, -100, -106a, -106b, -10b, -125b, -133a, -133b, 138-1*, -142-5p, -144, -146a, -155, -15b, -17, -187, -18a, -18b, -191, -193a-3p, -199a-5p, -19a, -19b, -208a, -208b, -21, -23a, -25, -26a, -26b, -29b, -30a, -30b, -328, -363, -451, -486-5p, -590-5p, -600, -92a, -92b, -93. Roche LC software was applied for analyses of amplification curves both in determining crossing points (Cp) and melting curves. Algorithms similar to the LinReg software were used to quantify amplification efficiency. Normalization was based on the average of 5 normalization assays detected in all samples. Results of miR expression after RT-qPCR are presented as normalized crossing point (dCp, refers to the crossing point (Cp) of the specific miR after subtracting the average Cp of the normalization miRs).

Statistical analysis

SPSS 21.0 for Mac (IBM SPSS Statistics, Chicago, Illinois) was used for statistical analyses. Paired students t-test was applied for comparison of RA versus LA within each patient. Pearson's Chi^2 and Fisher's exact test were used for categorical data. A two-tailed P-value <0.05 was considered significant. Hochberg–Bonferroni-correction was applied to statistical analyses of miR expression. GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA) was used for graphics.

Results

Table 1 provides an overview of clinical data. The groups were comparable with regards to clinical characteristics, with the exception of a statistically significant larger percentage of mitral valve disease in patients with AF as compared to SR. Information on LA diameter was available for 12 patients in SR and 13 in AF and indicated a nonsignificant tendency of LA dilatation in patients with AF. All patients diagnosed with cAF and one of the patients with pAF were in AF on preoperative electrocardiography.

Mitochondrial respiration

No significant differences were observed in paired comparison of mitochondrial respiration rates in samples from the RA versus LA within either SR or AF (Fig. 1), nor were there any differences in other parameters of mitochondrial function (Table 2).

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Table 1. Patient characteristics and perioperative parameters.

	SR (*n* = 16)	AF (*n* = 21)	*P*
Age, μ ± SD	71 ± 8	70 ± 8	ns
Female gender, %	25	24	ns
Aortic valve disease, %	31	43	ns
Mitral valve disease, %	44	76	<0.05
Chronic obstructive pulmonary disease, %	6	5	ns
Coronary artery disease, %	75	81	ns
Diabetes Mellitus, %	13	16	ns
Hypertension, %	50	48	ns
Previous cerebral insult, %	0	5	ns
Previous myocardial infarction, %	31	24	ns
Normal left atrial diameter (≤40 mm), %	83	46	ns
Dilated left atrium (≥41 mm), %	17	54	ns
LV EF, μ ± SD	49 ± 8	52 ± 8	ns
Combined CABG/AVR-surgery, %	13	24	ns
Isolated AVR-surgery, %	19	19	ns
Isolated CABG-surgery, %	63	48	ns
Isolated MVR-surgery, %	6	10	ns
ACE-inhibitor/ATII inhibitor, %	44	57	ns
Antiarrhythmic agents, %	6	5	ns
Beta blocker, %	63	76	ns
Calcium antagonist, %	31	24	ns
Diuretics, %	38	48	ns
Digitalis, %	0	5	ns

μ, mean; SD, standard deviation; SR, sinus rhythm; AF, atrial fibrillation; AVR, aortic valve replacement; MVR, mitral valve replacement; CABG, coronary artery bypass graft; LV EF, left ventricular ejection fraction; ACE, angiotensin converting enzyme; ATII, angiotensin II receptor.

MicroRNA

MiR array revealed 75 miRs that were differentially regulated between the LA and RA (data not shown). Array was used as a preliminary explorative study to identify miRs for investigation with qRT-PCR. qRT-PCR revealed increased LA expression of miR-10b, -133a, -133b, and -30b, and decreased expression of miR-100, -146a, -155, -199a-5p, and -208b as compared to RA in both the SR group and the AF group (Fig. 2).

MiR-93 displayed decreased expression in the LA as compared to the RA within patients with SR only (Fig. 2A).

In samples from AF patients, increased expression of miR-1 and -208a and decreased expression of miR-125b, -142-5p, and -92b was observed in the LA as compared to the RA in addition to the differences described in both patient groups (Fig. 2B).

Discussion

Comparison of the LA and RA revealed similar mitochondrial respiratory function in the two atria for patients with SR as well as in patients with AF. Mitochondrial respiration has never previously been compared between the LA and RA of patients with AF, and to our knowledge, only one study has previously assessed mitochondrial function in both human atria, where atrial tissue from two patients without known cardiac disease, and three patients with chronic heart failure were examined, demonstrating similar mitochondrial respiration rates in RA as compared to LA in agreement with our study (Lemieux et al. 2011). These results indicate that, although a difference of interatrial pressures normally exists between the RA and LA, the difference in workload does not appear to have detectable impact on mitochondrial respiratory capacity. These findings are of interest because they may increase our understanding of how results obtained from one myocardial location may relate to another, especially due to the increased attainability of RA appendage tissue

Table 2. Mitochondrial parameters of the right (RA) versus left atrium (LA) in patients with sinus rhythm (SR) or atrial fibrillation (AF).

	SR			AF		
	LA (*n* = 10) μ ± SD	RA (*n* = 16) μ ± SD	*P*	LA (*n* = 21) μ ± SD	RA (*n* = 21) μ ± SD	*P*
V_{amytal}/V_{max}	1.3 ± 0.5	1.1 ± 0.3	ns	1.0 ± 0.3	1.0 ± 0.3	ns
V_{ADP}/V_{max}	0.5 ± 0.3	0.4 ± 0.3	ns	0.5 ± 0.1	0.4 ± 0.2	ns
ACR	4.2 ± 2.4	4.9 ± 3.9	ns	4.5 ± 1.7	5.0 ± 1.9	ns
${}^{app}K_m{}^{(ADP-Cr)}$	210 ± 168	194 ± 149	ns	141 ± 77	158 ± 101	ns
${}^{app}K_m{}^{(ADP+Cr)}$	76 ± 80	101 ± 123	ns	43 ± 34	65 ± 39	ns
↑RR Cr	65 ± 50	73 ± 57	ns	69 ± 33	63 ± 35	ns

Data presented as mean ± SD. n, number of patients; SR, sinus rhythm; AF, atrial fibrillation; V_{amytal}/V_{max}, quantification of excess respiration of the cytochrome oxidase complex; V_{ADP}/V_{max}, ADP sensitivity ratio; ACR, acceptor control ratio; ${}^{app}K_m{}^{(ADP-Cr)}$ approximate Michaelis-Menten constant for ADP (μM) in the absence of creatine; ${}^{app}K_m{}^{(ADP+Cr)}$, apparent Michaelis-Menten constant for ADP (μM) in the presence of creatine; ↑RR Cr, increase in respiration rate after addition of creatine.

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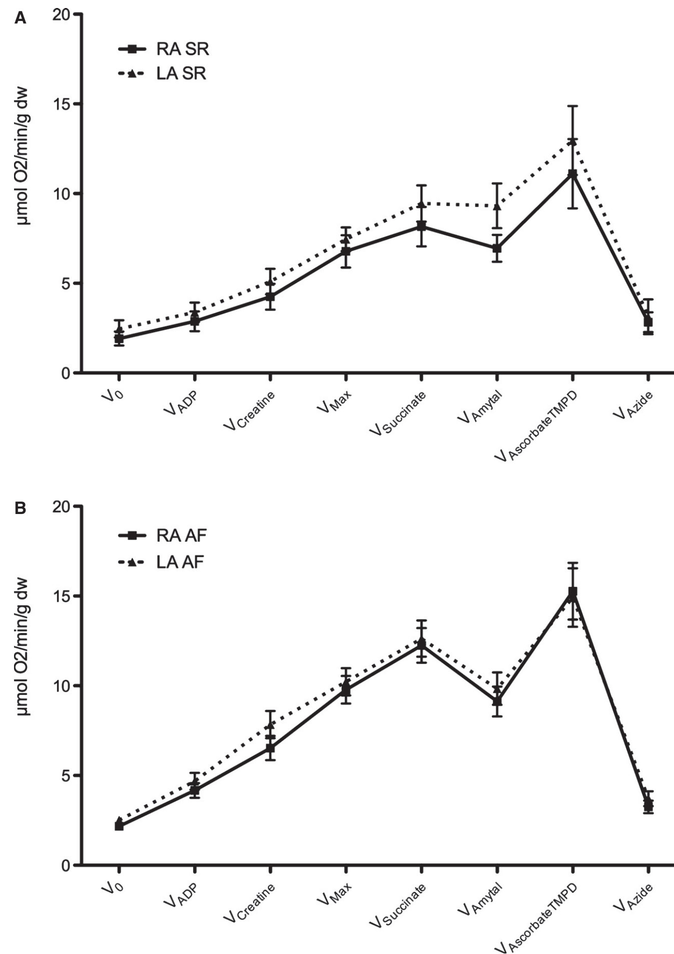

Fig. 1. Mitochondrial respiration rates within the left (LA) versus right atrium (RA) of patients with (A) sinus rhythm (SR) and (B) atrial fibrillation (AF). Respiratory rates are given as mean ± SEM (error bars) in µmol O_2/minute/g dryweight myocardial tissue, and are measured after subsequent addition of the following substrates and inhibitors: V_0, basal respiration with glutamate and malate as substrates for Complex I of the electron transport chain; V_{ADP}, respiration in the presence of a subsaturating amount of adenosine diphosphate (ADP); $V_{creatine}$, respiration rate after creatine supplement; V_{max}, maximal respiration rate in the presence of glutamate and malate with a saturating amount of ADP; $V_{succinate}$, respiration rate with Complex II substrate succinate; V_{amytal}, respiration during inhibition of complex I by amytal; $V_{ascorbateTMPD}$, respiratory rate with induction of complex IV activity through ascorbate (0.5 mmol/L) and N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD, 0.5 mmol/L), V_{azide}, respiration after irreversible inhibition of complex IV by azide.

in investigations involving the human myocardium (Ausma et al. 2000; Mihm et al. 2001; Kim et al. 2005; Reilly et al. 2011). It could have been expected that mitochondrial respiratory capacity would have been increased in the LA as compared to the RA of patients with AF due to an increased frequency of elevated LA pressure in this patient group (Stefanadis et al. 2001). However, our study does not support this notion. This may, however, depend on the extent of LA dilatation, and it must be kept in mind that our patient population did not reveal significant differences in LA diameter between the patient populations in AF versus SR; thus it cannot

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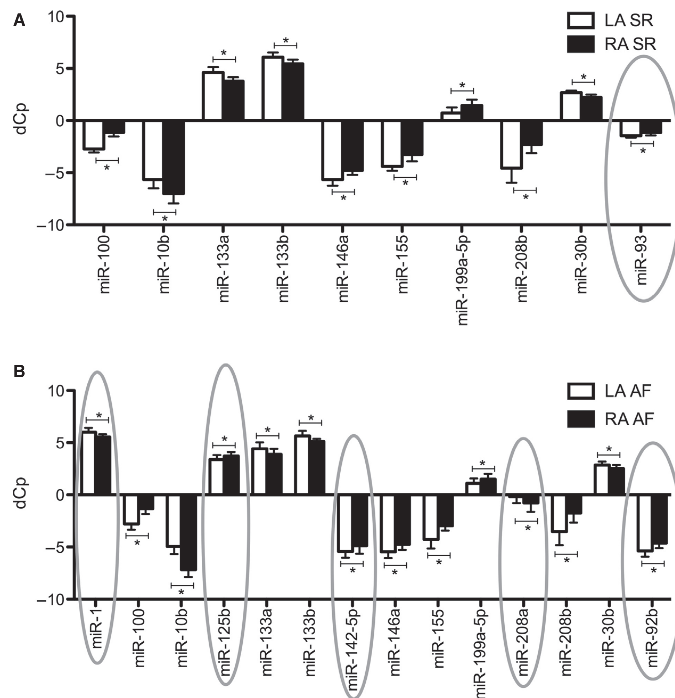

Fig. 2. Significant differences in microRNA (miR) expression between the left (LA) versus right atrium (RA) within patients with (A) sinus rhythm (SR) and patients with (B) atrial fibrillation (AF). dCp, normalized crossing point. Circles indicate interatrial differences in miR expression that are found exclusively within either SR or AF patients. Error bars indicate standard deviation. *$P < 0.05$.

be excluded that the results may differ in other patient populations with greater differences in LA diameter.

We observed a number of interatrial differences in miR expression that are common to both patient groups, whereas there are other interatrial differences in miR expression that differ between SR and AF patients. Differences in miR expression between LA and RA that are found in both patients groups may be ascribed to anatomic and physiological differences in the RA as compared to the LA (increased LA expression of miR-10b, -133a, -133b, and -30b, and decreased expression of miR-100, -146a, -155, -199a-5p, – 208b, as compared to RA).

The reduced expression of miR-93 in the LA versus RA of patients with normal SR may also be related to a difference of anatomical and physiological regions. It has been demonstrated that miR-93 has proangiogenic effects, promotes perfusion recovery after ischemia, and is associated with improved cell survival after hypoxia and serum starvation in endothelial and skeletal muscle cells (Hazarika et al. 2013).

Interatrial differences found in AF patients only include increased LA expression of miR-1 and -208a and decreased expression of miR-125b, -142-5p, and -92b as compared to the RA. Whereas electrophysiological mapping has indicated that myocardial areas in both the RA and LA may be responsible for activating atrial premature complexes in patients with AF (Natale et al. 2000), there are indications that electrical remodeling is altered differentially in RA as compared to LA in patients with AF (Caballero et al. 2010). Overexpression of miR-1 has been implicated in promoting arrhythmogenesis (Terentyev et al. 2009). Both overexpression and underexpression of the cardiospecific miR-208a have been demonstrated to induce cardiac arrhythmia by disturbing atrial electrophysiology in mice (Callis et al. 2009). Blocking cardiac miR-208a expression prevented cardiac remodeling and dysfunction and promoted health and survival of rats in a study of chronic hypertension and diastolic heart failure (Montgomery et al. 2011). In our previous report where we compared atrial miR expression between patients with

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SR versus AF, miR-208a expression was significantly lower in AF patients as compared to patients with SR in both atria (Slagsvold et al. 2014). MiR-125b is upregulated under hypoxia and upon stimulation with VEGF in skeletal muscle (Muramatsu et al. 2013). It has been demonstrated that miR-142-5p regulates genes involved in cell cycle control and increases proliferation and DNA synthesis in vascular smooth muscle cells of rats (Kee et al. 2013). Reduced myocardial expression of miR-92b has been demonstrated in mice with experimentally induced heart failure (Dirkx et al. 2013).

Investigations of miR expression in human myocardium are still at an early stage, and this study provides information of myocardial miR expression that is strictly descriptive in nature. Moreover, these data do not provide information as to whether any direct link exists between miR expression and mitochondrial function. Many unknown factors still need clarification, and even associative observations are valuable in the process of obtaining an understanding of miR in cardiovascular pathophysiology. Although direct comparison of the results are precluded due to differences in patient characteristics, we identified two other studies reporting comparative assessment of miR expression in the LA versus RA, one investigating patients with AF and valvular heart disease (Cooley et al. 2012), and the other patients with rheumatic mitral valve disease and either SR or AF (Liu et al. 2014). It is evident that continued research is necessary to establish representativeness of miR expression for specific patient populations, especially due to the common occurrence of concurrent heart disease in patients with AF.

Study limitations

The atrial appendage is selected for investigation as it constitutes the most accessible location for sampling human myocardial tissue. Whether AF-related alterations on the cellular level prevail throughout the atrium has not yet been assessed in humans, thus it has not been ascertained that tissue from the atrial appendage is representative to the remaining atrial myocardium of patients. However, it has been demonstrated that structural AF-related alterations in the atrial appendages of goats corresponded to that of the remaining atrial myocardium (Ausma et al. 1997).

Due to the frequent coexistence of cardiac disease in AF, distinguishing AF-related alterations from those of concomitant heart disease presents a challenge in investigations of AF. Although the clinical characteristics of the patients groups in our study were comparable in most aspects, there was a difference in prevalence of mitral valve disease. Although investigations of "lone" AF have been demonstrated in animal models (Morillo et al. 1995; Ausma et al. 1997), a potential interference of comorbidity cannot be excluded in our clinical study and further research is required to verify reproducibility.

Our investigation provides a description of associative alterations in miR expression and mitochondrial function. Further investigations are required to explore causal relations between mitochondrial respiration, miR, and AF.

Conclusion

Mitochondrial respiration is similar between the RA and LA in patients with normal SR as well as in AF. Interatrial differences in miR expression are observed within both patients with SR and patients with AF, and the interatrial differences in miR expression profiles diverge between patients with normal SR and patients with AF.

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Conflict of Interest

None declared.

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