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**THE ROLE OF ENERGY METABOLISM IN CARDIOPROTECTION INDUCED BY THE
ADAPTATION TO CHRONIC HYPOXIA**

**ÚLOHA ENERGETICKÉHO METABOLISMU V KARDIOPROTEKCI INDUKOVANÉ
ADAPTACÍ NA CHRONICKOU HYPOXII**

Doctoral thesis

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2018

DECLARATION OF THE AUTHOR:

I hereby declare that I have completed this thesis independently, except where explicitly indicated. It documents my own work, which was carried under the supervision of Dr. Jitka Žurmanová, Ph.D. Throughout, I have properly acknowledged and cited all sources used. Neither this thesis nor its substantial part has been submitted to obtain this or other academic degree.

In Prague

.....

Mgr. David Kolář

DECLARATION OF THE CO-AUTHOR

On behalf of all co-authors, I hereby declare that Mgr. David Kolář has essentially contributed to the formation of the articles which represent an integral part of this dissertation thesis. He has performed most of the experiments, especially in the paper where he stands as the first author and significantly contributed to the study where he stays as a second author for his active participation in the set-up of the experiments, in the interpretation of the data, and in the preparation of the manuscripts.

In Prague

.....

RNDr. Jitka Žurmanová, Ph.D.

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One can gaze and see a wonder in it. Two can gaze together and share this wonder. I believe that we are ever-emitting sparks of imagination, so let's spark together as only together, we can push back the boundaries of the stellar horizon.

Dedicated to my mother, Ivana, for everything and more.

ABSTRACT:

Cardiac energy metabolism is the one of the most complex system in the body. To sustain life, but also to respond quickly to any sudden changes (e.g. running, emotional stress), the heart has developed a unique ability and has become a metabolic “omnivore”. At physiological conditions, long chain fatty acids (LCFAs) present the major energetic source for the adult myocardium. However, the cardiac energy metabolism may be compromised during pathophysiological states. One of the most dangerous is, undoubtedly, ischaemia-reperfusion injury with its acute form, myocardial infarction. However, the adaptation to chronic hypoxia has been known for decades for its cardioprotective effect against I/R. Changes of cardiac energy metabolism induced by the adaptation have not been fully explored and the system conceals still too many secrets. This thesis has aimed to determine how adaptation to chronic hypoxia affects the cardiac metabolism of the rat LVs in the following set-ups:

1. The effect of chronic normobaric hypoxia (CNH; 3 weeks, 5500m) during a brief I/R protocol *in vitro* on the protein kinase B/hexokinase (Akt/HK) pathway, including the expression and phosphorylation of Akt, the expression and localization of HK, the expression of mitochondrial creatine kinase (mtCKS), and the level of Bcl-2 family proteins as well as the amount of activated adenosine monophosphate-dependent protein kinase (pAMPK α Thr¹⁷²).
2. The effect of CNH (3 weeks, 5500m) on the expression of major cardiac Akt isoforms, Akt1 and Akt2 with subsequent determination of HK enzyme activity in LVs of spontaneously hypertensive rat (SHR) and conplastic strain harbouring mitochondrial genome from Brown Norway strain, SHR-mtBN.
3. The effect of intermittent hypobaric hypoxia (IHH; 8h/day, 35 exposures, 7000m) on LV β -oxidation rates with respect to subcellular domains, on the expression of electron transport system (ETS) complexes (CI-CV), on respiratory rates, on glycolytic apparatus, NAD(H) pools, CK system, and finally, on mitochondrial susceptibility to Ca²⁺ overload.

The results have shown that:

1. Adaptation to CNH evoked an increase in HK1, HK2 expression and HK activity in ischaemia, concomitantly with pAkt Ser⁴⁷³. HK colocalization with mitochondria was not affected in CNH, whereas I/R cause HK disruption in N LVs. CNH prevented downregulation of mtCKS during I/R and decreased Bax/Bcl-2 ratio, suggesting a lower probability of apoptosis. Importantly, pAMPK α Thr¹⁷² levels were lower in CNH LVs during ischaemia, suggesting energetic advancement.
2. Adaptation to CNH upregulated Akt2 isoform in SHR-mtBN only. In contrast, no alterations of Akt1 expression in both strains neither Akt2 in CNH adapted SHR could be observed. Nevertheless, CNH upregulated the activity of HK in SHR-mtBN homogenates more profoundly than in SHR littermates. Mitochondrial HK activity was elevated in both strains similarly after CNH.
3. Adaptation to IHH decreased the β -oxidation rates in subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria with concomitant reduction of CI+CII-stimulated respiration. The expression of CI and CIV was downregulated in IHH LVs. The activity of CI and citrate synthase, but not that of CII dropped after IHH. The downregulated CI expression and activity probably cause the elevated NADH/NAD⁺ ratio in IHH. Surprisingly, acyl-CoA oxidase (ACOX) as well as peroxisomal lactate dehydrogenase (LDHBx) were upregulated, suggesting that peroxisomes partially overtake FAs processing in IHH LV. The enzyme activity of glycolytic apparatus, phosphofructokinase (PFK) and LDH, were upregulated after

the adaptation. IHH evoked a complete remodelling of CK system with CKB upregulation and translocation to M-line of sarcomere. The susceptibility to mitochondrial permeability transition pore opening was improved after IHH.

These data support the view that cardiac energy system remodelling presents the basis of cardioprotection against I/R induced by the adaptation to chronic hypoxia. Our data, showing the improvement of carbohydrate metabolism with concomitant downregulation of FA metabolism, and CK system remodelling, may uncover the important aspects of the protective adaptation and thus contribute to the development of future treatment of myocardial I/R injury. Nevertheless, although the research may not lead to the kind of knowledge that can be expected to give immediate practical benefits, its importance in spite of its theoretical character is immense.

Key words: heart, energy metabolism, mitochondria, peroxisome, glycolysis, β -oxidation, chronic hypoxia

ABSTRAKT:

Energetický metabolismus srdce představuje jeden z nejkompexnějších systémů těla vůbec. Nejen k zachování samotného života, ale také k tomu, aby bylo dokonale připravené na akutní změny (např. fyzická aktivita, emoční stres), si srdce vytvořilo jedinečnou schopnost využívat veškerých energetických substrátů (je tzv. metabolický „všežravec“). Za fyziologického stavu představují mastné kyseliny s dlouhým řetězcem primární substrát pracovního myokardu dospělého srdce. Nicméně, energetický metabolismus může být ovlivněn řadou patofyziologických stavů, které provázejí různá onemocnění. Jedním z nejnebezpečnějších onemocnění je bezesporu ischemicko-reperfúzní (I/R) poškození srdce a jeho akutní forma, infarkt myokardu. V několika posledních letech se v boji proti I/R osvědčily různé formy ischemického preconditioningu, avšak adaptace na chronickou hypoxii je pro svůj benefiční účinek v I/R známá již několik desetiletí. Změny energetického stavu srdce vyvolané adaptací na chronickou hypoxii nejsou zcela prozkoumány, a tak se v systému stále skrývá velké množství tajemství. Tato práce si klade za cíl zjistit, jakým způsobem ovlivňuje adaptace na chronickou hypoxii energetický metabolismus levé komory (LV) potkana v následujících modelech:

1. Vliv chronické normobarické hypoxie (CNH, 3 týdny, 5500 m) během krátkého I/R protokolu *ex vivo* na signalizační dráhu protein kináza B/hexokináza (Akt/HK) včetně vlivu na expresi a míru fosforylace Akt, expresi, lokalizaci a aktivitu HK, expresi mitochondriální kreatin kinázy (mtCKS) a hladin protein z rodiny Bcl-2. Dále pak množství aktivované adenosin dependentní protein kinázy (pAMPK α Thr¹⁷²).
2. Vliv CNH (3 týdny, 5500 m) na expresi majoritních srdečních isoform Akt, Akt1 a Akt2 s následnou analýzou enzymatické aktivity HK v homogenátu a mitochondriální frakci LV spontánně hypertenzního potkana (SHR) a konplastického kmene přechovávajícího mitochondriální genom kmene Brown Norway, SHR-mtBN
3. Vliv intermitentní hypobarické hypoxie (IHH, 8 h denně, 35 expozic, 7000 m) na rychlost β -oxidace subsarkolemálních (SSM) a interfibrilárních (IFM) mitochondrií, expresi komplexů (CI-CV) mitochondriálního elektron-transportního systému (ETS), míru spotřebu kyslíku u SSM a IFM, glykolytický aparát, specifické koncentrace NAD(H), CK systém. Konečně byl sledován vliv IHH na náchylnost mitochondrií k Ca²⁺ přetížení.

Výsledky ukázaly, že:

1. Adaptace na CNH vyvolala navýšení exprese HK1, HK2 a zvýšila celkovou aktivitu HK v ischemii, podobně jako vyvolala vyšší míru fosforylace pAkt Ser⁴⁷³. Lokalizace HK s mitochondriemi byla v průběhu I/R protokolu zachována jen u CNH, zatímco u kontrolních zvířat došlo v průběhu I/R k její translokaci. CNH zabránila snížení exprese mtCKs během ischemie a též snížila poměr Bax/Bcl-2, což nasvědčuje snížené pravděpodobnosti apoptózy. U CNH zvířat nebyla pozorována aktivace pAMPK α Thr¹⁷² na takové úrovni, jako tomu bylo u kontrolních LV.
2. Adaptace na CNH způsobila navýšení isoformy Akt2 pouze u SHR-mtBN. CNH tak neměla vliv ani na expresi Akt1 ani na expresi Akt2 u SHR. Nicméně, po adaptaci na CNH došlo k nárůstu aktivity HK v homogenátu SHR-mtBN více než tomu bylo u SHR. Na druhou stranu, CNH vyvolala navýšení HK aktivity mitochondriální frakce u obou kmenů obdobně.
3. Adaptace na IHH vedla ke snížení míry β -oxidace v SSM i IFM, a též k nižší míře respirace indukované substráty pro CI a CII. IHH snížila expresi CI a CIV v

homogenátu LV. Enzymatické aktivity CI a citrát syntázy byly sníženy, avšak IHH neměla vliv na aktivitu CII. Snížená aktivita CI zřejmě stojí na pozadí navýšeného poměru NADH/NAD^+ pozorovaného v IHH LV. V kontrastu s mitochondriálním metabolismem, exprese acyl-CoA oxidázy (ACOX) stejně jako peroxisomální laktát dehydrogenázy (LDHBx) byly navýšeny, což nasvědčuje částečné kompenzaci peroxisomy v utilizaci mastných kyselin po IHH. Enzymy glykolytického aparátu, fosfofruktokináza a LDH, navýšily po IHH svou aktivitu. Po IHH došlo k masivní přestavbě CK systému s navýšením exprese CKB a její translokací do M-linie sarkomery. Pravděpodobnost otevření mitochondriálního póru byla po adaptaci na IHH snížena.

Tato data naznačují a zároveň podporují skutečnost, že transformace energetického metabolismu srdce představuje základ kardioprotekce indukované adaptací na chronickou hypoxii v boji proti I/R. Prvky navýšení cukerného metabolismu, útlumu metabolismu mastných kyselin a remodelace CK systému, ale i úloha peroxisomů a metabolická komunikace jednotlivých kompartmentů mohou pomoci odhalit důležité činitele, a tak přispět k prevenci a léčbě I/R poškození. Nicméně, ačkoliv tento výzkum nemusí vyústit v druh poznání, který bude mít okamžité praktické využití, jeho důležitost v rámci teoretického poznání je nezměrná.

Klíčová slova: srdce, energetický metabolismus, mitochondrie, peroxisomy, glykolýza, β -oxidace, chronická hypoxie

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LIST OF ABBREVIATIONS

AA	Amino acid
Ab	Albumin
AB	Assay buffer
ACO	Aconitase
ACOX	Acyl-CoA oxidase
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AKAP1	A kinase anchor protein
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-dependent protein kinase
ANT	Adenine nucleotide translocase
ATP	Adenosine triphosphate
BN	Brown Norway strain
CAC	Carnitine/Acylcarnitine carrier
CaK	Cassein kinase
CaMKK	Calmoduline dependent kinase kinase
CAT	Catalase
CDC	Centre for disease control and prevention
CK _μ	Creatine kinase, brain-type (CKB); muscle-type (CKM)
CNH	Chronic normobaric hypoxia
CoA	Coenzyme A
CPT	Carnitine-palmitoyl transferase
CS	Citrate synthase
Drp1	Dynamin-related protein 1
ETS	Electron transport system
FA	Fatty acid
FABP	Fatty acid binding protein
FADH ₂	Flavine adenine dinucleotide
FAT	Fatty acid translocase
FBP	Fructose-2,6-bisphosphatase
FH	Fumarate hydratase
FoxO	Forkhead BoxO
Fru-2,6-PP	Fructose-2,6-bisphosphate
FUNDC1	FUN4-containing domain 1
G-6-P	Glucose-6-phosphate

GAPDH	Glyceraldehydephosphate dehydrogenase
GLUT	Glucose transporter
GPD	Glycerol-3-phosphate dehydrogenase
GTP	Guanosine triphosphate
H-FABP	Heart type of fatty acid binding protein
HIF	Hypoxia-inducible factor
HK	Hexokinase
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
CHH	Chronic hypobaric hypoxia
ChREBP	Carbohydrate responsible element binding protein
I/R	Ischaemia-reperfusion
ICD	Isocitrate dehydrogenase
IFM	Interfibrillar mitochondria
IHH	Intermittent hypobaric hypoxia
IMM	Inner mitochondrial membrane
INH	Intermittent normobaric hypoxia
K ⁺ _{ATP}	ATP-sensitive K ⁺ channel
KC	Krebs cycle
LCFA	Long-chain fatty acid
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LKB1	Liver kinase B1
MCFA	Medium-chain fatty acid
MCT	Monocarboxylate transporter
MDH	Malate dehydrogenase
MPC	Mitochondrial pyruvate carrier
mPTP	Mitochondrial permeability transition pore
mtCKS	Mitochondrial creatine kinase (sarcomeric)
mTOR(C)	Mammalian target of rapamycin (complex)
MUFA	Monounsaturated fatty acid
N	Normoxia
NADH	Nicotinamide adenine dinucleotide
NCX	Sodium-calcium exchanger
NHE	Sodium-hydrogen exchanger
OGDC	2-oxoglutarate dehydrogenase complex
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation

PC	Palmitoyl-carnitine
PCr	Phosphocreatine
PDH	Pyruvate dehydrogenase
PDHC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PDP	Pyruvate dehydrogenase phosphatase
PMCA	Plasma membrane Ca^{2+} -pump
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
PGC-1 α	Peroxisome proliferator activated receptor γ coactivator-1 α
PI3K	Phosphatidylinositol-dependent protein kinase
PK_	Protein kinase, A (PAK); C (PKC)
PNM	Perinuclear mitochondria
PPAR_	Peroxisome proliferator activated receptor α , (PPAR α); β/δ (PPAR β/δ); γ (PPAR γ)
PUFA	Polyunsaturated fatty acid
PYC	Pyruvate carboxylase
QH2	Ubiquinone
Rab	The Rab family of protein
RISK	Reperfusion Injury Salvage Kinase pathway
ROS	Reactive oxygen species
RXR	Retinoid X receptor
RyR	Ryanodine receptor
SCFA	Short-chain fatty acid
SCOS	Succinyl-CoA synthase
SERCA	Sarcoplasmic reticulum Ca^{2+} pump
SHR	Spontaneously hypertensive rat
SHR-mtBN	Spontaneously hypertensive rat harbouring mitochondrial genome of BN
SOD	Superoxide dismutase
sREBP	Sterol responsible element binding protein
SS	Starting solution
SSM	Subsarcolemmal mitochondria
TAG	Triacylglycerol
TCA	Tricarboxylic acid
VDAC	Voltage-dependent anion channel
VLCFA	Very long-chain fatty acid
VLDL	Very low density lipoprotein
WHO	World Health Organization

LIST OF PUBLICATIONS

DOCTORAL THESIS-RELATED PUBLICATIONS

Kolar, D., Gresikova, M., Waskova-Arnostova, P., Elsnicova, B., Kohutova, J., Hornikova, D., Vebr, P., Neckar, J., Blahova, T., Kasparova, D., et al. (2017). Adaptation to chronic continuous hypoxia potentiates Akt/HK2 anti-apoptotic pathway during brief myocardial ischemia/reperfusion insult. *Mol. Cell. Biochem.* 432, 99–108.

IF = 2.669

Nedvedova, I., **Kolar, D.**, Elsnicova, B., Hornikova, D., Novotny, J., Kalous, M., Pravenec, M., Neckar, J., Kolar, F., and Zurmanova, J.M. (2018). Mitochondrial genome modulates myocardial Akt/GLUT/HK salvage pathway in spontaneously hypertensive rats adapted to chronic hypoxia. *Physiol. Genomics* physiolgenomics.00040.2017.

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IF = 4.936

Manakov, D., **Kolar, D.**, Zurmanova, J., Pravenec, M., and Novotny J. (2018). Changes in the activity of some metabolic enzymes in the heart of SHR rat incurred by transgenic expression of CD36. *Journal of Physiology and Biochemistry*

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1. INTRODUCTION

“IMAGINATION IS EVERYTHING. IT IS THE PREVIEW OF LIFE’S COMING ATTRACTIONS “.

ALBERT EINSTEIN

As the circulation is an evolutionary consequence of the increasing body size and complexity, higher eukaryotes including human, require this system in their development and life to work as accurate as possible. A wonderful example depicting the essential role of cardiovascular system is the fact that the heart is the first organ to form during the development of the body (from the functional point of view). Cardiac development begins during early gastrulation and the heart is almost completely formed *in utero* (from the structural standpoint). And since only minor architectural remodelling occurs at birth, the cardiac morphogenesis may be essentially considered as an embryonic phenomenon [1,2]. However, to develop, to differentiate either pre- or postnatally, and to sustain continuous work, the heart relies mainly on its energy metabolism [3–6].

Interestingly, cardiac energy metabolism also undergoes a progressive development during the pre- [3,7], and early postnatal life [8–10]. The adult heart reaches the highest ATP requirement of ~30 kg daily in humans. To fulfil these demands, almost all of ATP is derived from mitochondrial OXPHOS with only minor provision of glycolysis and GTP generation by Krebs cycle (KC). As the relative ATP content within the cardiomyocytes is low and the ATP hydrolysis rates therein are high, the heart acts as a “omnivore” metabolizing a spectacular assortment of carbon substrates as energy sources. Fatty acid (FA) β -oxidation process, however, represents the major pathway how the adult heart replenishes ATP molecules [11]. Therefore, beside a continuous influx of variety of substrates, the heart insists upon a continuous supply of oxygen for its proper function.

Not only the developmental changes associate with myocardial energy metabolism alterations, so do the cardiac and cardiovascular diseases. Indeed, a general decrease in oxidative capacity and down-regulation of FA oxidation enzymes have been reported in different models of heart failure [12]. Cardiac mitochondria isolated from diabetic models show a decreased capacity for fatty-acid supported respiration [13]. In mouse models of obesity and diabetes, mitochondria have displayed a reduced oxidative capacity and so is in the diabetic model of cardiomyopathy [14,15]. Hypertension, hypertrophy, and ischaemia-reperfusion (I/R) all cause metabolic disturbances within the heart [16–19]. The ever-increasing number of cardiovascular diseases morbidity and mortality rates (WHO data) together with the studies above furtherly underline the

importance of the most accurate cardiac energy metabolism knowledge. Hence, uncovering the metabolic remodelling which occurs at any of these pathophysiological conditions may reveal essential targets in future interventions and treatment.

The scientific history of cardiac energetics goes to the ancient Greek culture. For Greeks the heartbeat was perceptible, thus taken as a sign of life and vitality. One of the earliest hypothesis was proclaimed by Aristotle: “*Animation and the possession of vitality are necessarily associated with heat...in whatever region of the body and in whatever organ the principle of heat is primarily located, there also must be the original vital principle of nutrition. Nature kindles the nutritive function in this fire of nature. In the heart, there is a continual accession of liquids from the nourishment; and their expansion by heat extending to the walls of this organ causes its pulsations* “. The hypothesis was the first to point towards the fact that for regular cardiac function there needs to be an energy turnover with continuous supply. After, a long lag phase had occurred until the beginning of 16th century, where new ideas and hypotheses concerning mainly cardiac anatomy and to less extent physiology were postulated. The 18th and 19th century may be seen as the physiologically dominated era. In these centuries, the development of isolated heart preparations started by works of Ringer, Martin, and Langendorff. In 1895, a classic study concerning the dynamics of heart muscle was published by Frank, which was approved and extended by works of Wiggers, Straub, and Starling several years later. However, studies on myocardial energy metabolisms itself started later with the works of Petenkofer, von Voit, and Rubner who approved the validity of the 1st law of thermodynamics in biological systems (excellently reviewed in [406]). Since then, biochemical analyses have profoundly deepened our understanding of the cardiac energy system which is summarized in Chapter 2.

2. CARDIAC ENERGY METABOLISM

“TO SUPPOSE THAT CHEMISTRY AND POETRY ARE INCOMPATIBLE, OR THAT BIOLOGY IS INCONSISTENT WITH A RELIGIOUS OUTLOOK ON THE WORLD (NOT WITH THEOLOGY) IS TO MISUNDERSTAND ENTIRELY WHAT THE HUMAN MIND, BY CONTEMPLATION AND EXPERIMENT, HAS ACHIEVED. BY EXTREME SPECIALIZATION AT INTERVALS, BY OVERLOADING THE MACHINE TO ITS LIMIT, DISCOVERIES AND PROGRESS ARE MADE: BUT THEIR BEARING IS BEST SEEN BY LETTING THE ENGINE IDLE, AND GIVING ONESELF TIME TO LOOK AROUND ”.

ARCHIBALD VIVIAN HILL

Since 1929, when German scientist Karl Lohmann isolated the molecules of adenosine triphosphate for the first time [20], many major discoveries concerning energy metabolism have been made [21–24] plus those of Krebs and Boyer [407, 408] and more. Although we now know, besides many more, that the cardiac muscle energy metabolism lies within the mitochondria where FAs are being metabolized as major substrate, decades of “paving the path to knowledge” have passed.

Cardiac ATP store levels are not high enough to assure beating which lasts longer than few seconds. For this reason, the cardiomyocytes are dependent on a continuous supply of oxygen and metabolites. However, cardiac muscle can adapt very quickly to increased energy demand or compromised energy supply [25,26]. The cardiomyocytes thus may shift from almost 100% FA-derived energy production to states in which there is a higher dependence on pyruvate oxidation, ketone bodies uptake, or glycolysis. The ability of using all energy substrates is unique, on the other hand - in concept of its constant and vital function - also very reasonable. Nevertheless, it may also reflect the cardiac development stages during pre- and postnatal life. The early embryonic heart depends strongly on glycolysis. In mouse, it was shown that pyruvate is the main source utilized oxidatively by pre-implanted embryo and that glucose and glycolysis becomes predominant energy sources by the stage of blastocyst [27]. Addition of iodoacetate (inhibitor of glyceraldehyde-3-phosphate dehydrogenase) to 11 and 12 days old rat embryos induced contractile dysfunction with a complete disappearance of contractile activity within 2 hours after administration [28]. Taken from the other end, glycogen stores in mouse fertilized eggs are relatively low but as soon as the embryo reaches the one-cell stage, substantial amount of glycogen can be measured [29]. The described event is valid for other rodent models [30] but also for human [31]. Following implantation, due to the establishment of blood flow to the embryo, mitochondrial biogenesis and

maturation partakes in oxidative metabolism reconciliation. So concomitantly with these alterations in mitochondrial biology, the expression of glycolytic enzymes drops whereas the enzymes of mitochondrial electron transport system (ETS) and those of Krebs cycle (KC) increases (summarized in Figure 1). The importance of glycolysis even in this stage is highlighted by studies where mutations in several glycolytic enzymes result in abnormal cardiac development or post-implantation lethality [29,32,33]. Although there is almost a 3-fold increase in maternal plasma TAG levels between 1st and 3rd trimester [34], FA catabolism becomes the predominant source of energy not sooner than postnatally [3,9,35]. In this time, the final maturation of the cardiomyocyte occurs modulated by biochemical, paracrine but also mechanical factors [36]. The following lines aim to describe the basics of mature cardiomyocyte high complex energy system.

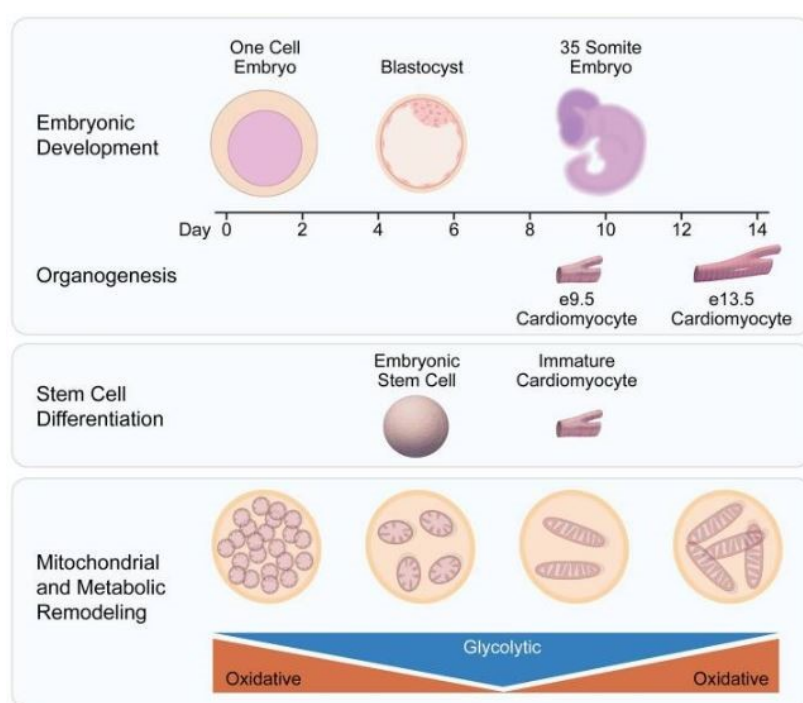


Figure 1: Prenatal development of cardiac metabolism. One cell embryo possesses the inherited mitochondrial genome from mother. The subsequent division of one cell embryo to blastocyst is followed by a decrease in mitochondrial mass, thus in oxidative metabolism. The embryonic dependence on glycolysis increases up to the point when mitochondria start to differentiate and replicate. Between e9.5 and e13.5, a steep increase in oxidative capacity can be observed. From [7]

2.1. Carbohydrate – fatty acid competition

Although the mature cardiomyocytes derive most of their energy from FA utilization, a competition between glucose and fatty acids and ketone bodies for their oxidation and uptake exists [37]. The first person who described the occurrence of an alternating interaction between glucose and FA metabolism in muscle and adipose tissue was Sir Philip J. Randle [24]. By the

discoverer, the phenomenon was called Randle cycle (although it has nothing in common with metabolic cycles like Krebs cycle, yet it describes the dynamic interaction between substrates) demonstrated in Figure 2. Since Randle's discovery, introducing a new dimension of control in cellular metabolism, much has been learned about the energy metabolism substrate cross-talk [38].

Figure 2: Glucose fatty-acid cycle: This is the original scheme from [24] showing an inter-competition of carbohydrate and FA metabolism between skeletal muscle and adipose tissue.

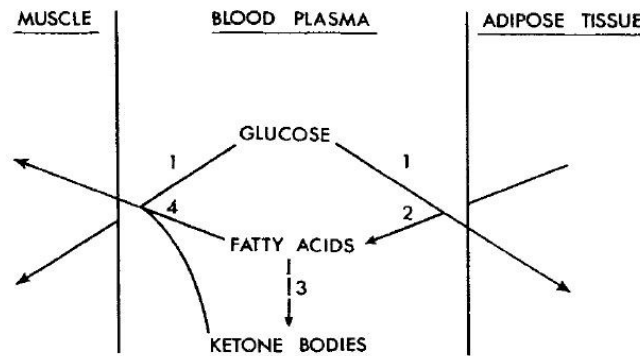


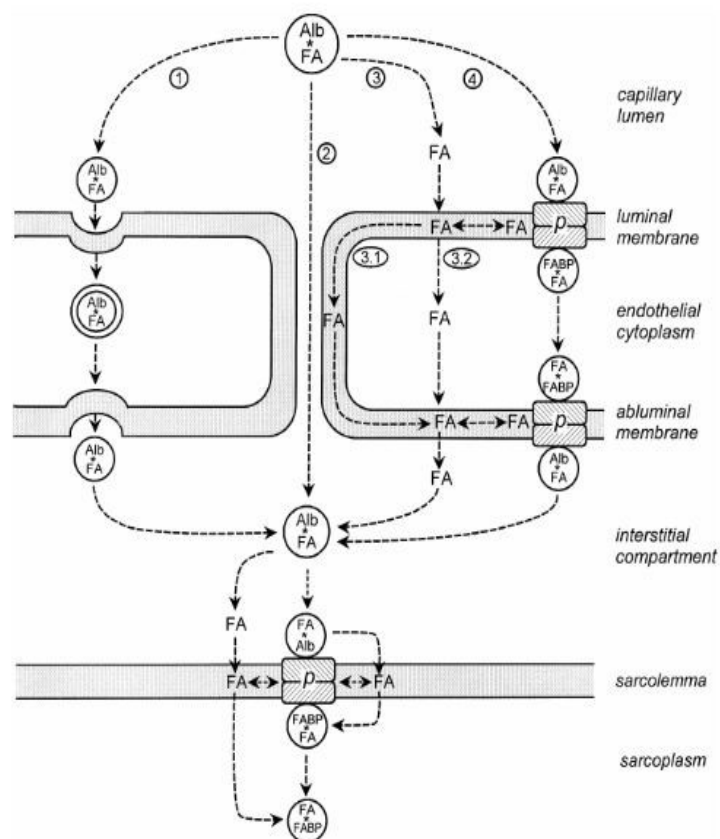
Fig. 2—Glucose fatty-acid cycle, blood phase.
 1. Uptake of glucose by muscle and adipose tissue.
 2. Release of fatty acids from adipose tissue to plasma albumin.
 3. Formation of ketone bodies from fatty acids by liver.
 4. Uptake of fatty acids and ketone bodies by muscle.

2.2. Fatty acid metabolism of the heart

Due to their hydrophobic character, even the delivery of FAs to the cardiac tissue is intricate. The extracted FAs from food intake attached either to plasma albumin or bound to TAG core of lipoprotein particles (LDL and VLDL) are delivered to the heart [11,39]. After FAs release from either albumin complex or by hydrolysis of TAG, a sequence of barriers blocks their way until they can be finally utilized by the cardiomyocytes. These barriers are represented by the capillary endothelium (blocking their transfer from capillary lumen), abluminal membrane of the endothelium (representing a barrier between the endothelial cell and interstitial space), and sarcolemma (interstitial space-cardiomyocyte border). The hydrophobicity of FAs allows them to diffuse through the phospholipid membrane of both – the endothelium and sarcolemma. On the other hand, the observed cardiomyocyte flux of FAs greatly exceeds the flux provided by simple diffusion [40]. Therefore, FAs are suggested to transverse a phospholipid bilayer by facilitated mechanism in which specific membrane associated proteins responsible for binding and carrying FAs are involved. This suggestion is furtherly supported by the identification of variety of proteins with these functions [41–44]. The complex mechanism of FA transport is depicted in Figure 3.

Figure 3: Possible intake of FA from the capillary lumen to the cardiomyocyte. Original from [40].

Transport of the albumin-fatty acid complex through the inter-endothelial clefts (2); various transport routes of the albumin-free fatty acids through the endothelial cells (1, 4) or of the dissociated fatty acids through the endothelial cells (3). Ab, albumin; FABP, cytoplasmic fatty acid - binding protein; FA – long chain fatty acid; p, membrane associated complex of proteins.



As soon as FAs enter the cardiomyocyte via FABP/CD36 complex or by diffusion¹, their fate is determined mainly by the properties of the acyl chain. The very long chain (VLC-), long chain (LC-), dicarboxylic, poly- (PU-) and monounsaturated (MU-) FAs but also prostaglandins, xenobiotic FAs, and CoA esters of eicosanoids are processed by peroxisomes, while the short-chain (SC-), medium-chain (MC-) and LCFAs are preferentially processed within cardiac mitochondria [11,45–48]. The heart expresses an intracellular fatty acid binding protein, H-FABP [41,49], which binds the incoming FAs and transports them towards mitochondria or peroxisomes. However, not all FAs that have been translocated to the cardiomyocyte are strictly oxidized. In fact, cardiomyocytes of the intact heart possess a TAG pool that can represent from 11 to 30% of heart energy expenditure depending on the perfusate [39]. The whole pool was traditionally viewed as inert, but a recent study by Carley *et al.* showed this pool to be very dynamic. Overexpression of CD36 in their rat model induced by adenoviral infection led to accelerated TAG turnover without a significant enlargement of intra-myocardial lipid pool. From their observations they presented a

¹ Recent studies suggest that the diffusion takes place in the case of SCFA and MCFA, but not in the case of LCFA and VLCFA [400]

reciprocal regulation among LCFA uptake, TAG synthesis, and TAG lipolysis to ensure tight control [50].

Before any FA can be oxidized in β -oxidation cycle it has to be activated by an enzyme called acyl-CoA synthase (ACS). The reaction is ATP-dependent and can be divided into two separate parts. In the first part, the FA molecule is being adenylated by ATP releasing inorganic pyrophosphate. In the second part, CoA displaces AMP and the resultant fatty acyl-CoA is formed with AMP as side product [51,52]. One of the pioneer work by Aas showed that at least 5 distinguishable ACS isoforms exist within the organism. He pointed out that for SCFAs, MCFAs, and LCFAs there is a unique set of these synthases localized in mitochondrial matrix, on the outer mitochondrial membrane (OMM) or in the microsomes [53]. By today, 26 ACS isoforms have been identified branching from the already mentioned ones towards VLCFAs ACS and so called “bubble-gum” isoforms. Many of these were characterized to be strictly peroxisomal, although their previous subcellular localization thought to be elsewhere (mainly microsomal) [54]. The corollary to this notion is that FAs may be activated either in the sarcoplasm, in the mitochondria or in the peroxisomes. Contrary to FAs, the newly created CoA esters cannot cross any lipid bilayer by a simple diffusion due to the physicochemical properties of covalently bound coenzyme A. Several transport mechanisms thus must exist in the mitochondria, a well-described multi-enzymatic complex has been characterized composed of carnitine-palmitoyl-transferase (CPT) 1, carnitine/acylcarnitine carrier (CAC), and CPT2 [55–57]. This system is required mainly for the mitochondrial uptake of LC-acyl-CoA. At first, acyl-group of LCFA is transferred to carnitine and subsequently, the acyl-carnitine molecule is shuttled to the mitochondria where it is converted back to acyl-CoA by CPT2 [11]. On the other hand, peroxisomal membrane seemingly consists of two separate players. The first is the ABC translocase 1 which oversees the acyl-CoA uptake to peroxisome. The carnitine-dependent mechanism takes place to escort the shortened acyl-CoA, propionyl-CoA, and acetyl-CoA molecules out of the peroxisome. However, not through the same system like in mitochondria. In peroxisomes, carnitine-acetyl-transferase (responsible for short chain acyl-CoA transfer) and carnitine-octanoyl-transferase (responsible for medium chain acyl-CoA transfer) were introduced [58]. The current mechanism how they reach mitochondria is unknown, although it most likely involves mitochondrial CAC [45,47,59]. It has been already mentioned that any facilitated transfer pronouncedly increases the uptake rates, thus might be taken as the rate-limiting step. Indeed, the activity of CPT1 represents the rate-limiting step in cardiac β -oxidation rates [60–62].

In both organelles, degradation of straight-chain saturated fatty acids requires the participation of four enzymes comprising the core of the β -oxidation cycle [45]. In the

mitochondria, β -oxidation pathway initiates with dehydrogenation of acyl-CoA molecule catalysed by acyl-CoA dehydrogenase. FAD is a required co-factor. This reaction is coupled with electron transfer flavoprotein (a part of ETS) which oxidizes FADH_2 back to FAD (donating electrons to ETS). However, in the peroxisomes, acyl-CoA oxidase (ACOX) is responsible for the initial step of β -oxidation. Due to the lack of ETS within peroxisomes, electrons from desaturation are directly donated to molecular oxygen, thereby producing hydrogen peroxide. Also, the third reaction (catalysed by 3-hydroxyacyl-CoA dehydrogenase) differs between these compartments. In mitochondria, NADH produced by this reaction is used in ETS producing ATP by reducing O_2 to H_2O . On the other hand, peroxisomal 3-hydroxyacyl-CoA dehydrogenase is coupled to NADH utilizing reaction, e.g. lactate dehydrogenase [63]. The peroxisomal β -oxidation end-products (acetyl-CoA, propionyl-CoA, chain-shortened acyl-CoA) are processed in other cellular compartments, mainly in mitochondria [47,64], see Figure 4.

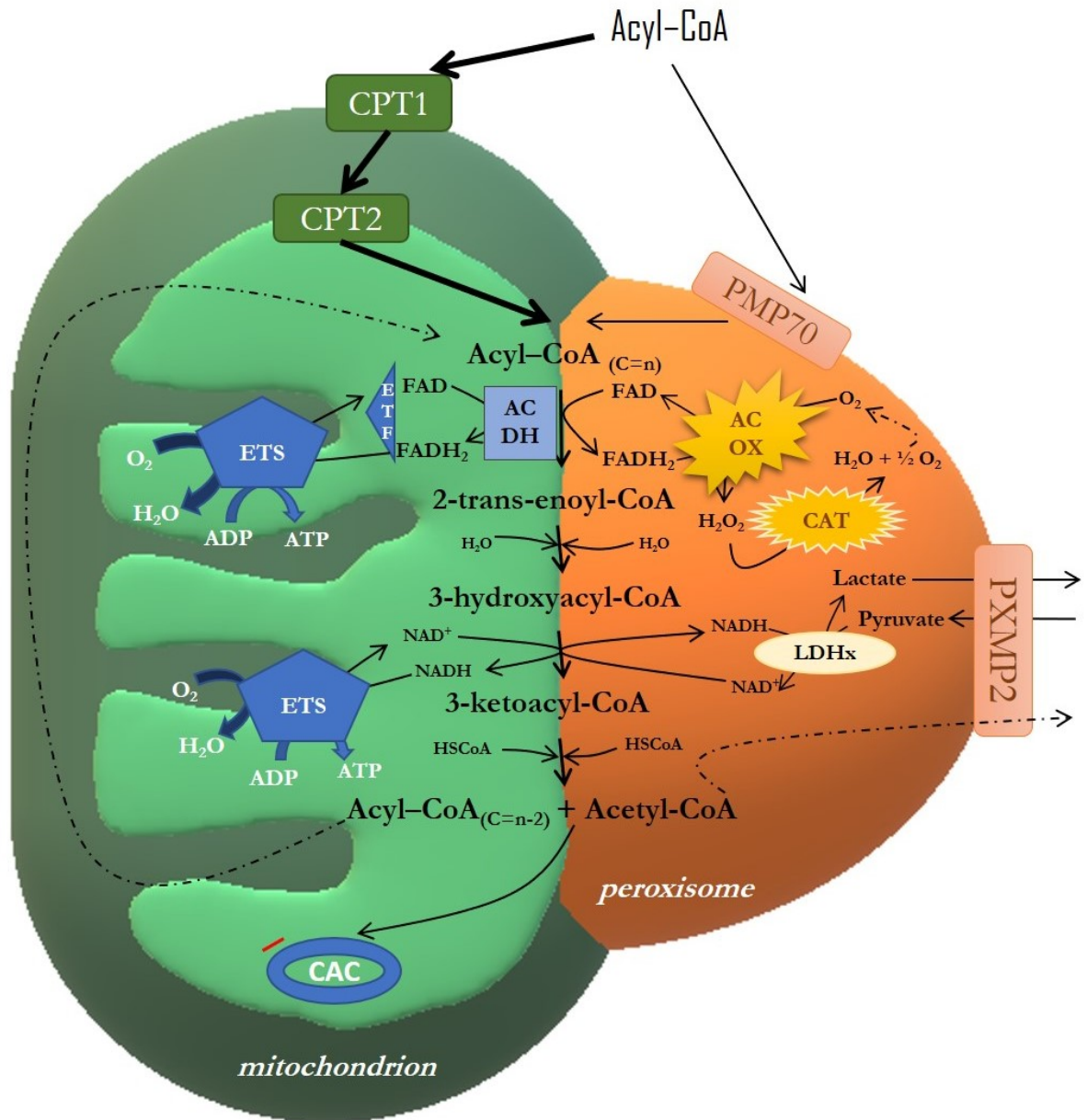


Figure 4: β -oxidation of long chain fatty acids. Created acyl-CoA enter mitochondria via carnitine dependent mechanism or peroxisomes which is mediated by ABC translocase, PMP70. In both organelles, 4 sequential reactions – dehydrogenation, hydration, dehydrogenation and thiolation – shorten the acyl-CoA chain, generating 2C shorter acyl-CoA molecule and acetyl-CoA in each cycle. There are several differences between these compartments – first, due the lack of ETS in peroxisomes, the first and the third reaction have to be mediated either by another enzyme (first reaction in peroxisome is facilitated by acyl-CoA molecule, ACOX which is coupled to catalase, CAT) or coupled to another reaction (3rd reaction interplays with peroxisomal lactate dehydrogenase, LDHx). Acetyl-CoA molecules enters the citric acid cycle (CAC) only in mitochondria. Peroxisomal Acetyl-CoA has to be transferred to mitochondrial compartment first. Original from (Kolar et al., 2018, Submitted).

2.3. Carbohydrate metabolism

Soon after Locke and Rosenheim were the first to establish an isolated perfused heart preparation, they showed that glucose is readily utilized as energy substrate in 1907. The fact that the heart is a metabolic omnivore was demonstrated several years later [65,66], for more interesting historical information see [406]. However, the discovery of individual glycolytic reactions transforming molecules of glucose to pyruvate and the complete glycolytic pathway was elucidated by many scientists including Otto Warburg, Jacob Parnas, Carl Neuberg, Gerti and Carl Cori, Hans von Euler, Otto Fritz Meyerhof and Gustav Embden (thus glycolytic pathway is sometimes referred as Embden-Meyerhof pathway) by 1940 [405].

Randle's observation in 1963 [24] provided the world with a novel insight between metabolic substrate cross-talk. He observed a short-term inhibition of several glycolytic steps by FA oxidation. These included the glucose transport, hexokinase (HK) activity, phosphofructokinase (PFK) 1 and pyruvate dehydrogenase (PDH) revealing the most important regulatory steps in carbohydrate metabolism. Moreover, he identified that the inhibitory effect of FAs is not distributed among the glycolytic pathway equally. Whereas PDH activity was affected the most and the inhibitory effect reached almost 100%, the glucose transport rate was altered the least.

Circulating glucose or fructose is transported across the blood-tissue barrier through ubiquitously distributed glucose transporter (GLUT) 1 [67] or by GLUT11, respectively [18]. Cardiomyocyte expresses two major GLUTs undergoing changes mirroring the maturation process [68]. Whereas the ubiquitously expressed GLUT1 is the major isoform of the foetal heart and the expression is relatively high, GLUT4 becomes the major isoform expressed in the adult heart having a physiological role in insulin-stimulated glucose uptake [69,70]. Both of GLUTs possess a similar affinity to glucose with K_m values of ~ 3.4 mM and ~ 5 mM for GLUT1 and GLUT4, respectively. The notion of similar and quite low K_m values of GLUT 1 and 4 led to two corollaries – first, due to observed concentration of circulating glucose, GLUTs represent the rate-limiting step in the cardiac glucose utilization at physiological conditions, second – to upgrade the glucose uptake, much more of GLUTs have to be translocated onto the sarcolemma [38,71]. In 1997, Fischer *et al.* reported to determine distinct GLUT4 pools within the heart as these pools were mobilized by different stimuli/signalling pathway which was validated later [72,73]. So besides insulin, it was shown that exercise [74], hypoxia [75] and stress conditions like ischaemia [76,77] or diabetes [78] may affect the sarcolemmal density and/or the expression of GLUTs. The trafficking

of GLUT vesicles is mediated by individual GTPases of the Rab family, a possible explanation why different signalling pathways may be taken into account [79,80].

Immediately upon entering the cell, glucose is rapidly utilized by one of the two major isoforms of cardiac HK. This step is very important to keep intracellular sources of carbohydrates steady because the created glucose-6-phosphate (G-6-P) is impermeable to sarcolemma. Again, like in the case of ACS, this reaction is ATP-dependent. The major isoform of the adult heart is HK2, whereas the foetal and the new-born heart express mainly HK1 [81,82]. In contrast to other HK isoforms expressed in mammalian tissue, HK1 and HK2 contain a special hydrophobic region of 21 amino acids (AA) at the N-terminus allowing them to attach to mitochondrial voltage dependent anion channel (VDAC) [83–86]. The subcellular localization thus play an important role in regulation of HK activity. Indeed, it is suggested that the localization of HK reflects its metabolic role – when HK is bound to the OMM via VDAC, it catalyses the conversion of glucose to G-6-P which is further metabolised in the glycolytic flux. On the other hand, soluble forms of HK represent the anabolic routes where G-6-P serves as a precursor of glycogen [82,87] or in lipid synthesis via pentose-phosphate pathway [82,88,89]. The increasing G-6-P concentration affects glycogen synthase (GS) activity which can be demonstrated by the effect of insulin [90,91]. Moreover, it was shown that it is [G-6-P] which promotes the HK2 dissociation from mitochondrial OMM as a response to elevated glucose uptake [92,93]. Except G-6-P, the value of pH was also shown to regulate HK-OMM interaction affecting HK2 rather than HK1 [94]. Even HK reaction may become the rate-limiting step in cardiac carbohydrate metabolism. This situation can be depicted by elevated inotropic stimulations of the heart (e.g. during acute exercise or stress) during which the accumulated glucose inhibits the glycogen phosphorylase (GP) [38,95].

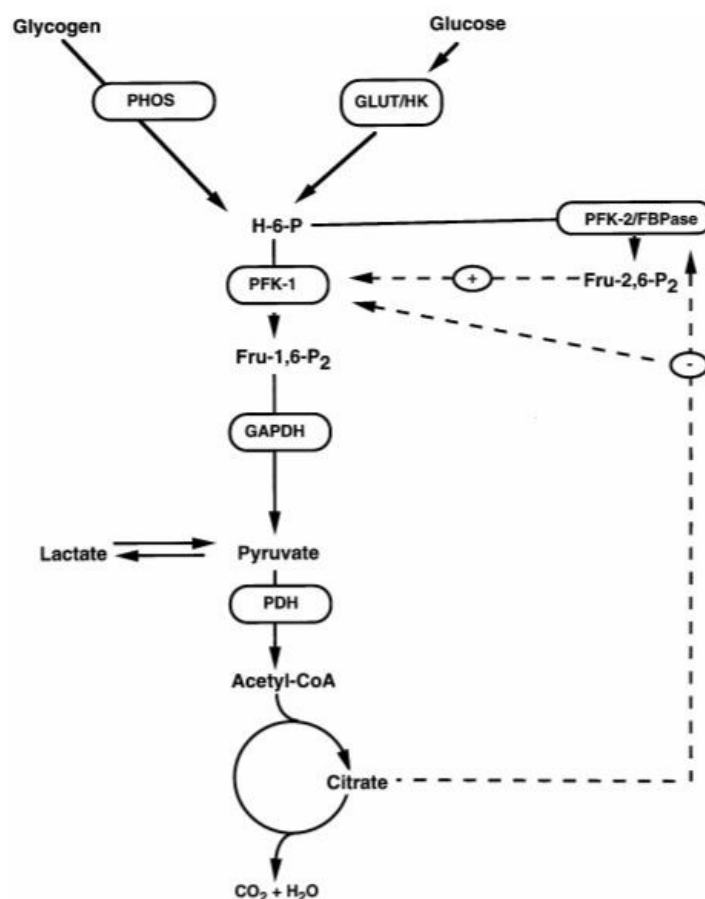
G-6-P transformed to fructose-6-phosphate (Fru-6-P) is subsequently metabolised in glycolytic pathway and enters a reaction catalysed by PFK to form Fru-1,6-PP. PFK1/PFK2 are ones of the most important enzymes in terms of regulation of cardiac glycolysis. Although PFKs are not the rate-limiting step in all situations, the ability is gained when the cardiac tissue is exposed to insulin (e.g. post-prandially) [96]. The activity of PFK1 (generating fructose-1,6-PP) is under a tight control of PFK2 (bifunctional enzyme – PFK2/fructose-2,6-bisphosphatase - FBP) which controls the synthesis and degradation of fructose-2,6-bisphosphate (Fru-2,6-PP). Cardiac PFK2 contains about 100-times more PFK2 than FBP2 at rest. Whereas Fru-1-6-PP operates as an allosteric inhibitor of PFK1, elevated concentration of Fru-2,6-PP strongly potentiates PFK1 activity. Other stimulatory or inhibitory effects are provided by ions, metabolic intermediates and ADP/ATP ratio. PFK1 reaction may thus be inhibited by H^+ , citrate and ATP, whereas PFK2 activity may be stimulated by Ca^{2+} or ADP [97]. Several kinases play role in PFK activation as well

(e.g. AMPK, Akt) [98,99]. Additionally, it was shown quite recently that PFK is being degraded in diabetic mice when its substrate is absent suggesting that the loss is a result of reduced insulin signalling [100].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the next regulatory enzyme standing in the line. GAPDH catalyses the reaction converting glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate while NAD^+ is being reduced to NADH. The NADH created in this reaction is shuttled to mitochondria to be processed in ETS. However, NADH is impermeable for mitochondrial membranous system. Therefore, NADH is being shuttled to mitochondria via either malate/aspartate or glycerol-3-phosphate pathway [87,101,102]. GAPDH was identified as an accessory protein of cardiac K^+_{ATP} channel situated on the sarcolemma coupling glycolysis with the channel activity [103]. A connection between insulin signalling (via Akt) and GAPDH has been revealed recently. [104]. In this study, the phosphorylated Akt interacted with $\sim 40\text{kDa}$ protein (called p40 by the authors) that was also phosphorylated. By peptide mass mapping technique, they identified p40 to be phosphorylated GAPDH. However, they could not find a correlation between the phosphorylation status of GAPDH and its activity although an increase in activity was observed. Cardiac ischaemia or any situation when mitochondrial network function is compromised ultimately lead to a decreased oxidative rate with reduction of NADH/ NAD^+ ratio which evokes a reduction in enzyme activity making this reaction the rate-limiting step. Few years ago, it was shown that GAPDH is also a multi-functional protein. Under various of stress conditions (especially the oxidative ones), GAPDH translocate to nucleus after its cysteine residue is S-nitrosylated by nitric oxide in a complex with Siah1 which results in cytotoxicity [105]. Nitric oxide was also shown to induce GAPDH aggregation at OMM which was accompanied by mitochondrial swelling, dysfunction, mitochondrial permeability transition pore (mPTP) opening and cell death [106]. The aggregation of GAPDH with mitochondria was also observed after ischaemia-reperfusion (I/R)-induced injury directly promoting mitochondrial micromitophagy [107]. The regulatory steps in glycolysis are summarized in Figure 5.

Figure 5: Regulatory mechanisms of glycolytic flux.

Original from [97]. The control steps are shown in boxes. PHOS, glycogen phosphorylase; GLUT, glucose transporter; HK, hexokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. The dotted lines represent the allosteric control of activity of phosphofructokinases, PFK1 and PFK2



The final regulatory enzyme, and possibly the most important one, determining the fate of pyruvate is pyruvate dehydrogenase (PDH) situated in mitochondria. Pyruvate needs to be transported to mitochondrial matrix via mitochondrial pyruvate carrier (MPC) [108]. PDH was the most inhibited enzyme by upregulated FA oxidation observed by Randle *et al.* [24] uncoupling glycolysis from OXPHOS. PDH is an integral part of a massive pyruvate dehydrogenase complex (PDHC). PDHC consists of – PDH (sometimes referred as E1), dihydrolipoamide acetyltransferase (sometimes referred as E2) with dihydrolipoamide dehydrogenase (sometimes referred as E3). But there's more, the PDHC catalytic core is made of tens of copies of E1, E2, and E3. E1 is under a tight control of 4 isoforms of pyruvate dehydrogenase kinase (PDK) a 2 isoforms of pyruvate dehydrogenase phosphatase (PDP). Up to three serine residues (creating Site 1, Site 2, and Site 3) of E1 are being phosphorylated by PDK which inhibits E1 activity and thus the whole PDH reaction. Only Site 1 can be phosphorylated by all PDKs, whereas the rest (i.e. Site 2 and Site 3) is being phosphorylated by distinct isoforms introducing hierarchical control. The control is provided by retarding Site 1 dephosphorylation induced by PDPs thus keeping PDH in its inactivated state. Distribution of PDKs and PDPs is tissue-specific and the heart possesses all PDK isoforms (PDK1-4) and both PDP isoforms (PDP1 and PDP2) (excellently reviewed in

[109]). The role of PDHC in cardiac pathophysiology was reviewed recently [110]. The complex regulation of PDHC with the catalytic reaction is depicted in Figure 6.

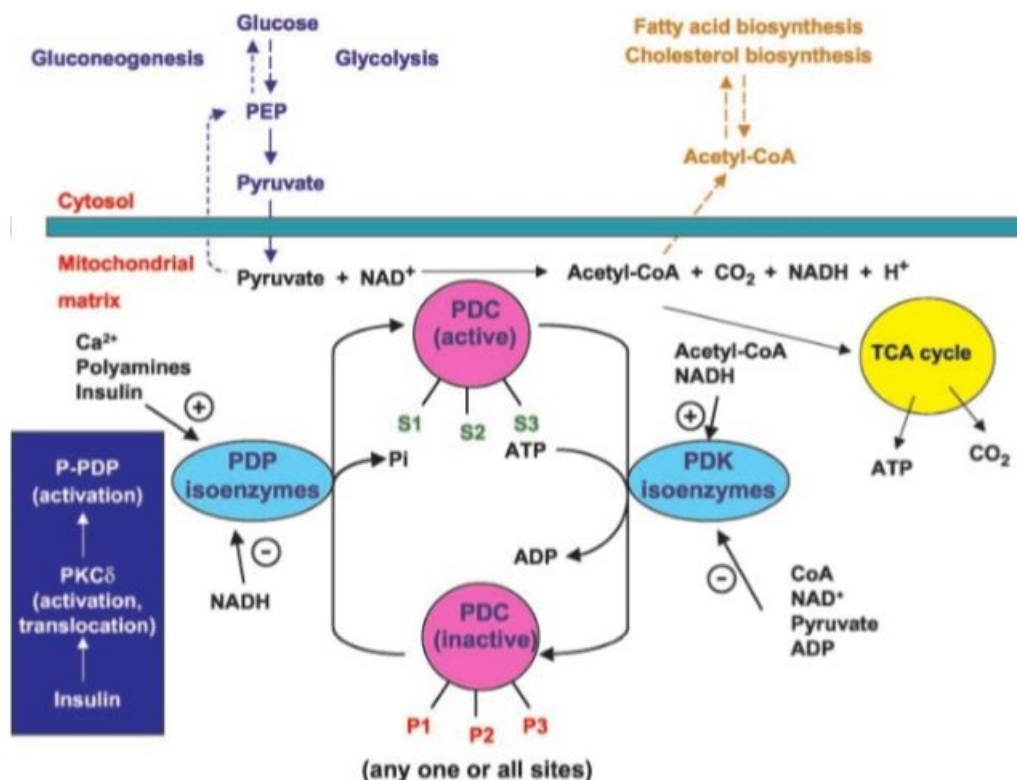


Figure 6: Pyruvate dehydrogenase complex and its regulation of glucose oxidation: Original from [109]. PDH activity is regulated by phosphorylation/dephosphorylation sites catalysed by PDKs and PDPs. Three serine residues of E1 phosphorylation sites are shown as S1, S2, and S3 in dephosphorylated conditions and as P1, P2, and P3 when phosphorylated. PDC, pyruvate dehydrogenase complex; PDP, pyruvate dehydrogenase phosphatase; PDK, pyruvate dehydrogenase kinase; TCA – tricarboxylic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NAD(H), nicotinamide adenine dinucleotide; PEP, phosphoenolpyruvate; CoA – coenzyme A; PKC, protein kinase C.

Nevertheless, in case of PDH inactivity, pyruvate molecules may be metabolized in several other chemical reactions. Besides pyruvate carboxylase (PYC) and alanine aminotransferase (ALT), the reaction used by the heart excessively is mediated by LDH [111]. Until Gerty and Carl Cori proposed Cori's cycle, lactate was taken as an end product, and soon after Cori's, it was recognized as an important metabolic side product that can be utilized either by liver in gluconeogenesis or in oxidative tissues, e.g. neuronal, muscular and cardiac [112–114]. The ability of lactate and other monocarboxylate compounds (e.g. AAs, iodothyronine) to cross membranous bilayer is due to

monocarboxylate transporter (MCT) family of proteins with 14 isoforms identified until today [115–117]. Cardiac LDH is encoded by two different genes, *LDHA* and *LDHB*. These genes give rise to muscle, M- and heart, H- subunit, respectively. The catalytically active LDH is made up 4 subunits and the isoforms thus range from M4 through M3H1, M2H2, M1H3 to H4 isoform, which the last being the major adult cardiac isoform. LDH reaction catalyses the conversion of pyruvate to lactate with subsequent oxidation of NADH to NAD⁺ but also the opposite reaction - oxidation of lactate to pyruvate with NAD⁺ being converted to NADH. The former idea that lactate is created only in anaerobic settings was refuted by Warburg's observation in 1924 which we now call the “Warburg's effect” [118] - pointing on the fact that “anaerobic glycolysis” may, in certain conditions, be set on even though the oxygenation is sufficient.

The cardiac muscle thus can operate either with pyruvate incoming from glycolytic flux or lactate incoming from the extracellular fluid through MCT1 or other MCTs. Another important factor of LDH role in metabolism is its subcellular localization. Similarly, like GAPDH, sarcoplasmic isoforms of LDH are parts of ATP-sensitive K⁺_{ATP} channel associating with Kir 6.2 and SUR2A subunits at channel's N- and C-terminus, respectively. This localization enables a very effective NAD⁺/NADH coupling between GAPDH and LDH reactions, but importantly, it also enables the myocardium to couple K⁺_{ATP} channel activity with the metabolic status of the cell [119]. In rat cardiac tissue, the presence of mitochondrial LDH was observed in many studies [120,121] but the LDH role therein is still a matter of debate [122]. Despite the attractiveness of “lactate shuttle” between sarcoplasmic and mitochondrial compartment, there are several theoretical arguments against it as postulated by Halestrap and Wilson. At first, the LDH reaction within mitochondria matrix is both energetically unfavourable and incompatible with the known NADH redox compartmentalization. Secondly, pyruvate carrier and MCT differ in ultrastructure, inhibition profile, and substrate affinity. And finally, all significant mitochondrial MCT and LDH was removed from samples when careful density gradient centrifugation was applied [117]. Another LDH localization within the cell was reported within peroxisomes (mentioned in chapter 2.2) where it serves as NAD⁺ donor. The expression and localization of LDH may be affected by various conditions like hypoxia via HIF-1 α signalling. Indeed, in our newest study, we have shown that adaptation to intermittent hypobaric hypoxia (IHH; see Results, Study C) led to upregulation of peroxisomal LDHB protein with concomitant upregulation of the rate limiting enzyme of peroxisomal β -oxidation, ACOX1 (Kolar et al., 2018; Submitted).

However, and also in context to cardiac metabolism, PDH reaction which is taking place in mitochondria yields much more energy from pyruvate than LDH reaction itself. At physiological conditions, pyruvate is being oxidized by PDH, but the pyruvate is formed from both sources (i.e.

glycolysis and lactate oxidation). In the situation when mitochondrial metabolism is somehow inhibited (ischaemia, oxidative stress, intoxication), LDH starts producing lactate and the heart becomes a net producer of lactate.

2.4. Cardiac mitochondria

Before we continue on our journey, which could be defined as “the fate of acetyl-CoA”, several important notes have to be added.

- The mature cardiomyocyte is a highly-organized cell, in fact its complexity and hierarchy are reflected into energy compartmentalization (which has already been partially depicted). At least three distinguishable mitochondrial subpopulations can be found within the cardiac cell – i) subsarcolemmal mitochondria (SSM) that are distributed underneath the sarcolemma, ii) interfibrillar mitochondria (IFM) that are spread between the sarcomeres and iii) perinuclear mitochondria (PNM) which lie at peri- and juxtannuclear region (for more information about cardiac ultrastructure and mitochondrial families see [123]). Each family possess a unique role in cardiac energy metabolism, e.g. while SSM are suggested to participate in homeostasis, IFM and PNM are thought to power contractility and nucleus, respectively. In fact, cardiomyocyte maturation is dependent on a proper mitochondrial development as arresting mitochondrial maturation impairs cardiomyocyte differentiation [124].
- Besides FAs and carbohydrates, acetyl-CoA molecules can be created also from metabolic reactions using ketone bodies (like acetoacetate or β -hydroxybutyrate) or AAs as substrates. Although these routes do not represent the major way how the cardiomyocytes derive their energy, in particular situations like acute and chronic fasting or diabetes [125], ketone bodies utilization by the myocardium may rise. AAs serve as main precursors in anaplerosis of KC [126,127] but can be utilized in pathological conditions [128].
- The ancient view depicting mitochondria like rigid, cigar-shaped organelles is, in context of the recent knowledge, pathetic. Mitochondria are shaped perfectly to maximize their productivity creating a very dynamic mitochondrial reticulum undergoing mitochondrial fission and fusion processes, which in turn follow cellular disturbances in metabolic or ionic homeostasis [129,130]. Mitochondrial morphology may thus vary affecting also cristae morphology [131].

- In concordance to the notes, mitochondrial metabolism will be described in its very basics, i.e. not reflecting the developmental stage nor the subcellular localization of mitochondria. The reader is referred elsewhere to find more information about KC, ETS and OXPHOS [129,132–139].

Acetyl-CoA molecules which were created from carbohydrates, FAs, AAs or ketone bodies and reached mitochondrial matrix (in case of peroxisomal acetyl-CoA) enter the KC. The cycle is present in all aerobic and some anoxygenic organisms and has 2 basic functions. In the 8 sequential linear irreversible and reversible reactions, mitochondria harvest energy and store it in the form of reducing equivalents (i.e. NADH, FADH₂) and high-energy phosphate (GTP). Moreover, there are many entry points to this pathway but as the name “cycle” implies, every product of a reaction is a substrate for the next one eventually leading to regeneration of each one of them. As Hans Krebs noted himself on his Nobel banquet lecture: “It is indeed remarkable that all foodstuffs are burnt through a common terminal pathway...The biological significance of the common route may lie in the fact that such an arrangement represents an economy of chemical tools.” (Krebs, 1953). KC reactions can be divided into irreversible (catalysed by CS, Isocitrate Dehydrogenase; ICD, 2-oxoglutarate dehydrogenase complex; OGDC) and reversible (catalysed by aconitase; ACO, succinyl-CoA synthetase; SCOS, succinate dehydrogenase, fumarate hydratase; FH, malate dehydrogenase; MDH). In the first step, acetyl-CoA condensate due the activity of CS with a molecule of oxaloacetate creating citrate with a concomitant release of coenzyme A (CoA). This step is also the rate-limiting. After this reaction, the citrate is metabolized creating 3 NADH, 1 GTP, QH₂ and oxaloacetate in each of these metabolic “Merry-Go-Round” rides [140]. However, some of the citrate escapes KC, entering sarcoplasm in which citrate serves as acetyl donor for malonyl-CoA [141]. The increasing concentration of malonyl-CoA inhibits mitochondrial carnitine-dependent transport thus blocking mitochondrial FAs uptake [62]. Malonyl-CoA is a precursor in FA synthesis, so the inhibitory effect on CPT is logic as it uncouples a process in which FAs are being built from the one utilizing them. This also explains the inhibitory effect of upregulated glucose oxidation observed by Randle [24,142]. It is of note that glycolysis (via GAPDH), β -oxidation (via Acyl-CoA Dehydrogenase and 3-hydroxyacyl-CoA Dehydrogenase), β -hydroxybutyrate $\rightarrow$ acetoacetate transformation, and several AAs enzymes (e.g. Glutamate Dehydrogenase) also create reducing equivalents either in the form of NADH or FADH₂. See Figure 7.

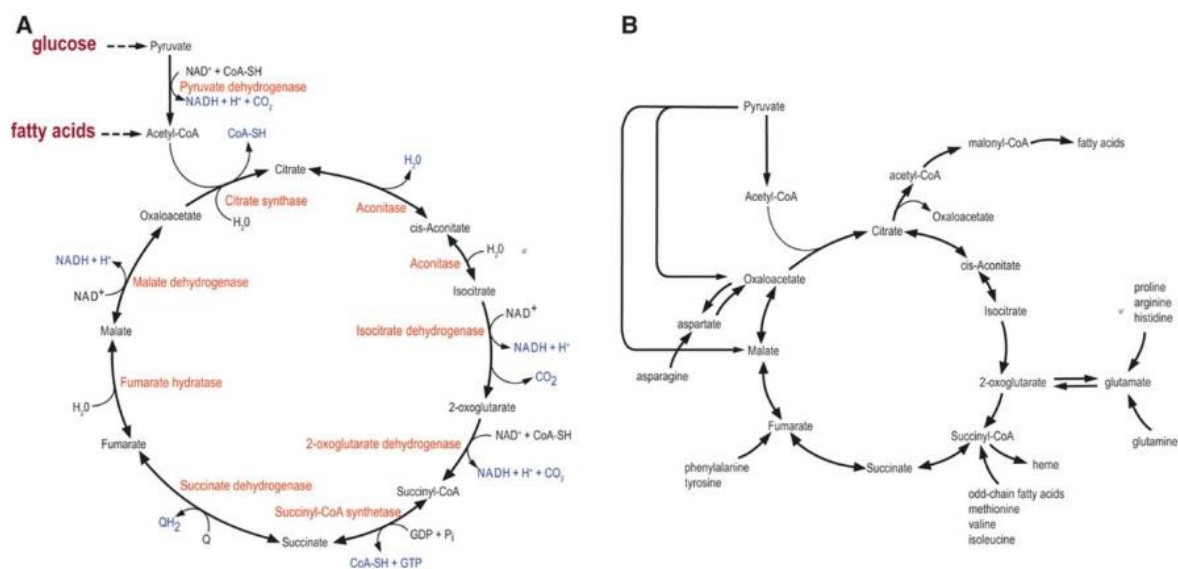


Figure 7: Krebs cycle, cata- and anaplerosis: Original from [140]. **A:** An overview of Krebs cycle reactions. Observe that apart from 3 irreversible steps (citrate synthase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase), the rest of the KC reactions are reversible allowing reverse flux in certain conditions. **B:** Cataplerosis and anaplerosis production of various biosynthetic precursors making the CAC as central metabolic hub, connecting diverse metabolic pathways. CoA-SH indicates reduced coenzyme A; FADH₂, reduced flavin adenine dinucleotide; GTP, guanosine triphosphate; NAD⁺/NADH, oxidized/reduced nicotinamide adenine dinucleotide, QH₂ – reduced coenzyme Q.

Mitchell's chemiosmotic theory revealed that these reducing equivalents, via the ETS, synthesise ATP [23]. The process, now called oxidative phosphorylation, consists of the flows of two sets of particles: the oxido-reduction and hydro-dehydration particles. Chemiosmosis is the movement of ions, across a semipermeable membrane down their electrochemical gradient. ATP synthase (F₀F₁-ATPase, also called Complex V) is the last part of ETS that is employing chemiosmosis (H⁺ electrochemical gradient) to generate ATP. The high gradient between matrix and mitochondrial intermembrane space is kept high due the activity of ETS complexes – NADH dehydrogenase (NADH:ubiquinone Oxidoreductase; Complex I), Succinate Dehydrogenase (succinate:ubiquinone oxidoreductase; Complex II, part of KC, this enzyme itself is not H⁺ translocating), coenzyme Q:cytochrome c oxidoreductase (Complex III, also known as cytochrome *bc₁* complex), and cytochrome c oxidase (Complex IV, COX). All of these enzymes catalyse the transfer of electrons from reducing equivalents through the ETS using electron carriers, i.e. ubiquinone (coenzyme Q) and cytochrome c. ETS is often called electron transport chain, however, this does not reflect the reality, as the electron flow to the ubiquinone is convergent, see Figure 8. Four electrons are carried towards COX that are used in reduction of O₂ to H₂O, while 4 H⁺ are being translocated across OMM. However, during oxidative metabolism of mitochondria, and more in pathological conditions, reactive oxygen species (ROS), such as superoxide anion (O₂⁻), hydrogen peroxide, and hydroxyl radical (HO•) may be generated [143–145]. The proton circuit

can be partially uncoupled by proton leaks (small amounts of H^+ are not used via Complex V but translocate back to mitochondrial matrix which is mediated by uncoupling proteins for instance) and a relationship between proton leak and ROS production was suggested [146]. From respiratory/metabolic states of mitochondria defined by Chance and Williams is clear that the expression and activity of ETS complexes, [ADP], $[O_2]$ and [substrates] may affect the respiration rates [409]. The presence/absence of super-complexes like respirasomes affect the respiratory rates [138,147,148]. Taken from the other end, any alteration either on the protein or enzyme activity levels of each of the ETS complexes may, in turn, affect KC rates and the rates of other reactions generating reducing equivalents. This sort of regulations is due alterations of $[NADH]/[NAD^+]$ and $[FADH_2]/[FAD]$ ratios [149].

Another player affecting mitochondrial metabolic rates in the heart is calcium, in fact $[Ca^{2+}]$. The increase in intramitochondrial $[Ca^{2+}]$ stimulated PDH (by direct PDP stimulation), ICD, and OGDC activities through K_m reduction [150]. Interestingly, all three enzymes are also regulated by $[ATP]/[ADP]$ and $[NADH]/[NAD^+]$. Moreover, $[Ca^{2+}]$ was shown to stimulate glycerol-3-phosphate dehydrogenase (GPD, GPD is a part of GPD shuttle which is similar to malate/aspartate shuttle, shuttling reducing equivalents from sarcoplasmic NADH into mitochondria) in flight-muscle [151]. Ca^{2+} transport within the cell is restricted and a constant extrusion of Ca^{2+} to the external milieu or uptake by intracellular stores (in sarcoplasmic reticulum; SR) maintain cytosolic Ca^{2+} concentrations low under basal conditions [152]. The specificity of sarcoplasmic Ca^{2+} signalling is controlled by sophisticated “toolset” involving a variety of ion channels, ion pumps, and exchangers that mediate the trans-membrane conveyance [153]. Mitochondrial uptake and extrusion mechanisms contain – mitochondrial calcium uniporter (mCU), Letm1 Ca^{2+}/H^+ antiporter, Sodium-Calcium exchangers (NCX), but also mPTP. Mitochondria may also store Ca^{2+} in matrix department. However, when the intramitochondrial calcium stores are too high for too long, mitochondrial dysfunction with subsequent swelling (cristae de-condensation), mPTP opening, release of Cytochrome c, and apoptotic cell death occur [154]. Thus the entire cellular life depends on the proper energy balance.

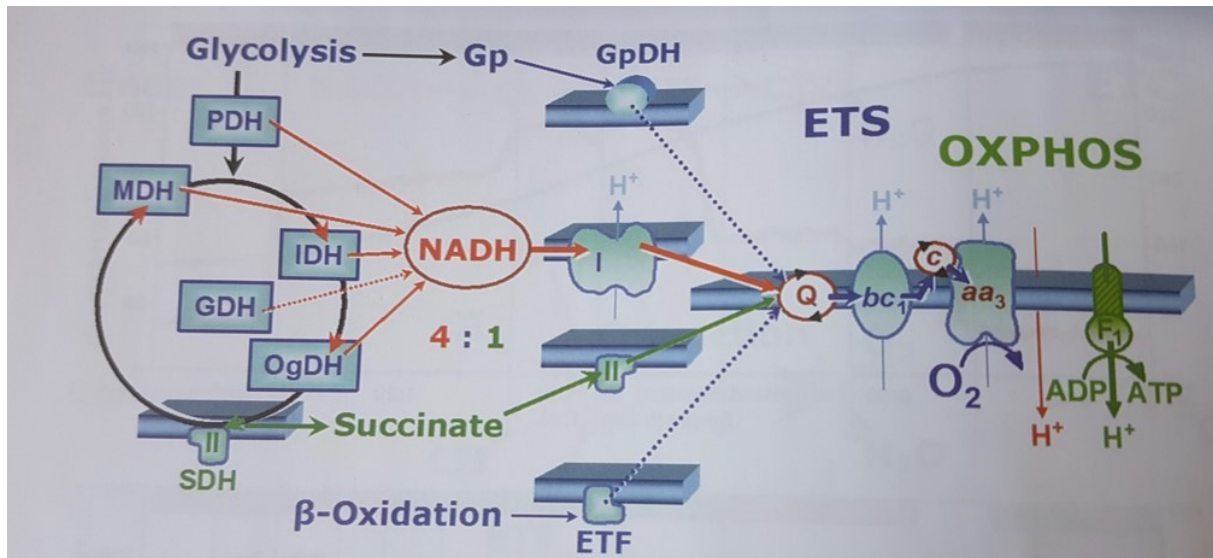


Figure 8: Convergent electron transfer at the levels of the Q-junction (receiving electrons from Complexes I and II, glycerol-3-phosphate dehydrogenase, GpDH, and electron transfer flavoprotein, ETF) and at the level of the NADH pool. Dehydrogenases are PDH, pyruvate DH; MDH, malate DH; IDH, isocitrate dehydrogenase; OgDH, oxoglutarate dehydrogenase; SDH, succinate dehydrogenase – yielding a NADH:succinate ratio of 4:1 in the full KC. GDH; glutamate dehydrogenase; OXPHOS – oxidative phosphorylation; ETS, electron transport system; Q, ubiquinone; c, cytochrome c; bc₁; redox centre of Complex III, aa₃ redox centre of Complex V; F₁ subunit of Complex V. Original from [409].

2.5. The phosphocreatine – creatine kinase system

The evidence shows the existence of distinct cellular ATP microdomains which has been recently referred to as cardiac energetic unit (CEU) [4]. The microcompartments are localized within mitochondria and at the sites where ATP is being utilized. CEUs are connected by phospho-transfer network, particularly phosphocreatine (PCr) – creatine kinase (CK) pathway [155]. The pathway works to bypass and overcome the relatively slow adenine nucleotide diffusion rates and works as both, the temporal and spatial energy buffer. CKs catalyse the following reversible reaction: $\text{ATP} + \text{Cr} \leftrightarrow \text{ADP} + \text{PCr} + \text{H}^+$. The reaction is vital for cardiac physiology as i) it keeps the [ATP] steady in the closest vicinity to ATPases and ii) by PCr generation, it also stimulates mitochondrial respiration (via [ADP]) [4,155–157]. In other words, any acute increase in workload that would affect the activity of myosin ATPases, any ionic disturbance that would stimulate the activity of ion pumps (SERCA, PMCA, Na^+/K^+ ATPase) would ultimately lead to decreased [PCr]/[Cr] with Cr being shuttled (mainly) towards mitochondria, where PCr would be re-phosphorylated by CK and the created ADP would subsequently stimulate OXPHOS.

Importantly, almost 90% of total cardiac ATP turnover is represented by this system and works with another phosphotransferase enzyme, adenylate kinase [158,159].

Two sarcoplasmic types of CK (muscle-type, M and brain-type, B; forming -MM, -MB, and -BB dimers) and one mitochondrial (sarcomeric, mtCKs; forming octamers) are the major isoforms expressed in the cardiomyocytes. The localization of sarcoplasmic CKs varies, whereas CKM is attached mainly in M-line of the sarcomere [160] in the close vicinity to myosin ATPases or SERCA [161], CKB is solubilized in the sarcoplasm and represents the minor isoform in the adult heart. However, even in this energetic system, massive remodelling may be observed during cardiac development, in which the foetal-CKBB dimers are gradually replaced - at first by CK-MB and then by CK-MM dimers [162,163]. This reflects the mentioned transition towards oxidative metabolism and mitochondrial development during embryogenesis. It is of interest that mtCKS isoform upregulation is seen after the onset of CKM expression. Mitochondrial CKs are located in the mitochondrial intermembrane space, suggesting that mtCKS is involved in OMM-IMM connection (between VDAC and adenine nucleotide translocase; ANT) and inside the mitochondria, along the cristae near ANTs [164–166]. The only prerequisite for mtCKS binding is cardiolipin-enriched membrane. Each of the sarcoplasmic isoforms has distinct enzyme properties [167] which may be modified by many stimuli, including adrenergic stimulation [168], AMPK [167,169], PKC [170]. The relationship between AMPK and Akt [171] may be the reason why insulin stimulates the activity of CKBB and CKMB dimers, but not that of CKMM [172]. On the other hand, CKM association with M-line was reported to be pH-dependent [173,174] and all isoforms of CK were shown to be very sensitive to oxidative damage [175,176] with mtCKS being the most vulnerable one thanks to its localization. This situation can be also depicted by two studies of our laboratories – during the adaptation to less severe chronic normobaric hypoxia (CNH, 5000m) in which mtCKS and CKB expression rose and only CKM isoform decreased [177], however during adaptation to severe intermittent hypobaric hypoxia (IHH, 7000m, 8h/day), CKM with mtCKS decreased while CKB was upregulated (Kolar et al., 2018; Submitted). The shared compensatory effect of CKB was also seen in mice whose either CKM or both CKM and mtCKS isoforms were genetically ablated [178].

The corollary from the described mechanism is that the CK-PCr system possess an essential role in cardiac energy metabolism. Its ability to adapt either by modulation of CK activity, or CK isoform expression, in other words – to align the momentous energy demands with the supply to keep the energy balance as much constant as possible. The CK-PCr system is depicted in Figure 9. It should be clear by now that cardiac energy metabolism responds to changes instantly, e.g. by fatty-acid cycle, changes in mitochondrial metabolism and CK system. These changes are also under a tight control of hormonal stimuli (e.g. glucagon, insulin) or catecholamines (e.g. adrenaline), but also of transcription factors which facilitate the middle- and long-term adaptations of cardiac energy metabolism.

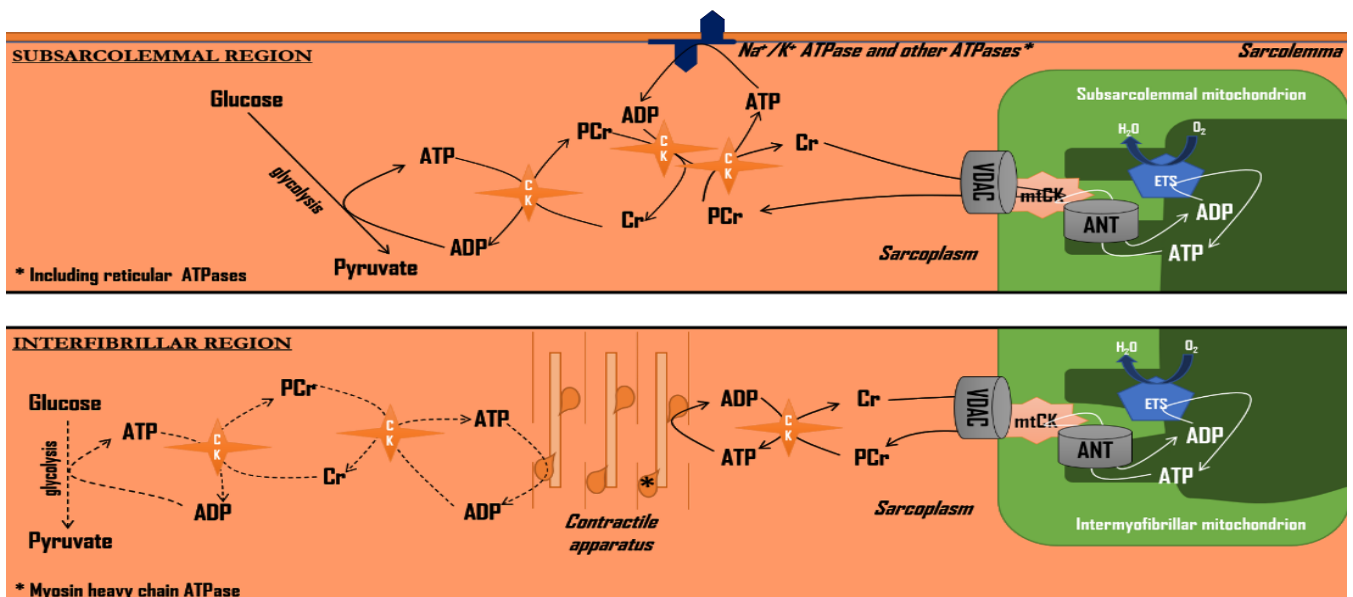


Figure 9: Cardiac PCr – CK system: The upper part of the picture demonstrates subsarcolemmal region, the lower shows the interfibrillar part. Imported creatine (Cr) is charged to the high-energy compound phosphocreatine (PCr) by the action of either CK coupled to glycolysis (all represented by 4-pointed stars) or by mitochondrial CK (mtCK, 8-pointed stars) coupled to electron transport system (ETS). PCr then drives ATPases in the subsarcolemmal region. These ATPases may include: ATP-dependent ion pumps and transporters (Sarcoplasmic reticulum Ca²⁺ pump, Na⁺/K⁺ ATPase and others), ATP-gated ion channels, ATP-requiring metabolic enzymes and protein kinases. In the interfibrillar region however, PCr mainly serves to drive myosin (Mg²⁺)-ATPase for muscle contraction, in this region at normoxic conditions glycolysis does not represent a major route for PCr to regenerate (interrupted route). Adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), voltage dependent anion channel (VDAC), adenine nucleotide translocator (ANT). Original from (Kolar et al., 2018; Submitted).

2.6. Energy regulation and transcriptional control

The dramatic changes in cardiac growth and development, the first inhale of the neonate, the initiation of high-fat diet in the form of breast milk are the factors likely determining the final, i.e. mature cardiomyocyte phenotype. Although the sudden changes are met by an acute response,

at longer time, however, the metabolism is determined by a spectrum of transcription factors and their coactivators.

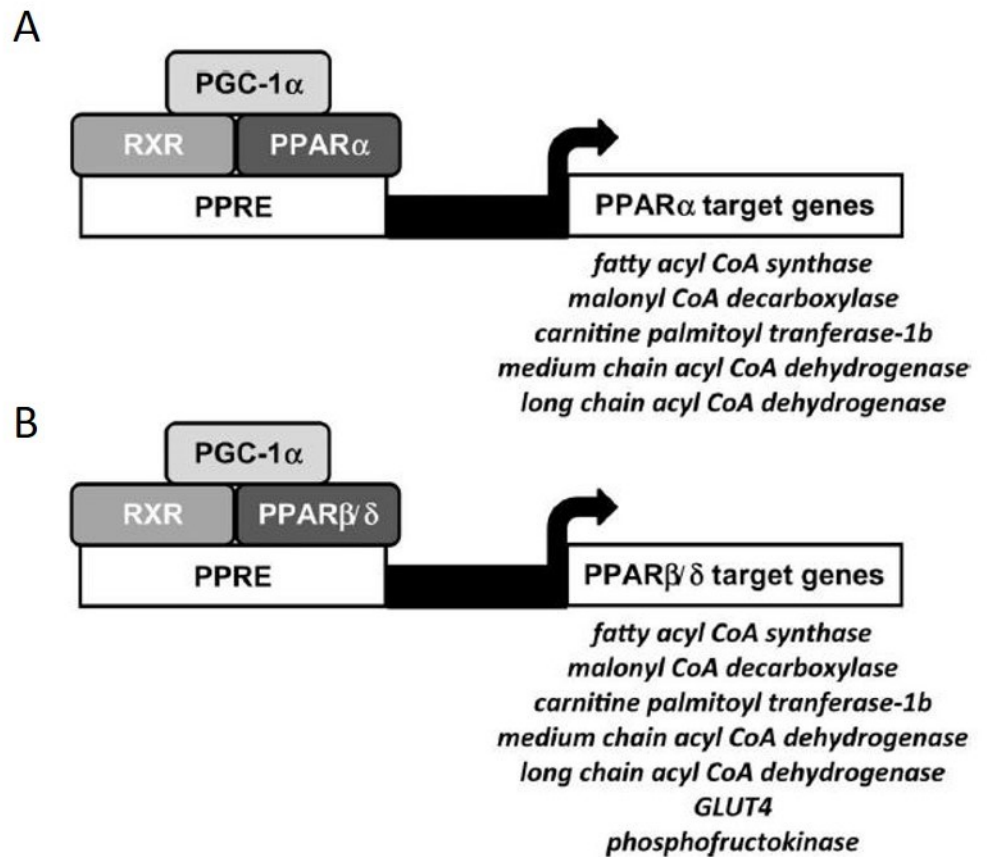
Mature cardiomyocyte O_2 consumption rates are extreme [179], therefore the majority of these transcription pathways control ETS and OXPHOS together with mitochondrial biogenesis [26]. Acute alterations in [AMP] are ultimately recognized by Adenosine monophosphate-activated Kinase, AMPK. AMPK is a serine/threonine protein kinase with a highly conserved motif in eukaryotes and has one purpose – to restore ATP levels. It is composed of three subunits, one catalytic subunit α and two accessory subunits β and γ . These subunits form 12 different isoforms and the resultant isoform is likely to possess distinct properties, e.g. activity, localization, tissue distribution, but also regulation [180–183]. AMP binds to γ -accessory subunit and promotes AMPK activity, nevertheless there are other stimuli that may boost it. Calmodulin-dependent protein kinase kinase (CaMKK; Ca^{2+} -dependent signalling cascade) has been shown to phosphorylate Thr¹⁷² residue of α -catalytic core which activated AMPK [184,185]. Another upstream regulator of AMPK is activated liver kinase B1 (LKB1) and many pharmacological agents were also reported as stimulants [186]. Upon activation, AMPK mediates the upregulation of both – the carbohydrate and the FAs metabolism - by translocation of GLUT4 vesicles towards sarcolemma [187,188], but also by the sarcolemmal recruitment of CD36 [189], by inhibition of acetyl-CoA carboxylase producing malonyl-CoA [190], by PFK activation [98] and regulation of PDHC [191,192]. AMPK was reported to phosphorylated peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) [182,193]. AMPK overrides Randle's cycle and the inhibition of glucose uptake by FAs no longer prevails (but the inhibitory effect on glucose oxidation persists via activation and expression of PDK) [38]. It is of note, however, that AMPK represents only a transient solution as its prolonged, i.e. chronic activation was shown deleterious [19,194].

At molecular level, an interesting relationship between AMPK and another serine/threonine kinase, protein kinase B/Akt is present. Akt, as insulin mediator, affects cardiac energy metabolism by glucose uptake stimulation (via GLUT4, HK2 and PFK2) [99], mTOR pathway [195], and the Forkhead Box O (FoxO) transcription factory family of proteins were also reported to be regulated by Akt-mediated phosphorylation [196,197]. Besides role in metabolism, Akt performs in survival, proliferation, growth and hypertrophy, for more information please read an excellent review by [198]. In context to myocardial energy metabolism – AMPK has been reported to regulate insulin receptor substrate 1 (IRS-1) and Akt, whereas Akt with insulin had negative impact on AMPK activation. [199]. Interestingly a study by [200] showed that during energy depletion the induction of apoptosis is due the inhibitory effect of AMPK on Akt signalling.

Cardiac tissue also derives information about the incoming food sources by two more recently found families of proteins. The first, sterol regulatory element-binding proteins (SREBPs) which are tightly bound to membrane and sense FAs. Its N-terminal active domain translocates to nucleus to start transcription of its target genes. There are 3 isoforms, SREBP1-a, SREBP1-c, and SREBP-2 with distinct lipid gene targets [201]. The second, carbohydrate response-element binding protein (ChREBP), was first thought to be expressed only in liver, but it was shown that it can be also expressed in the muscles. ChREBP contains several functional domains including nuclear export and import signals. As it senses mainly the alteration in carbs, target genes are involved in glucose metabolism [202,203].

A complex system of modulators is represented by the peroxisome proliferator activated receptors (PPARs) and its coactivator, mainly the one already mentioned – PGC-1 α . The heart mainly expresses PPAR α and PPAR β/δ [3]. These receptors, showing distinct function in cardiac metabolism, hetero-dimerize with retinoid X receptor (RXR) upon activation and regulates the genes involved in FA (PPAR α) or in both - i.e. FA and carbohydrate metabolism (PPAR β/δ) – in the case of PPAR β/δ , besides GLUT4 recruitment and PFK stimulation, its upregulation also leads to higher glucose oxidation rates. PPAR β/δ also regulates the expression of PPAR α and PGC-1 α , thus upregulation FAs metabolism and oxidation rates. On the other hand, binding of either of these heterodimers (α -RXR or β/δ -RXR) to PGC-1 α significantly increases the transcriptional activity. Moreover, PGC-1 α may co-activate other regulatory pathways – oestrogen-like receptor pathway and nuclear-respiratory factor pathways. With concomitant regulation of both major cardiac PPARs, the activity and expression of PGC-1 α may control the entire metabolic phenotype of the cardiomyocyte [204–206]. The transcription activity of PPAR α and PPAR β/δ is illustrated in Figure 10. These central regulators of gene expression are complemented with factors, such as hypoxia inducible factor 1 (HIF-1), which will be the topic of Chapter 3.

Figure 10: PPAR signalling in the heart. Original from [3]. The binding of the PPAR α /RXR heterodimer to PPAR response elements (PPRE) increases the expression of various genes involved in FA metabolism. The binding of PGC-1 α to the PPAR α /RXR heterodimer or to PPAR β / δ /RXR heterodimer increases their activity. Depicted are genes encoding proteins involved in FA metabolism (**A**, **B**). PPAR β / δ (**B**) also increases the expression of GLUT4 and phosphofructokinase.



3: HYPOXIA

“WE KNOW THAT ONCE WE STOP LEARNING AND CALL OURSELVES LEARNED, WE BECOME USELESS MEMBERS OF THE SCIENTIFIC SOCIETY”.

CHRISTIAN DE DUVE

“KNOWLEDGE IS THE DEATH OF RESEARCH”.

WALTHER HERMANN NERNST

When Cieza de Leon (1553) was describing the life among Inca during the Spanish conquest, some spectacular notes were added. After the first Spanish colonists reached the area of Andes, they were shocked how high fecundity rate the locals had. In fact, it took about 53 long years of infertility and stillbirths until finally, the first child was born in one of the Spanish families. The inability to give birth was ascribed to the cold Andes climate and obviously, the colonists had no idea that it is the oxygen concentration in the inhaled air that was responsible for their problems [207].

These lines were probably one of the first documenting that the reduced oxygen levels may significantly affect human physiology. Moreover, it also documented the first note about some kind of “adaptation² to hypoxic³ conditions”. However, although I would not like to underestimate the problem Spanish colonists had, modern society is facing much terrific problem. World health organization (WHO) reports that 15.2 million people died either because of ischaemic heart disease or it’s acute form, stroke, in 2016. And although massive progress in our understanding of these diseases has been made, these diseases have remained the leading causes of death globally in the last 15 years (WHO data). Shockingly, Centre for Disease Control and Prevention (CDC) released a report in 2010 estimating that by the year 2050, as many as 1 in 3 U.S. adult citizens could be diagnosed diabetic⁴ (CDC data). It thus seems that we have not “reached the top” yet, especially if the current trends continue. The next section briefly describes ischaemia, reperfusion, I/R injury and then the focus will be on the possibilities how to protect the heart against I/R (all in the context of cardiac energy metabolism).

² Adaptation as was defined by [401]: „ Adaptations are modifications of organisms that occur in the presence of particular environments and circumstances and as such are not limited to modifications that seem favourable to the individual“.

³ Note the difference between hypoxia (i.e. reduced partial pressure of O₂) and ischaemia (defined as „no flow“, thus anoxia)

⁴ According to WHO, diabetes killed another 1.6 million people in 2016 (WHO data)

3.1. Ischaemia

Ischaemic heart disease describes a group of clinical syndromes (e.g. stable and unstable angina, myocardial infarction, sudden cardiac death) that all are characterized by myocardial ischaemia. This word has its origin in Greek/Latin – first Greeks started using *ischaimos* (from *ischein* + *haima* = to restrain + blood) and then Roman empire took it and made the word *ischaemus* which means “styptic”. It is a situation describing an imbalance between cardiac blood supply and demand. Contrary to hypoxia, the inadequate perfusion not only reduces oxygen supply to the myocardium, ischaemia is also associated with reduced availability of nutrients and inadequate removal of metabolic end products [208,209]. Complete elimination of flow quickly results in high energy phosphate drop, lactate accumulation and contractile dysfunction which, if unrestrained, leads to cardiac necrosis and myocardial infarction⁵ [210].

In the first seconds of ischaemia, due to a sudden drop of [O₂] electron transport in ETS slows down and a decrease in NAD⁺/NADH ratio can be observed. This ultimately results in reduced ATP formation by OXPHOS in the mitochondria [211]. High-energy PCr is quickly regenerating ATP molecules, but as the complete turnover of ATP in a healthy myocardium takes approximately 10 seconds and the PCr storage levels are not high enough [11,212], PCr drops significantly at the onset of ischaemia [213]. Due to elevated [AMP]/[ATP], AMPK becomes activated [214]⁶. Activated AMPK phosphorylates all targets (mentioned in section 2.6.) and mediates the translocation of GLUT onto the sarcolemma, PFK2 activity upregulation, inhibition of acetyl-CoA carboxylase (to avoid FA anabolism) – in other words, the ATP-utilizing reactions are minimized, whereas the ATP-generating ones are speeded up. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR; stimulator of AMPK) was also shown to induce glycogenolysis by activating glycogen phosphorylase in rat skeletal muscle [215]. However, as the ischaemia switches from the acute to transient form, the lack of oxygen furtherly inhibits OXPHOS with concomitant increase in ROS production by almost 2-fold in SSM and IFM [216]. Glucose and glycogen become very important sources of energy but both are processed glycolytically as the heart keeps deriving most of its energy from FA β -oxidation (Stanley, 2001). The inability to couple glycolysis to OXPHOS via malate/aspartate shuttle (thanks to high [NADH]) accelerates LDH reaction and the heart becomes a net lactate producer. Unfortunately, as the blood flow is restricted, metabolic by-products cannot be washed away and accumulate, particularly NADH, lactate, and

⁵ in 1680's Latin started using the word *infarcire* (“to stuff”), Latin of 1870-1875 developed *infartus* (“stuffed into”)

⁶ Besides AMPK, another signalling pathways are activated in ischaemia: i) Grb2-Sos-Ras ii) GPCR-PLC γ -DAG-PKC ϵ iii) EGFR-PI3K-PDK1/2-Akt and iv) TNFR-ASK-MAPK – for more information see [402]

H⁺ [218]. Therefore, intracellular pH starts to drop⁷. Sarcoplasmic acidification impedes cardiac muscle capability to maintain Ca²⁺ homeostasis. The reduction in pH was shown to increase the ATP requirements for SERCA [219] as well as the amount of Ca²⁺ necessary to induce myocyte force generation is increased at ischaemic pH levels [220]. Therefore, the energy provided by ATP hydrolysis is excessively used in chemical work (i.e. to support ionic homeostasis) and less to generate power. Furthermore, as the intracellular levels of [H⁺] rise, it accelerates the Na⁺-H⁺ exchange across the sarcolemma. And, sadly, the vicious cycle begins. The energy disbalance and the loss of ATP slows down the activity of Na⁺/K⁺ ATPase, which in turn leads to intracellular [Na⁺] overload. This leads to reverse mode of NCX⁸ activation, exchanging 3Na⁺ for 1Ca²⁺ [221]. ROS production from ETS furtherly inhibits activity of SERCA [222], which in turn leads to even higher increase in sarcoplasmic [Ca²⁺]. Although the FAT/CD36 translocated from the sarcolemma at the onset of ischaemia, the intramyocardial lipid accumulation is persisted [223] and oxidation of FAs dominates as the main residual source of ATP production. According to Randle's cycle, the upregulated FA oxidation inhibits pyruvate oxidation, thus furtherly uncoupling glycolysis from OXPHOS which leads to higher myocardial acidification. In the prolonged/severe ischaemia, cardiac tissue is totally dependent on anaerobic glycolysis using endogenous substrates and eventually, even glycolysis becomes inhibited due PFK inhibition by [H⁺] [87]. As indicated above, the ischaemia-induced metabolic shift in the heart is complex and strongly depends upon the type and duration of ischaemia.

3.2. Reperfusion

During reperfusion, the oxidative rates are progressively returning to the pre-ischaemic values, however the contractile power is transiently impaired and due to its gradual recovering, the heart is often referred to as “stunned myocardium” [224]. Moreover, after the restoration of blood flow, metabolites like lactate can be effectively excluded. The incoming oxygen accelerates OXPHOS with subsequent ROS production [225] that exaggerates myocardial injury. These ROS are not only of mitochondrial origin – xanthine oxidase is an important extramitochondrial source of ROS during reperfusion, as allopurinol (xanthine oxidase inhibitor) is already being used as therapeutic intervention to mitigate I/R injury in human [226]. The acute increase in the level of oxidative stress impairs many cardiac signalling pathways – for instance, in healthy heart D-AKAP1/PKA pathway cooperate to regulate mitochondrial structure and function. D-

⁷ It is noteworthy to add that accumulation of H⁺ is likely due the activity of ATPases and cardiac inability to replenish ATP stores. A study of [403] showed that the decrease in [HCO₃⁻] is much greater than the increase of lactic acid during ischaemia in the dog myocardium.

⁸ Na⁺/Ca²⁺ exchanger

AKAP1/PKA phosphorylates dynamin-related protein 1 (Drp1) at the OMM which inhibits Drp1 fission activity. At high ROS levels, the PKA regulation of D-AKAP1 is impaired and mitochondrial stability and OXPHOS function is compromised [227,228]. The restoration of blood flow stimulates the activity of NHE and, especially if the myocardium is not reperfused in a timely manner, also the activity (reversed) NCX which leads to a further Ca^{2+} overload. In this moment, however, as the change is sudden, Ca^{2+} concentration may rise even above the ischaemic levels and induce calpain-mediated proteolysis with sarcoplasmic reticulum stress in which eventually the activation of PKC δ may lead to the terminal response [229–231]. Because of the OXPHOS restoration, mitochondrial ionic and metabolic exchange, including the shuttle pathways restore. As sarcoplasm is overloaded with Ca^{2+} , the intramitochondrial stores of Ca^{2+} may become unbearable (in combination with depleted adenine nucleotide pool and oxidative stress). Mitochondria undergo massive swelling, thanks to a non-specific mPTP opening [232,233]. ATP generation rates of swollen mitochondria are pitiful when compared to the healthy mitochondria and if this situation is not fixed, i.e. if the mPTP opening persists, the cell death occurs via either apoptosis or necrosis [234]. Recently, GAPDH accumulation with subsequent oligomerization was seen in mitochondria that were damaged after I/R. GAPDH was reported to facilitate mitochondria into multi-organelle lysosomal-like (LL) structures for their elimination [107]. Reperfusion also upregulates the levels of casein kinase (CaK) 2 α which enhances the FUN4-domain containing 1 (FUND C1) protein by phosphorylation effectively inhibiting mitophagy [235]. So except cardiac necrosis, apoptosis, and necroptosis, cardiac I/R induced injury also induce spurring [230,231,236] in and/or alterations in autophagy [235]. It is of note that the major determinant of the resultant myocardial injury is the ischaemic period interval length and its severity, but also by the myocardial preparedness for ischaemic insult (as will be discussed later). Indeed, the interval in which the cardiac injury changes from reversible to irreversible state was estimated to be 20 min, as seen in normothermic global ischaemia of isolated rat heart [237]. The pathophysiologic mechanisms of myocardial I/R injury are summarized in Table 1.

Pathogenesis associated with I/R injury and the high mortality and morbidity rates of cardiovascular diseases drive the scientific research which has made a significant progress in understanding the disease, therefore many discoveries regarding cardioprotection against I/R emerged. Among the best-known strategies, ischaemic forms of pre- [238] and postconditioning [239,240], physical exercise [241], and adaptation to chronic hypoxia [209] have been shown to significantly reduce the I/R injury. The last is the subject of the next subchapter.

Table 1: Pathophysiologic mechanisms of myocardial I/R injury, re-draw from [242]

Mechanism	In particular
Ion accumulation	Intracellular calcium overload Increased intracellular sodium Drop in pH with rapid normalization upon reperfusion
Dissipation of mitochondrial membrane potential	mPTP
Free radical formation/ROS	Generation from Xanthine oxidase Release of reactive mitochondrial intermediates Neutrophil infiltration ROS-induced ROS
Dysregulated NO metabolism	Loss of NO-vasodilation Accumulation of reactive peroxynitrite
Apoptosis and autophagy	
Endothelial dysfunction	Cytokine and chemokine signalling Expression of cellular adhesion markers Impaired vasodilation
Platelet aggregation and microembolization	
Immune activation	Innate immunity (e.g. complement activation, expression of Toll-like receptors) Neutrophil accumulation Cell-mediated damage (macrophage and T-cell)

3.3. Hypoxia in terms of adaptation

Hypoxia determines a state in which there is a disproportion between oxygen supply and oxygen demand. Contrary to ischaemia, however, the blood flow is persisted, so the metabolites may be effectively washed away. According to [209] there are several elements that can lead to a unique type of hypoxia – i) when haemoglobin function is impaired or when there's an insufficient erythrocyte density, the hypoxia may be referred as anaemic; ii) when there's enough nutrients, physiological pO_2 and haemoglobin levels in the blood, but the flow is restricted (e.g. by atherosclerosis) the hypoxia may be referred as circulatory; iii) when tissue's ability to utilize oxygen is compromised due to a cytotoxic agent, the hypoxia may be referred as histotoxic; and iv) the hypoxemic hypoxia which occurs when the pO_2 of the arterial blood is low (e.g. *in utero* development, life at high-altitude). Although de Leon's notes about Spanish colonist was

remarkable, it was not sooner than in 1960, when Hurtado [243] showed in his study that high-altitude inhabitants had a lower incidence of myocardial infarction (i.e. loss of cardiac myocytes caused by prolonged ischaemia). However, in 1958, Kopecky and Daum showed (possibly as one of the first ones) in Prague that the heart's contractile recovery after I/R is improved in the hearts previously exposed to anoxic conditions when compared to control (Kopecky and Daum, 1958). Since then, many beneficial effects of adaptation to high-altitude hypoxia have been revealed – reduced infarct size [245,246], improved post-ischaemic recovery [247], reduced incidence of arrhythmias during I/R [248,249] and more [209].

There is a certain time delay in the development of adaptation which distinguishes it from the acute physiological response. If the environmental change is sufficient to evoke an immediate physiological response (e.g. sweating in hot climate), adaptive processes have been initiated and a subsequent exposure, provided it is not unduly delayed, will evoke qualitatively similar physiological responses. However, quantitatively, they will be altered [250]. Since the use of human subjects in experiments studying cardiac I/R injury in context to high-altitude adaptation *in vitro* and *in vivo* would be unethical in many cases, diverse animal models have been adopted in experimental studies. The most frequently used experimental models in research of adaptation to continuous hypoxia are either held in normobaric⁹ or hypobaric¹⁰ chambers under laboratory conditions or (*via* Spanish conquerors) in natural high-altitude environment (i.e. mountains). And in terms of exposure, we may distinguish between continuous and intermittent type of chronic hypoxia, as these reflect the fact that the hypoxic conditions may not be of permanent nature (e.g. asthma, chronic pulmonary obstruction, metabolic syndromes, sleep apnoe). Therefore, 4 basic types of adaptation exist – continuous normobaric hypoxia (CNH), continuous hypobaric hypoxia (CHH), intermittent normobaric hypoxia (INH), and intermittent hypobaric hypoxia (IHH) [209]. All four types are associated with the cardioprotective effect against I/R as shown in rat heart models [251–254]. Contrary, a study of Savourey [255] demonstrated that while CNH adaptation led to a lower pO₂ values in coronary blood, greater hypoxemia, and hypocapnia, the effect of IHH in these aspects was not so pronounced. Another notable corollary arises from the effect of IHH on the heart antioxidant system – whereas the guinea pig heart's antioxidant system is empowered by superoxide dismutase (SOD) and CAT [256], the rat heart showed SOD to be upregulated but not CAT [257] which corresponds with our newest study where CAT was not affected by IHH adaptation (Kolar et al. 2018; Submitted). At last, several studies have shown adaptation to chronic

⁹ The concentration of oxygen is reduced on a desirable value but the air pressure corresponds to the low altitude.

¹⁰ The concentration of oxygen is reduced on a desirable value through the reduction of air pressure (simulating high-altitude).

hypoxia as detrimental, failing in protection against I/R [258,259]. It is obvious that the various experimental hypoxic models, the species of experimental animals, but also the methodological approaches do allow to compare the common mechanisms on one hand, however, the detailed involvement of individual factors is genus- and tissue-specific and as such may differ in different types of adaptation.

3.3.1. Mechanism of adaptation to chronic hypoxia

Hypoxic conditions are immediately recognized by hypoxia inducible factor 1 (HIF-1). This is possible due to 3 factors – i) the transcription factor is composed of two subunits; subunit 1 α is constantly degraded by the action of prolyl and asparagyl hydroxylases under normoxic conditions in the sarcoplasm [260,261], whereas the 1 β is located in the nucleus and is generally regarded as constitutively expressed [262]. The decreased concentration of oxygen inhibits the activity of prolyl and asparagyl hydroxylases which subsequently leads to HIF-1 α accumulation and translocation to nucleus [263,264] or ii) the increase amount of ROS produced by complex III in hypoxic conditions stabilizes it [265]; and iii) 2-oxoglutarate regulates binding of hydroxylated HIF-1 α to prolyl hydroxylase domain 2 [266], so energy depletion may in turn lead to HIF-1 α accumulation. Upon arrival into nucleus, HIF-1 α creates a heterodimer with HIF-1 β . This heterodimer is transcriptionally active and has over 100 target genes that are functionally involved in angiogenesis, cell differentiation, tumour metastasis, apoptosis and energy metabolism [267,268]. The effect of HIF-1 α on cardiac energy metabolism will be addressed in the next section. However, adaptations to hypoxia were reported to affect many other signalling pathways. As the adaptations affect the whole system and not the heart alone (contrary to ischaemia which causes a local distress), the process is accompanied with higher adrenergic stimulation at the onset (Ostadal et al., 1984, from the Book: Catecholamines and Stress 1984). Adrenergic stimulation *via* cAMP may induces the activity of PKA, which was seen in IHH rat heart [269]. PI3K/Akt inhibitor, LY294002 before I/R protocol abolished the IHH-induced cardioprotective effect [270] and similar effect was observed when wortmannin (specific Akt inhibitor) in mice adapted to CNH was applied [271]. Moreover, we observed that the activation of PI3K/Akt pathway is enhanced in a brief I/R of CNH rat heart when compared to their normoxic littermates [272]. Notably, PI3K/Akt pathway, as a part of RISK (**R**eperfusion **I**njury **S**alvage **K**inase) pathway, is inextricably connected with ischaemic-preconditioning [273]. It is interesting that RISK pathway is involved in both cardioprotective interventions (i.e. chronic hypoxia and conditioning). In 2002, Neckar *et al.* demonstrated that both cardioprotections are not additive, in other words – that the preconditioning does not furtherly improve the effect of chronic hypoxia [246]. However, the same group reported that the cardioprotective effect of chronic hypoxia lasts longer when compared to

conditioning [274]. Apart from PI3K/Akt signalling, extracellular signal-related kinase, ERK1/2, are also parts of preconditioning, which has also been reported to be activated in IHH adaptation [271,275]. At last but not least, PKC has an indisputable role in cardioprotection induced by chronic hypoxia. In neonatal rat ventricular myocytes, hypoxia alters the distribution of PKC isoforms, particularly PKC α , PKC ϵ and PKC δ isoforms [276,277]. Hence PKC activation and translocation from sarcoplasm to membrane fraction by hypoxia was observed, and inhibition of PKC ϵ by a specific inhibitor, ϵ V1-2 peptide, abolished the protective effect [278], PKC was implicated also in the adaptation to hypoxia which was confirmed many times [277,279]. Hlavackova *et al.* showed in their study that the IHH (7000m, 8h/day) upregulated and increased p-PKC ϵ ¹¹/ PKC ϵ ratio, but also PKC δ levels as well as the amount of phosphorylated PKC δ on Ser⁶⁴³ residue [280]. It was proposed that the upregulation of PKC δ is due to higher oxidative stress, as demonstrated by N-acetylcysteine treatment of IHH adapted rats which abolished the cardioprotective effect [252]. Importantly, PKC ϵ was identified as one of components of mTORC2¹² Rictor [281], whose activation was shown in Akt phosphorylation on Ser⁴⁷³ residue, revealing an important relationship between two kinases. We observed a higher amount of Akt phosphorylated on Ser⁴⁷³ in I/R protocol of CNH adapted rats when compared to the control, but not that on Thr³⁰⁸ residue [272]. Another evidence that approves the effect of Akt and PKC in the adaptation to hypoxia is provided by study of Wang *et al.*, who showed that both wortmannin, ϵ V1-2 peptide and 5-hydroxydecanoate (inhibitor of mitochondrial K⁺_{ATP} channels) all inhibited the cardioprotective effect [282]. The role of mitochondrial K⁺_{ATP} channel was shown in the IHH 7000 m regimen whereas 5000 m was associated with sarcolemmal K⁺_{ATP} channel [283]. These channels control the membrane potential and regulate membrane swelling, see [284,285] for more information. The I/R injury is caused by Ca²⁺ overload, any process maintaining the Ca²⁺ homeostasis could be a strong cardioprotective tool. Indeed, adaptation to chronic hypoxia was shown to affect several proteins – phospholamban [269] and CaMKK II [286] – these two proteins present two major regulators of Ca²⁺ homeostasis – activated CaMKK II phosphorylates phospholamban (inhibition) which, subsequently, activates SERCA and Ca²⁺ reuptake into sarcoplasmic reticulum [287]. Similarly to these effects, IHH adapted rats showed a higher [NADH]/[NAD⁺] (Kolar et al., 2018; Submitted) and high [NADH] was shown to inhibit the ryanodine receptors (RyRs) activity [288], which may in turn improve the Ca²⁺ handling during I/R.

¹¹ Phosphorylated PKC ϵ

¹² Mammalian Target Of Rapamycin Complex 2

3.3.2. Cardiac energy alterations induced by chronic hypoxia and its advantages

From the text in 3.1. and 3.2., any pharmacological effectors or interventions against I/R injury should generally focus on:

- Increasing myocardial glycolytic rates by increasing glucose uptake during ischaemia or by glycogen store increasement prior to ischaemic insult
- Inhibiting myocardial FA oxidation with concomitant upregulation of pyruvate oxidation
- Direct stimulation of PDH activity
- Improving myocardial FA utilization in ischaemia
- Improving PCr-CK system
- Stabilizing mitochondrial membrane potential
- Protecting mPTP opening

Any improvement in cardiac energetics during ischaemia and the consecutive reperfusion would not only aid the contraction sustainment but it will also help to maintain the ionic balance even during prolonged ischaemic episodes as the ion pumps function also depends on ATP. Any pre-preparations of metabolic apparatus for the conditions of limited oxygen before the situation supervenes have two advantages at once – first, the metabolic shift is not so dramatic/gradual and second, as the myocardium does not have to change the proteome (i.e. create new enzymes and disintegrate the one that are not necessary at the moment), the pre-preparations are energy-saving themselves.

HIF-1 α activation during the adaptation to chronic hypoxia has a major impact on cardiac metabolism. HIF-1 is a key regulator of genes expressing proteins involved in the anaerobic metabolism of glucose (e.g. GLUT, HK, Aldolase c, LDHA, and PDK) [268,289,290] thus with activated Akt may significantly contribute to the upregulated glucose metabolism in the adapted hearts [75,272,291]. This effect is important in tissues which derives most of energy from carbohydrate oxidation (as it fights against exaggerated ROS production). Nevertheless, cardiac tissue uses mainly FA oxidation to provide energy, so this effect alone does not minimize ROS generation therein. In context to FA oxidation rates, HIF-1 interacts with PPAR α and downregulates its transcription [292,293]. But HIF-1 also downregulates RXR-PPAR α heterodimerization, thus impeding PPAR α transcription activity [294]. As PPAR α is the major determinant of FA oxidation within the cardiomyocyte, this ultimately leads to a decreased rates of FA oxidation as seen in both cardiac mitochondrial subpopulations of IHH adapted animals (Kolar

et al., 2018; Submitted) and in whole LV tissue samples [295]. This, in turn, decreases FA oxidation rates through downregulation of targeted genes like CPT1 and medium-chain acyl-CoA dehydrogenase like observed after 1 week of IHH in mice [296]. Moreover, HIF-1 also modifies ETS complexes. It was reported that HIF-1 downregulates Complex I activity adopting NDUFA4L2 [297] which we approved in IHH adapted rats where NDUFB8 (a labile subunit of Complex I) was downregulated (Kolar et al. 2018; Submitted). Another example of HIF-1 action is the switch from COX 4.1 to COX 4.2 subunit (Complex IV) which enhances the enzyme activity [298], however, in our recent studies we were neither able to detect an increase in COX 4.2. mRNA levels in SHR and conplastic SHR-mtBN¹³ adapted to CNH (in fact, both COX 4.1 and 4.2 mRNA levels were downregulated) [291] nor did we in IHH adapted rats where MTCO1 protein expression (the most labile subunit of Complex IV) was downregulated (Kolar et al., 2018; Submitted). But upon these pathways, HIF-1 activation also regulates other factors to assure higher oxygen supply, e.g. neovascularization or greater coronary flow [299].

Activation of either Akt or AMPK will furtherly improve the glycolytic rates, but the later will also influence the oxidative pathways activating PDH. Moreover, activated Akt can phosphorylate HK2 on Thr⁴⁷³ residue. Phosphorylated HK2 binds to OMM promoting advanced metabolic channelling [300,301]. HK2 associates with mitochondrial voltage dependent anion channel (VDAC) at the outer leaflet of OMM [83] and cooperates with mitochondrial mtCKS in the intermembrane space, and adenine nucleotide translocase (ANT) which is located in IMM [302]. This not only couples glycolytic rates to OXPHOS, but also stabilizes mitochondrial membrane potential as hexokinase disruption from mitochondrial membrane leads to mitochondrial membrane depolarization and cardiac necrosis [303,304]. Moreover, HK2 was shown to compete the VDAC binding site with pro-apoptotic protein Bax [85]. The phosphorylated HK2 is less sensitive to (G-6-P)-mediated sarcoplasmic recruitment with concomitant increase in HK enzyme activity [300]. HK2 activity and association with mitochondria was approved in IHH adapted rats [305]. The brief ischaemic insult did not cause any disruption of HK2 from OMM in CNH adapted rats which correlated with the amount of p-Akt Ser⁴⁷³ [272]. Interestingly, we also showed that the energy deprivation of CNH adapted LV myocardium is not as severe as in appropriate normoxic LV as demonstrated by lower AMPK activation in CNH [272]. The activated Akt may also be the reason of upregulated CKB isoform in CNH [177] and in IHH (Kolar et al., 2018; Submitted).

¹³ SHR (Spontaneously hypertensive rat); SHR-mtBN (Spontaneously hypertensive rat harbouring mitochondrial genome from a more ischaemia-resistant strain Brown Norway)

Both, activated PKA and/or PKC δ can affect mitochondrial morphology – for instance, activated PKA via D-AKAP1 improving mitochondrial bioenergetics [227], whereas PKC δ through phosphorylation of GAPDH at Thr²⁴⁶ that stops GAPDH-mediated micro-mitophagy [306]. Phosphorylated GAPDH may thus persevere as an integral part of sarcolemmal K⁺_{ATP} channel. Interestingly, GAPDH regulates this channel activity irrespectively of high intracellular ATP by producing 1,3-bisphosphoglycerate [103]. As LDHA is upregulated by the action of HIF-1 and likewise represents an integral part of K⁺_{ATP} channel, the metabolic channelling between these two reactions may not only help glycolytic flux in anoxic conditions, but also may induce K⁺_{ATP} channel opening. This particular situation reveals a non-canonical role of the energy metabolism enzymes.

Taken together, adaptation to chronic hypoxia represents an extremely powerful intervention rendering the heart less susceptible to I/R injury as it provides all the points mentioned in the beginning of this subchapter. However, there is a price for it. Except the beneficial effects, there are some adverse effects one needs to be aware of. For instance, pulmonary hypertension with concomitant RV hypertrophy (caused by increased resistance of pulmonary vessels), cerebral and coronary vascular problems, fatigue etc. [209,307,308].

Obviously, the list of proteins, enzymes, molecular pathways and their inter-play is so complex thus the given description is still far from complete. Many other enzymes (especially that of KC and ETC), receptors (oestrogen-like receptors, thyroid hormone receptors etc.) play role. However, the description of these enzymes and pathways needs to be found elsewhere.

4. RATIONALES

1) It was demonstrated previously that Akt (as a part of Reperfusion Injury Salvage Kinase pathway) plays role in cardiac ischaemic-preconditioning [273,309]. This procedure renders the heart less susceptible to I/R injury as does the adaptation to chronic hypoxia [209,274]. Moreover, it was shown that the cardioprotection induced by preconditioning and hypoxia are not additive indicating that some of the molecular mechanisms may be shared by both interventions [246]. Therefore, we hypothesized that the cardiac Akt/HK2 pathway could be affected differently in a brief ischaemia-reperfusion insult in CNH adapted animals when compared to their normoxic littermates.

2) Muravyeva *et al.* reported that mitochondrial genome modulated the ischaemic tolerance in diabetic rats [310]. SHR rats were reported to be predisposed to metabolic disturbances affecting many systems including the cardiovascular [311,312]. As mitochondrial proteins are encoded by two separate genomes (mitochondrial and nuclear), we proposed that the exchanged mitochondrial genome in uniquely developed conplastic strain SHR-mtBN may be a relief for cardiac energetic deficit observed during I/R protocol, thus provide a stronger cardioprotection.

3) Selectively replaced mitochondrial genome of SHR by BN potentiated the cardioprotective effect of CNH against myocardial I/R injury [291]. In this study, higher activity of HK in SHR-mtBN was reported. HK activity may be regulated by Akt by its translocation towards mitochondrial outer membrane [300,301]. Interestingly, it was also shown that activated Akt increases stability of HIF1 α [313,314]. Our goal was to determine the possible role of RISK pathway in CNH-afforded cardioprotection in both strains.

4) Hypoxia was shown to reduce the cardiomyocyte ability to process FAs [315]. The phenomenon was also shown in rat hearts adapted to IHH [295]. Foetal-gene reprogramming induced by hypoxia upregulates glycolytic genes [177,289,316,317]. The upregulated glycolytic flux may, in turn, promote changes in gene expression [318]. We hypothesized that both cardiac mitochondrial subpopulations (SSM and IFM) are affected differently by the adaptation to IHH as previous studies had indicated several metabolic alterations between them in normoxic conditions [319]. We also proposed that the changes of mitochondrial metabolism may be reflected within other energy domains of the cardiomyocyte.

5. AIMS OF THE THESIS

Major aim

- 1) In all parts of our study, we have aimed to depict the role of altered cardiac energy metabolism of rat LV in cardioprotection induced by adaptation to chronic hypoxia with special interest to mitochondrial physiology.

Fractional aims

- a) In the first part (Study A) of our investigation, we aimed to determine the possible role of Akt salvage pathway and HK translocation in the CNH adapted rat LV subjected to a brief I/R protocol.
- b) Concomitantly, we investigated its role in cardiac metabolism alterations (mtCKS, AMPK), the level of anti- (Bcl-2) and proapoptotic (Bax) proteins, and HK translocation during I/R.
- c) In the second part (Study B), we strived to describe the effect of adaptation to CNH on Akt pathway between SHR and SHR-mtBN rat LV, the possible changes in the expression of major cardiac Akt (Akt1 and Akt2), the alteration of carbohydrate metabolism (expression and localization of HK2 and GLUT4) as a potential inducer of cardioprotection.
- d) Concomitantly, we aimed to describe the affection of Akt pathway on HK enzyme activity in sarcoplasmic and mitochondrial compartment
- e) In the last part (Study C), the aims were to determine how IHH affects cardiac rat LV energetics with respect to cardiac energy domains (SSM, IFM, sarcoplasm, peroxisomes), the expression and activity of mitochondrial OXPHOS enzymes, the oxidative capacity and capability to process FA by SSM, IFM, and by the peroxisomal compartment.
- f) Parallely, the expression and activity of several glycolytic enzymes (PFK, LDH), NAD(H) pool, and cardiac CK system with respect to sarcoplasmic isoform localization were determined.
- g) Finally, we tested the susceptibility of cardiac mitochondria to Ca^{2+} .

6. MATERIALS AND METHODS

6.1. Animals

In the studies we used two types of animals:

- Adult male Wistar rats were obtained from breeding facility Velaz, Ltd., Czech Republic
- Spontaneous hypertensive rat (SHR; SHR/OlaIocv strain) and conplastic strain SHR-mtBN (SHR/OlaIpcv-mt^{BN/Crd}) harbouring the mitochondrial genome of a highly inbred Brown Norway. These were kindly provided by Ing. Michal Pravenec, DrSc. from Czech Academy of Sciences¹⁴.

All rats were kept at 12/12-h light/dark cycle and fed by a standard laboratory chow, water access *ad libidum*. The animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Academies Press [320]. The experimental protocol was approved by the Animal Care and Use Committee of the Institute of Physiology, Czech Academy of Sciences.

6.2. Model of chronic normobaric hypoxia

Animals (Wistar rats, SHR, and SHR-mtBN) were housed in a normobaric chamber equipped with hypoxic generators (Everest Summit, Hypoxico Inc., NY, USA), which reduced the percentage of oxygen in the ambient air to 10%, corresponding to altitude of 5500 m asl¹⁵. All animals were exposed to normobaric hypoxia for 3 weeks whereas the control group was kept at room air for the same period of time.

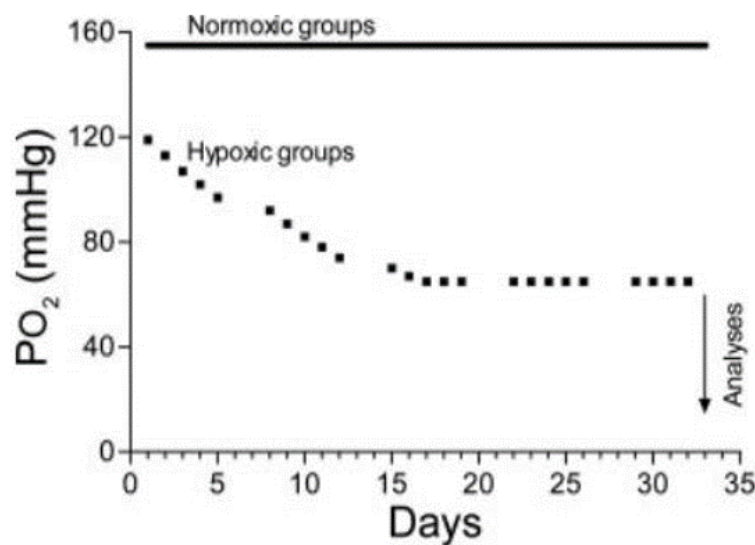
6.3. Model of intermittent hypobaric hypoxia

Adult male Wistar rats were exposed to intermittent hypobaric hypoxia (IHH) (7000 m asl, 8h/day; 35 exposures). Barometric pressure was lowered stepwise, so that the level equivalent to an altitude of 7,000 m was reached after 13 exposures (see Figure A). The control group, similarly to CNH, was kept at room air for the same time.

¹⁴ If you want to know more about the conplastic strain derivation, please see [404]

¹⁵ Asl = above sea level

Figure A: Model of chronic intermittent hypoxia and the experimental protocol. Hypoxic animals were subjected to hypobaric hypoxia starting at $pO_2 = 119$ mmHg (equivalent to 2400 m) and decreasing stepwise up to $pO_2 = 65$ mmHg (equivalent to 7000 m); this level of hypoxia was maintained for additional exposures until the heart was excised. Filled squares indicate daily exposures lasting 8 hours. Adapted from [252]



6.4. Ischaemia and reperfusion protocol

The animals were killed by cervical dislocation and the hearts were immediately excised and washed with ice cold saline. The hearts were then quickly placed on the Langendorff apparatus and perfused at constant flow (10 ml/min/g) by standard Krebs–Hanseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4 , 25 mM $NaHCO_3$, 11 mM glucose, pH 7.4) gassed with 95% O_2 and 5% CO_2 at 37 °C. The hearts from both normoxic (N) and hypoxic (CNH) groups were subsequently divided into three subgroups: (i) controls: perfused for 20 min; (ii) ischaemia: perfused for 20 min and then subjected to global (i.e. no flow) ischaemia for 10 min; (iii) ischaemia/reperfusion: perfused for 20 min, subjected to 10 min ischaemia followed by reperfusion for 10 min. At the end of each protocol, left (LVs) and right ventricles (RVs) were dissected, weightened, frozen, and stored in liquid nitrogen for further biochemical analyses.

6.5. Tissue preparation

All rats were sacrificed by cervical dislocation and the hearts were rapidly excised and washed in ice-cold saline. For biochemical analyses, the left ventricles (LV) were dissected, weighed, and used fresh or were immediately frozen in liquid nitrogen for further analyses. Please note that for biochemical analyses employing commercial kits, special tissue preparation was applied. However, the manufacturer's protocol was always strictly respected.

6.5.1. Tissue homogenization

a) For WB analyses of Wistar rats samples: Frozen tissue samples were pulverized in liquid nitrogen and subsequently homogenized in glass-Teflon homogenizer (Sartorius), using overhead stirrer (Ika, Eurostar). Homogenization buffer (in MM: 12.5 TRIS, 2.5 EGTA, 1 EDTA, 250 sucrose, 5 DL-dithiothreitol (DTT), protease (cOmplete, Roche/Sigma-Aldrich) and phosphatase (PhosSTOP, Roche/Sigma-Aldrich; pH = 7.4) inhibitors) in the ratio 1:8 (w/v) was used. Approximately 250uL of created homogenate was immediately stored at -80 °C. To solubilize the contractile apparatus protein, the rest of homogenate was resuspended in high-urea buffer (5 M urea, 2 M thiourea, 10 mM sodium pyrophosphate, and 0,13% (v/v) 2-mercaptoethanol, pH = 7.4).

b) For WB of SHR and SHR-mtBN samples: frozen tissue was slowly defrosted in ice-cold homogenization buffer, tissue was cut by ophthalmic scissors (normally used for tenotomy) into tiny pieces (< 1 mm³ each). The pieces were subsequently homogenized using glass-Teflon homogenizer (Sartorius) attached to overhead stirrer (Ika, Eurostar). The homogenates were either aliquoted and stored at -80 °C or used in sucrose density fractionation as described in [75,321].

6.6. Protein concentration analysis

Protein concentration of all samples was determined using Bradford protocol [322]. Briefly, samples were diluted (10-25x) in ultra-pure water and 10 µL was loaded into 96-well plate with blanks and standards of known concentration. The standards were created from BSA solution (Quick Start Bovine Serum Albumin, 2mg/mL; Bio-Rad). 250 µL of Bradford dye (Sigma-Aldrich) was added into each well and the absorbance of all samples was read using 96-well Multi-reader system Synergy HT (BioTek). The calibration samples create the calibration curve which serves to determine sample final concentration.

6.7. RNA isolation and Biomark chip analysis¹⁶

Frozen tissue pieces were pulverized in liquid nitrogen and subsequently homogenized in RNazol (Sigma-Aldrich) to extract the total cellular RNA. Concentration of extracted RNA was measured on NanoDrop (Thermo Fisher Scientific). RNA purity was thereafter tested on Agilent 2100. One µg of total RNA was loaded to the reverse transcription and the PCR reaction was performed using RevertAidTM H-minus First Strand cDNA Synthesis Kit with oligo(dT) primers (Fermentas/BioGen). Gene expression profiling (Biomark chip analysis) was performed by

¹⁶ In co-operation with RNDr. Vlasta Korenková, Ph.D., Mgr. Dita Sotáková, and Mgr. Iveta Nedvěďová

RNDr. Vlasta Korenkova, Ph.D. at the Institute of Biotechnology, Czech Academy of Sciences, with cDNA pre-amplification according to [323]. Transcript levels were normalized to the level of Hypoxanthine-guanine Phosphoribosyltransferase (HPRT) transcript. Specific primers sequences are listed in Table A.

TABLE A: Specific primers design.

Gene name:	L sequence (5'>3'):	R sequence (5'>3'):
HPRT	GACCGGTTCTGTCATGTCG	ACCTGGTTCATCATCACTAATCAC
CAT	CAGCGACCAGATGAAGCA	GGTCAGGACATCGGGTTTC
CKB	CCACTTCCTCTTCGACAAGC	GGAACGTCTTATTGTCATTGTGC
CKM	CCGCAGCATCAAGGGTTA	CCCGTCAGGCTGTTGAGA
mtCKs	GCCACCCCTTCATTAAGACTG	CAAAAAGGTCAGCAAACACCT
CS	AGACATCGGTTCTTGATCCTG	GCCAAAATAAGCCCTCAGGT
GLUT1	ACGTCCATTCTCCGTTTCAC	TCCCACGGCCAACATAAG
GLUT4	TGCAGTGCCTGAGTCTTCTTT	CCAGTCACTCGCTGCTGA
HIF1a	AAGCACTAGACAAAGCTCACCTG	TTGACCATATCGCTGTCCAC
HIF2a	GCAACTACCTGTTACCAACC	TTCATCAAAGTCTGGCTTCC
HK1	TCTGGGCTTCACCTTCTCAT	ATCAAGATTCCACAGTCCAGGT
HK2	CCAGCAGAACAGCCTAGACC	AGATGCCCTGAATCCCTTTG
LDHA	CACAATCAGCTGGTCCTTGAG	CACAATCAGCTGGTCCTTGAG
LDHB	TGGCTTGTGCTATCAGCATT	GACATCCACCAGGGCAAG
MCT1	TTCTGCATTGGTGTCATTGG	CATAGZGAGAGCTGGGTTCAAG
MCT2	TCAGAGCCAGAGCTGAGGAC	TGGCATTTCTGAGCTTCTACTG
MCT4	TGGACGTGAGGGTGGACT	TTTTCTCAGGCTCTGCCCTC
PDHA1	CAAAGATGAGGTCTGTTTGACATT	GCTTCAAATATCTGACCCTGGTT
PDK3	CATGTACTCAACTGCTCCTCGT	GCAAGCCATAACCAAATCCA
PFKFB2	CGTTATCTCTCACCAGGCTGT	GCACCTCAAGTACGGCAACT

Abbreviations: Hypoxanthine-guanine phosphoribosyltransferase (HPRT, housekeeping gene), Catalase (CAT), Creatine kinase brain-type (CKB), muscle-type (CKM), mitochondrial sarcomeric-type (mtCKs), Citrate synthase (CS), Glucose transporter 1/4 (GLUT1/4), Hypoxia-inducible factors (HIF1 α , HIF2 α), Hexokinase 1/2 (HK 1/2), Lactate dehydrogenase muscle-type (LDHA), heart-type (LDHB), Monocarboxylate transporter (MCT1/2/4), E1 α -subunit of Pyruvate dehydrogenase (PDHA1), Pyruvate dehydrogenase kinase 3 (PDK3), Phosphofructokinase/fructose-bisphosphatase 2 (PFKFB2)

6.8. SDS-PAGE and Western Blot (WB) analyses

LV homogenates and subcellular fractions were diluted to the same concentration ($\mu\text{g}/\mu\text{L}$) with high-urea buffer. Afterwards, 4x Laemmli sample loading buffer (Bio-Rad) and DTT were added to make a final concentration (1x Laemmli and 50 mM DTT). Diluted samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10-12% polyacrylamide gels (5% for the stacking part) (Acrylamide solutions provided by Sigma-Aldrich) at room temperature. The voltage was set to 100 V until the samples reached the separating part of the gel. Subsequently, the voltage was progressively increased to 175 V. The current was kept

still (350 mA) thorough the SDS-PAGE. The process was terminated at the moment when the vanguard samples left the most bottom part of the gel. The electro-transfer of the gel-resolved proteins onto nitrocellulose membrane (0.2 μ m, Bio-Rad) was done by TransBlot Turbo system (Bio-Rad) with the following parameters – 25 V, constant 1 A, 30 min. Membranes were blocked for 1 hour in TRIS-buffer saline (TBS) containing 0.05% (v/v) Tween 20 (Sigma-Aldrich) (TTBS) and 5% (w/v) non-fat dry milk at room temperature. Afterwards, the membranes were washed three times with TTBS (15 min each) and incubated overnight with the primary antibody. On the next day, the membranes were washed (3x15 min in TTBS) and incubated with an appropriate secondary anti-rabbit (GE Healthcare Amersham; Sigma-Aldrich), anti-mouse (Invitrogen) and anti-goat (Santa Cruz Biotechnologies) antibody conjugated with horseradish peroxidase. Proteins were visualized by enhance chemiluminescence (ECL) substrate (SuperSignal West Dura, Thermo Fisher Scientific) and quantified by the LAS-4000 imaging system (Genetica, FujiFilm). Changes in relative protein levels were quantified using Quantity One software (Bio-Rad). The same protein concentration of all samples was used and GAPDH was used as loading control to ensure that the whole process was not affected in any way. Table B shows the specific primary antibodies with the provider company.

Table B: The specific primary antibodies for the WB:

Protein	Lot	Provider
HK1	sc-28885 ab78420	Santa Cruz Biotechnologies Abcam
HK2	sc-28889 ab78259	Santa Cruz Biotechnologies Abcam
CKM	sc-15164	Santa Cruz Biotechnologies
CKB	sc-15157	Santa Cruz Biotechnologies
mtCKS	sc-15168	Santa Cruz Biotechnologies
Akt1	#2938	Cell Signalling
Akt2	#2962	Cell Signalling
pSer Akt	A00965	GenScript
pThr Akt	A00964	GenScript
OXPPOS	ab110412	Abcam
LDH A	2012S	Cell Signaling
LDH B	NB110-57160	Novus Biologicals
MCT 1	sc-14917	Santa Cruz Biotechnologies
PMP 70	SAB4200181	Sigma-Aldrich
Bax	ab7977	Abcam
Bcl-2	SAB4500003	Sigma-Aldrich
ACOX1	ab184032	Abcam
CAT	sc-271803	Santa Cruz Biotechnologies

Abbreviations: Hexokinase 1/2 (HK1/2); Creatine kinase brain-type (CKB); muscle-type (CKM); mitochondrial sarcomeric-type (mtCKs); Protein kinase B isoforms $\frac{1}{2}$ (Akt1 and Akt2); phosphorylated Akt on Ser473/Thr308 (pSer/pThr Akt); Mitochondrial ETS complexes I-V(OXPPOS), Lactate dehydrogenase muscle-type (LDHA), heart-type (LDHB), Monocarboxylate transporter 1 (MCT1), Peroxisomal membrane protein of 70kDa (PMP70), Pro- and anti-apoptotic member of Bcl-2 family proteins (Bax, Bcl-2); Acyl-CoA oxidase 1 (ACOX1), Catalase (CAT)

6.9. Subcellular fractionation:

6.9.1. Isolation of SSM and IFM

The two major subpopulation of mitochondria were isolated from fresh LV according to previous protocol [305]. In a few words, fresh LV samples from both experimental groups (i.e. control vs. adapted) were immediately placed in homogenization buffer (in mM: 250 sucrose, 10 TRIS, 2 EDTA, 2 EGTA, BSA (0.5 mg/ml), pH 7.2), cut into tiny pieces and homogenized in Teflon-glass homogenizer. The homogenate was centrifuged at 800 *g* (4 °C, 10 min). Supernatant (subsarcolemmal mitochondria, SSM) was filtered through nylon filter and centrifuged at 10,000 *g* (4 °C, 10 min). The 800 *g* pellet (interfibrillar mitochondria, IFM) was resuspended and stirred on ice in a high-potassium Tris buffer (in mM: 125 KCl, 250 M Sucrose, 10 Tris, pH 7.2). After stirring, the mixture was gently homogenized again, centrifuged at 800 *g* (4 °C, 10 min), the pellet was discarded, and the supernatant was filtered and centrifuged at 10,000 *g* (4 °C, 10 min). The resultant pellet was washed in sucrose-TRIS buffer [in mM: 250 sucrose and 10 TRIS, pH 7.2], gently homogenized and centrifuged again with the same conditions. The supernatant was discarded, and the pellet was resuspended in sucrose-TRIS buffer and used for further analyses.

6.9.2. Peroxisomal isolation

For the isolation of the cardiac peroxisomal fraction, a commercial peroxisome isolation kit was purchased from Sigma-Aldrich (PEROX1). The tissue preparation was in accordance to manufacturer's protocol with only slight modifications reflecting the tissue characteristics. *Via* successive centrifugation, this kit allowed us to obtain the following fractions: i) cellular debris (pellet of 1,000 *g*), ii) heavy mitochondrial fraction (pellet of 2,000 *g*) used for the swelling protocol, iii) cytoplasmic fraction (supernatant of 25,000 *g*), and iv) crude peroxisomal fraction (pellet of 25,000 *g*). The crude peroxisomal fraction was subsequently ultracentrifuged at 100,000 *g* (4 °C, 90 min) (Optima Max-XP, Beckman Coulter) to obtain purified mitochondrial and peroxisomal heart fractions. The latter subcellular fractions served in Western Blot analyses.

6.10. Enzyme activity and NAD(H) pool

6.10.1. Enzyme activity

The specific enzyme activities of HK, CK, PFK, malate dehydrogenase (MD), LDH, Complex I + Complex III (NADH DH- Cyt C OXRED), Complex II + Complex III (Succ DH – Cyt c OXRED), citrate synthase (CS) were measured spectrophotometrically using a 96-well Multireader system (HK, CK, PFK, LDH) or a spectrophotometer *Shimadzu-UV1601* (NADH/Succ DH – Cyt C OXRED, MDH, CS). Enzyme activities of CK (liquid acc. To IFCC, Roche Diagnostics), PFK (Sigma-Aldrich), and LDH (Sigma-Aldrich) was determined using the commercial kits.

The specific enzyme activity of HK was assessed by enzyme-coupled assay of slightly modified Worthington protocol. The assay buffer (AB) consisted of (in mM): 50 Tris, 13.3 MgCl₂, 0.8 NAD⁺,

0.8 ATP and 1U/mL glucose-6-phosphate dehydrogenase (Roche/Sigma-Aldrich); pH = 8.0. The starting solution (SS) contained (in mM): 1500 glucose, 50 Tris, 13.3 MgCl₂. Samples with the same concentration were loaded onto 96-well plate with positive control (purified HK by Roche/Sigma-Aldrich). 167 µL of AB was added, the samples were incubated at 30 °C for 2 minutes and then the reaction was initiated by the addition of 33 µL SS into the well. The reaction was set to 30 min at 30 °C while Multi-reader Synergy HT (BioTek) was measuring the absorbance increasement at 339 nm. CS specific activity was measured at 412 nm using 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB). DTNB reacts with the free sulfhydryl group of coenzyme A and produces mercaptide ions. The reaction was measured in Shimadzu-UV spectrophotometer and the AB and the SS can be seen in Table C. The specific activity was calculated as U/g protein using absorption coefficient of $13.6 \cdot 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ as described in [324].

The activity of CI+CIII and CII+CIII was measured spectrophotometrically in Shimadzu-UV. The ABs and SSs for both reactions are summarized in Table C. The specific activity was calculated as U/g protein using absorption coefficient of $19.1 \cdot 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$. Modified from [325]. MD specific enzyme activity was assessed similarly. The AB and SS can also be found in Table C. The specific activity was calculated as U/g protein using absorption coefficient of $6.22 \cdot 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$. Modified from [326]

Table C: Enzyme activity assays. The table continues on the next page.

Citrate synthase			
	Conditions:	Assay Buffer	Volume [µL]
λ	412 nm	Tris - HCl (100 mM, pH = 8.1)	920
ε	$13,6 \cdot 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$	Sample	10
t	80 s	Acetyl-CoA (12,2 mM)	10
		DTNB (10,1 mM)	10
		Starting solution:	Volume [µL]
		Oxaloacetate (10 mM)	50
NADH dehydrogenase - Cytochrome c Oxidoreductase (CI+CIII)			
	Conditions:	Assay Buffer	Volume [µL]
λ	550 nm	Phosphate buffer (100 mM, pH = 7.4)	940-945
ε	$19,1 \cdot 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$	cytochrome c (8,1 mM)	10
t	80 s	KCN (5 mM)	10
		Sample	15-20
		Starting solution:	Volume [µL]
		NADH (15,1 mM)	20

Succinate dehydrogenase - Cytochrome c Oxidoreductase (CII+CIII)			
Conditions:		Assay Buffer	Volume [μ L]
λ	550 nm	Phosphate buffer (100 mM, pH = 7.4)	950
ϵ	$19,1 * 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$	cytochrome c (8,1 mM)	10
t	80 s	KCN (5 mM)	10
		Sample	10
		Starting solution:	Volume [μ L]
		Succinate (500 mM)	20
Malate dehydrogenase:			
Conditions:		Assay Buffer	Volume [μ L]
λ	340 nm	Phosphate buffer (100 mM, pH = 7.4)	965
ϵ	$6,22 * 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$	Sample	5
t	120s	NADH (15,1 mM)	20
		Starting solution:	Volume [μ L]
		Oxaloacetate (151 mM)	10

6.10.2. NAD(H) pools

To determine the whole NAD(H) pool in LV (i.e. specific concentration of NAD⁺, NADH and the total NAD(H)) we purchased a commercial kit provided by Sigma-Aldrich. The results can be seen in the appropriate result section. However, it is notable to add that after the frozen LV tissue samples (~25 mg) were pulverized in the liquid nitrogen and resuspended in commercial homogenization medium (kit-included), the samples were centrifuged, and the supernatant collected. The supernatant was immediately placed into a 10kDa-spin off filter (to avoid NADH utilizing reactions; Sigma-Aldrich). The resultant filtrate was processed as indicated in manufacture's protocol.

6.11. Mitochondrial susceptibility to Ca²⁺ overload

Heavy mitochondrial fraction was obtained from fresh rat LV myocardium (see 7.8., section b). This fraction was resuspended in sucrose-Tris buffer (see 7.8., section a), gently homogenized, and centrifuged at 2,000 *g* (4 °C, 10min). The resultant pellet was resuspended again in sucrose-Tris buffer and underwent a swelling protocol using calcium salts described in [327] to analyse the mitochondrial susceptibility to mPTP opening. Briefly, approximately 100 μ g of mitochondrial fraction was applied into cuvette containing Swelling-buffer (in mM): 125 Sucrose, 65 KCl, 10 HEPES, 5 Succinate, 1 PO₄³⁻; pH = 7.2. The swelling (mPTP opening) was induced by 400 μ M

Ca²⁺ (infusion of 20 mM CaCl₂ into cuvette). The maximal swelling rates are expressed as [$\Delta A_{520} \cdot 10 \text{ s}^{-1} \cdot \text{mg}^{-1} \text{ protein}$].

6.12. High resolution respirometry

Respiration of LV SSM and IFM was determined using high-resolution respirometry (Oxygraph 2-k, Oroboros®) as described previously [291] using MiR05 (in mM: 0.5 EGTA, 3 MgCl₂, 60 K⁺-lactobionate, 20 taurine, 10 KH₂PO₄, 20 HEPES, 110 D-sucrose; BSA 1 g/l) as an incubation medium [328]. The medium with samples of LV SSM and/or IFM (~ 100 µg of protein) were added to the oxygraphy chambers and underwent the respiration protocols. Complex I and II state 3-stimulated mitochondrial respiration was achieved by adding [in mM: 2 malate, 10 glutamate, 5 ADP, 0.01 cytochrome C, 10 succinate and 2 KCN] to the incubation medium. Malate, ADP (in the same concentrations) with 0.0125 palmitoyl-carnitine (PC) were added to analyse mitochondrial β -oxidation rates. Specific O₂ consumption rate was calculated as [pmol/(s * mg protein)] using DatLab6 (Oroboros®).

6.13. Immunofluorescence analyses¹⁷

6.13.1. Preparation of cryo-sections

Hearts were rapidly excised and perfused under constant pressure with relaxing calcium free Tyrode's solution (in mM): 140 NaCl, 5.4 KCl, 1 Na₂HPO₄, 1 MgCl₂·6H₂O, 10 glucose, 5 HEPES, pH 7.4) for 2 min and then by 4% methanol-free formaldehyde (Thermo Fisher Scientific) for 3 min using Langendorff apparatus. Thereafter, hearts were immersed in 4% formaldehyde for the next 2 h and subsequently incubated in 20% sucrose overnight. LV were cut transversally in one-third from apex, separated, snap-frozen in liquid nitrogen, and then stored in -80 °C. Cryosections (5-7 µm) were prepared from frozen ventricles using a cryostat (Leica CM3050, Leica-microsystems).

6.13.2. Immunofluorescence staining

Cryo-sections were permeabilized in ice-cold methanol (p.a). Sections were rinsed in 1% SDS as an antigen retrieval step. Nonspecific binding sites were blocked for 1 h at room temperature in

¹⁷ In co-operation with RNDr. Barbara Elsnicová, Ph.D. and Mgr. Mário Helés

10% appropriate serum diluted in PBS containing 0.3% Triton X-100, 1% BSA and 0.3 M glycine (Sigma-Aldrich).

To assess HK1 and HK2 colocalization subcellular compartments, the cryo-sections were incubated with rabbit primary antibodies against HK1 and HK2 (Abcam) and the mitochondrial compartment was stained with mouse MitoProfile Total OXPHOS Blue Native Antibody Cocktail (Abcam). Primary antibodies were visualized by donkey anti-rabbit IgG Alexa Fluor 488 (Invitrogen) and goat anti-mouse IgG Alexa Fluor 647 secondary antibody, respectively.

To evaluate the degree of localization of CK within M-band, the cryo-sections were incubated either with rabbit polyclonal antibody against CKB (GenScript), or with rabbit polyclonal antibody against CKM (Abcam). Both primary antibodies were detected with donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (Thermo Fisher Scientific). The sarcomere compartments were visualized by Alexa Fluor 647 conjugate of phalloidin (Thermo Fisher Scientific). As phalloidin binds to the actin filaments, M-band was defined as regions with no phalloidin signal as reported previously [174].

To assess the co-localization of LDHB with peroxisomal compartment, the cryo-sections were incubated with primary rabbit polyclonal antibody against LDHB (Novus Biologicals) and anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific). Sections were counterstained with primary mouse monoclonal antibody against PMP70 (Sigma-Aldrich) with donkey anti-mouse IgG secondary antibody conjugated with Alexa Fluor 647 (Thermo Fisher Scientific). Sections were then mounted in ProLong Gold Antifade Reagent containing a nuclei marker 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) and stored at 4 °C.

6.13.3. Quantitative fluorescence microscopy

The images were acquired using a wide-field fluorescence microscope (Olympus Cell IX2-UCB, Olympus) equipped with Plan-Apochromat 100×/1.4 NA objective. Images were acquired using Hamamatsu ORCA camera C4742-80-12AG (Hamamatsu), and co-localization was calculated using Fiji ImageJ open source software [329]. Five positions on each sample were sequentially acquired for red and green channels. Each position was optically sectioned at 0.5-μm steps resulting in ~16 focal planes depending on specimen thickness. The background subtraction (sliding paraboloid, roll ball = 5 with disabled smoothing) and default auto threshold setting were applied before the Pearson's or Mander's correlation coefficient calculation.

To evaluate CK in sarcomeres exclusively, a non-phalloidin mask with a fluorescent signal corresponding to the M-bands of cardiomyocytes was applied. Focal planes of the red channel (phalloidin counterstaining actin filaments) were merged into one image, using FIJI software, Z Projection function with selected Average Intensity projection setting. Inversion of actin staining

created black background instead of a former actin signal and signal in the M-band region. Sarcomeres of central cardiomyocyte were selected and saved as a region of interest. Gaussian blur (sigma radius 12) was applied on duplicate of the green channel (CK), which was afterwards subtracted from the original image using FIJI's Image Calculator to reduce background noise. Mander's M1 coefficient used for co-localization analysis in selected regions of interest was calculated using ICA plugin.

6.14. Statistical analysis

At least five hearts from each experimental group were used for WB, enzyme activity, and Biomark Chip analyses, and five hearts were used for quantitative fluorescence analyses whether not implicated differently. Five hearts from N and IHH rats served for SSM and IFM isolation. Six hearts from each group were processed in peroxisomal isolation method. The WB, immunoreactivity and relative mRNA abundance were expressed as fold over control group or as in- or decrease of overall immunoreactivity. The statistical differences between two groups were determined by the unpaired Mann Whitney test ($P < 0.05$) using the GraphPad Prism 5.00 software. The statistical differences between more groups (e.g. mitochondrial sub-populations) were determined by ANOVA test with Newman-Keul's post-test ($P < 0.05$). All data are shown as means $\pm$ SEM.

7. RESULTS

STUDY A

7.1. Expression and phosphorylation of Akt

Both N and CNH groups did not show any significant changes in the expression of total Akt and the amount of Akt phosphorylated on threonine residue (pAkt Thr³⁰⁸) during the I/R insult (Fig. R1A, C). Interestingly, a significant decrease by 36 % was seen in Akt phosphorylated on serine residue (pAkt Ser⁴⁷³) after reperfusion compared to ischaemic N group. In the CNH group, there was a significant increase by 77 % in the level of pAkt Ser⁴⁷³ after ischaemia. Ischaemic level of pAkt Ser⁴⁷³ was higher by 22 % in CNH than in N group (Fig. R1B).

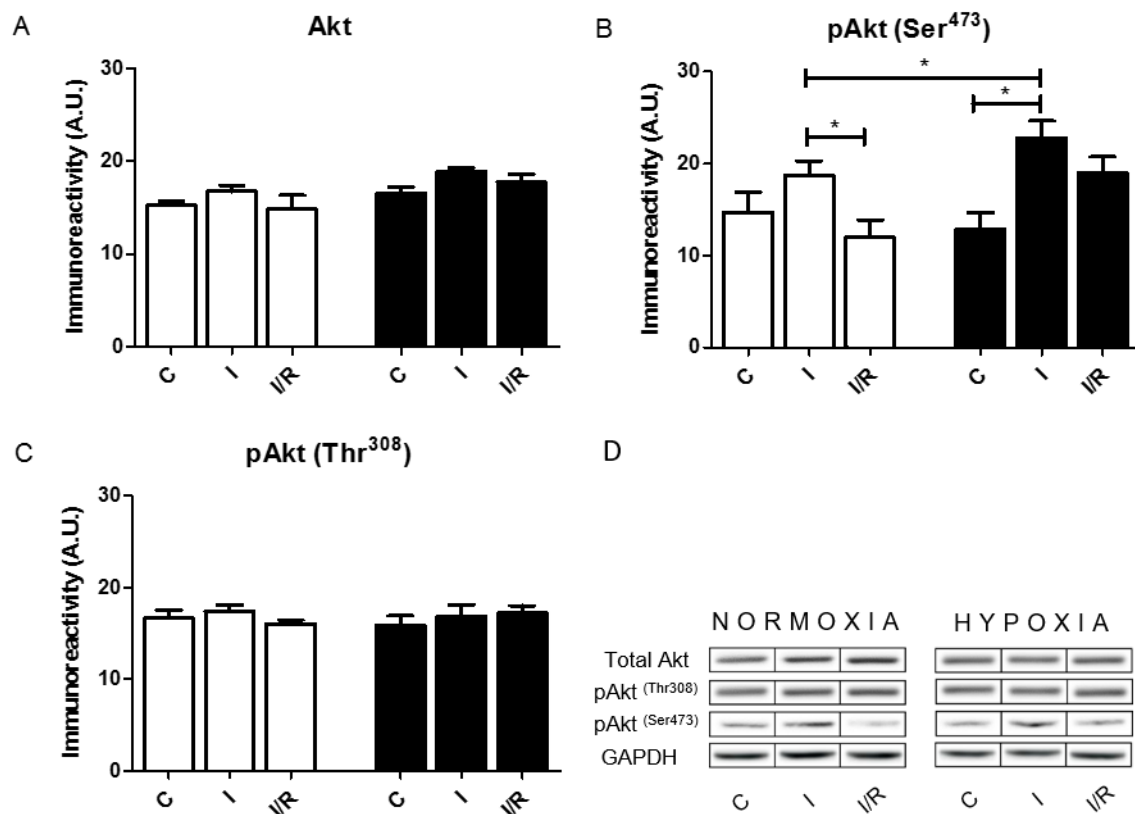


Figure R1: Effect of ischaemia and reperfusion on total and phosphorylated Akt in the rat heart. Relative protein levels of total Akt (a), phosphorylated Akt (Ser473) (b), and phosphorylated Akt (Thr308) (c) in the left ventricles of the perfused control (C), ischaemic (I), and reperfusion (I/R) hearts from normoxic (*white columns*) and CNH rats (*black columns*) were determined by Western blotting (d). Values are mean $\pm$ SEM, ($n = 6$), $*p < 0.05$

7.2. Expression and enzyme activity of HK

There was no effect of CNH on HK protein levels in control perfused hearts. Adaptation to CNH increased the level of both HK1 and HK2 after ischaemic period by 31 and 40 %, respectively, when compared to the corresponding controls (Fig. R2A, B). However, there was no effect of CNH on HK levels in control perfused hearts. The HK activity was elevated by CNH in the perfused controls by 23 %. On the other hand, ischaemia affected HK activity similarly in both groups (Fig. R2C).

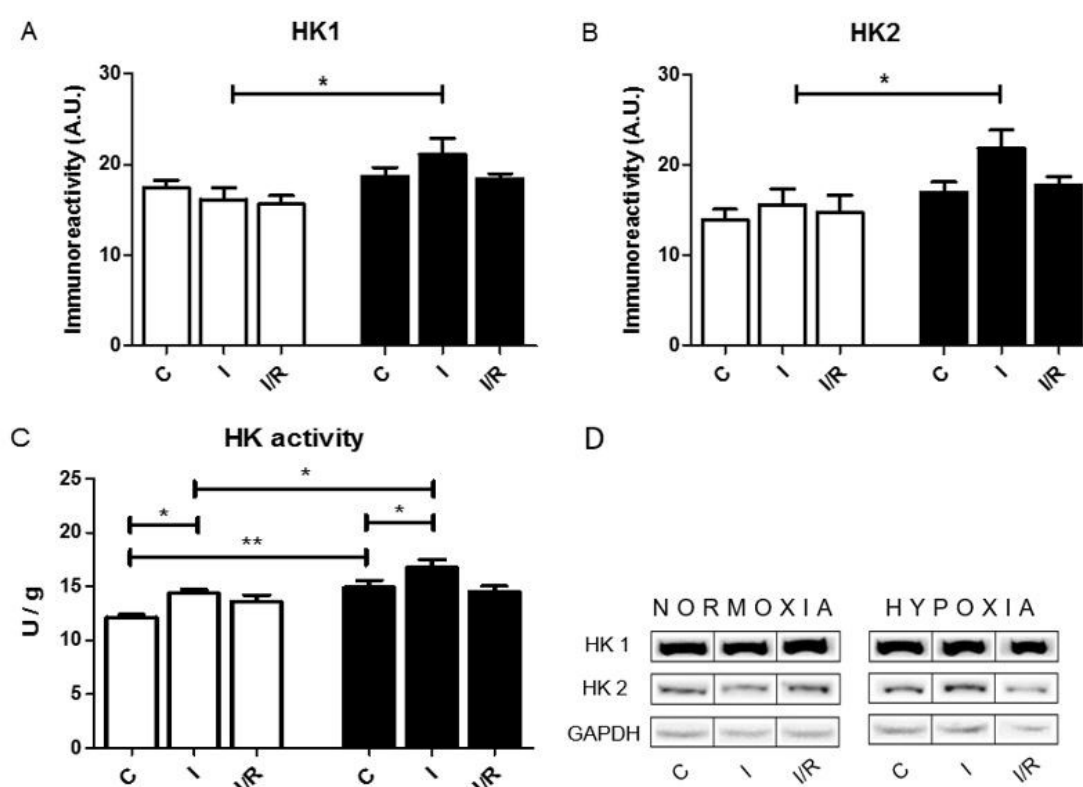
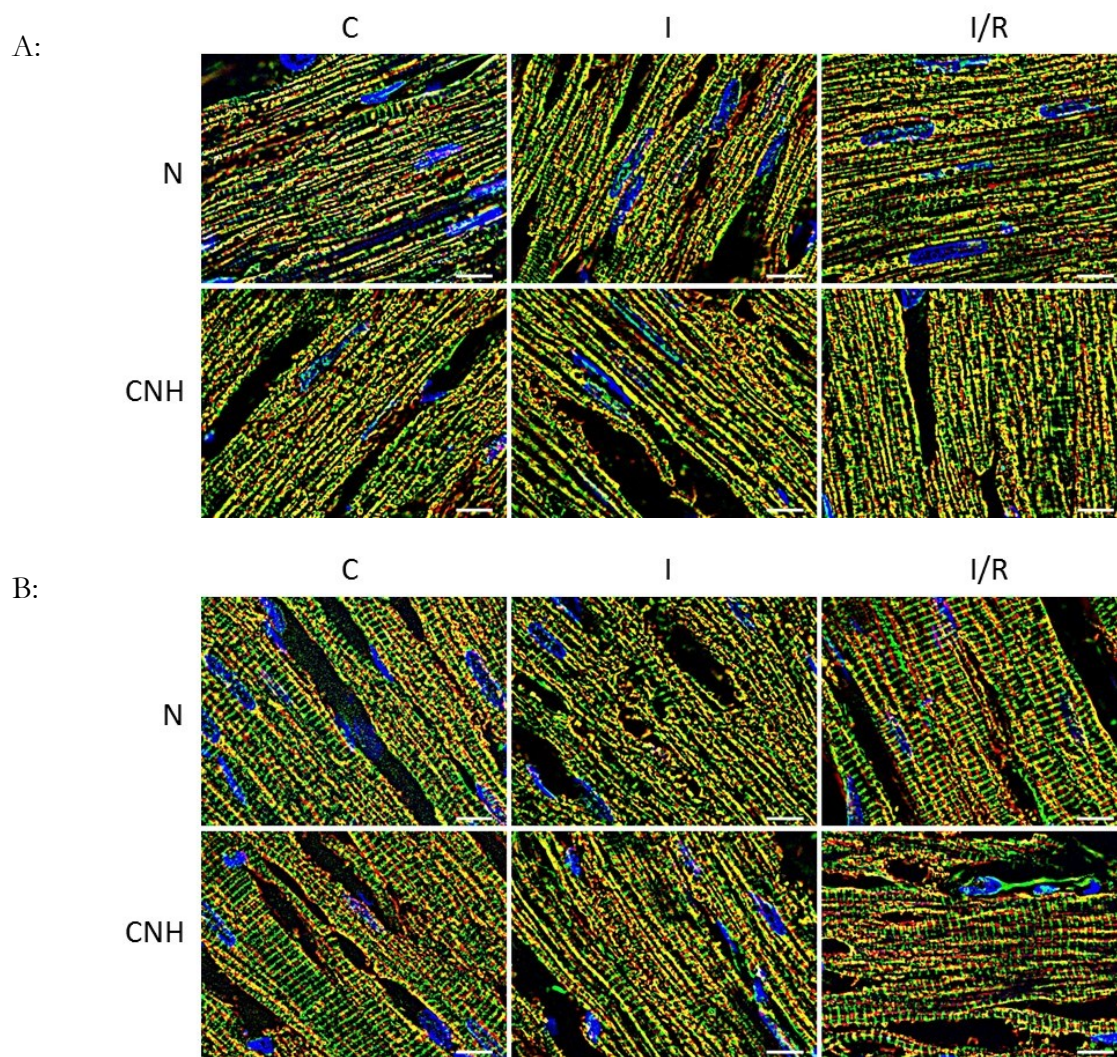


Figure R2: Effect of ischaemia and reperfusion on the expression and activity of HK isoforms in the rat heart. Relative protein levels of HK1 (A), HK2 (B), and HK specific enzymatic activity (C) were determined in control (C), ischaemic (I), and reperfusion (I/R) hearts from normoxic (white columns) and CNH rats (black columns). Representative image of western blotting included (D). Values are mean $\pm$ S.E.M. (n = 6), * p < 0.05, ** p < 0.01.

7.3.Co-localization of HK with mitochondria

The co-localization of HK1 with mitochondria evaluated by Pearson's correlation coefficient did not vary between the groups (Fig. R3C). In the N group, the co-localization of HK2 with mitochondria was significantly lowered after reperfusion compared to ischaemia, while only a tendency was observed in the CNH group (Fig. R3D). Interestingly, the same pattern of HK2 co-localization (decrease after reperfusion by 33, 32, and 37 %, respectively) was observed with all mitochondrial sub-populations (Fig. R3E, F, G), i.e. intermyofibrillar, perinuclear, and subsarcolemmal mitochondria.



Please note that Figure R3 continues on the next page

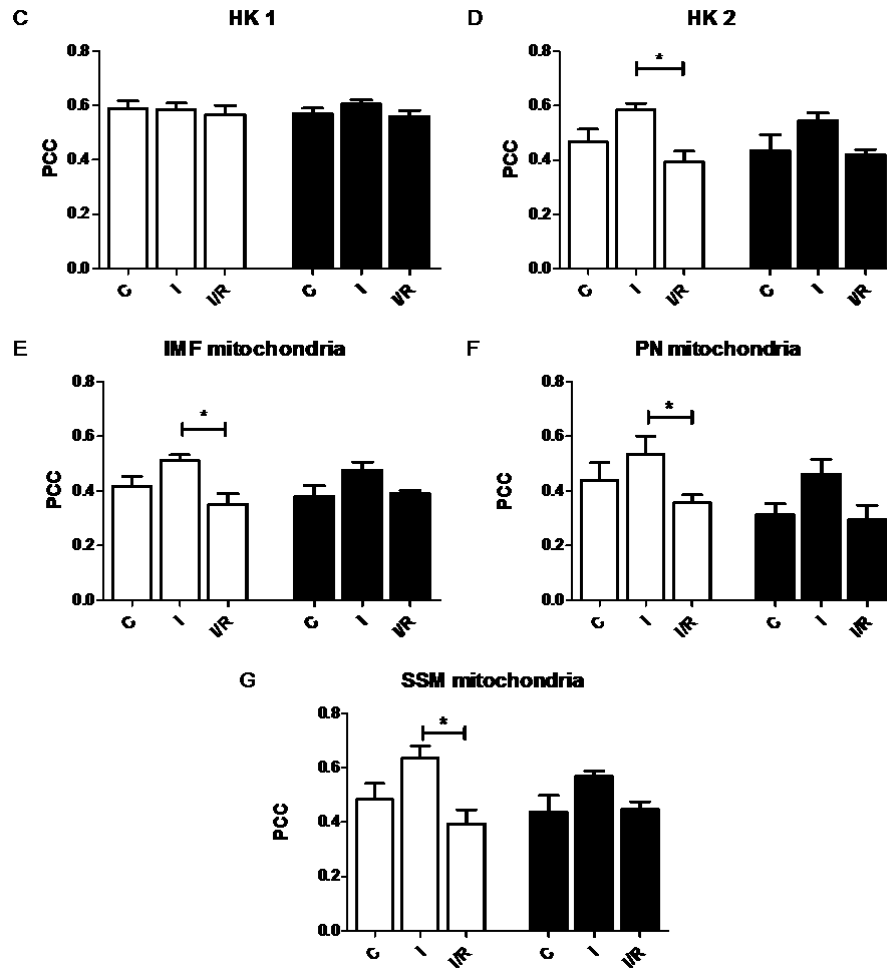


Figure R3: Effect of ischaemia and reperfusion on the co-localization of HK isoforms with mitochondria in the rat heart. Representative micrographs show the distribution of HK1 (A) and HK2 (B) in longitudinal cryo-sections of the left ventricles of control (C), ischaemic (I), and reperused (I/R) hearts from normoxic (upper panels) and CNH rats (lower panels). The green colour corresponds to specific HK staining, red colour represents mitochondrial compartment and the blue colour indicates nuclei DAPI staining. Scale bar represents 13 μ m. Co-localization of HK1 (C) and HK2 (D) with mitochondria was quantified by Pearson's correlation coefficient (PCC). Co-localization of HK2 with intermyofibrillar (IMF) (E), peri-nuclear (PN) (F), and subsarcolemmal (SSM) (G) mitochondrial sub-populations in the LV of normoxic (white columns) and CNH (black columns) rats. Values are mean $\pm$ SEM, (n=5), * $p < 0.05$.

7.4. Expression of mtCKS

In N group, mtCK expression slightly decreased by 12% after ischaemia, while no effect of I/R insult was observed in rats adapted to CNH (Fig.R4).

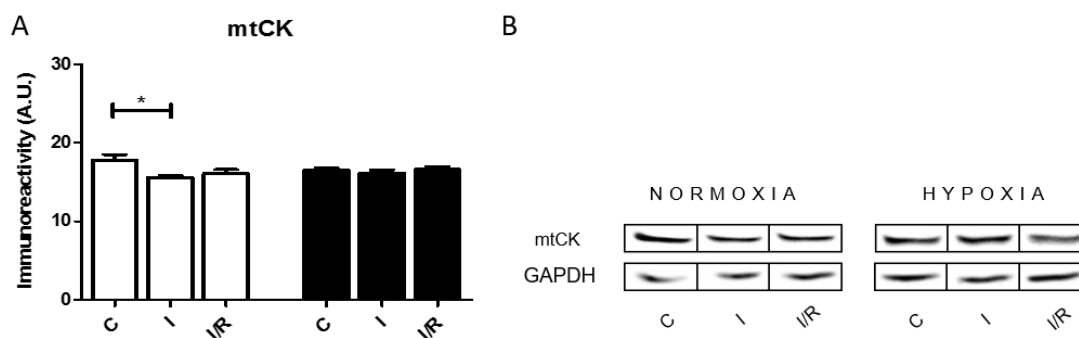


Figure R4: Expression of mtCKS isoform in the left ventricle after ischaemia and reperfusion in normoxic (white columns) and CNH rats (black columns) (A). The amount of mtCKS perfused control (C), ischaemic (I), and reperfusion (I/R) hearts determined by western blotting (B). Values are mean $\pm$ S.E.M., (n = 6), * p < 0.05.

7.5. Phosphorylation of AMPK α

Ischaemia markedly increased the level of phosphorylation of pAMPK α Thr¹⁷² in N and CNH groups by 319 and 236%, respectively, compared to corresponding controls (Fig. R5). The subsequent reperfusion that provided energy substrates returned the values back to control levels.

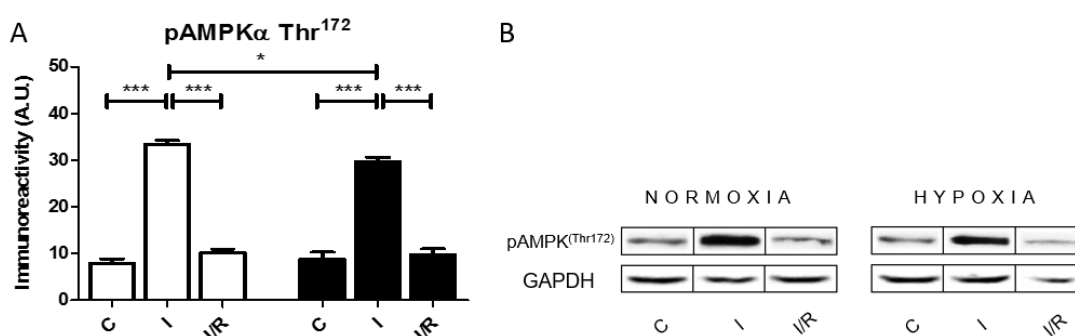


Figure R5: Determination of total phosphorylated AMPK α at Thr¹⁷² residue in the left ventricle after ischaemia and reperfusion of normoxic (white columns) and CNH rats (black columns) (A). The amount of p-AMPK α in perfused control (C), ischaemic (I), and reperfusion (I/R) hearts was determined by western blotting (B). Values are mean $\pm$ S.E.M. (n = 6), * p < 0.05, *** p < 0.001.

7.6. Expression of Bcl-2 family proteins:

Expression of the Bcl-2 during the I/R insult in any group (Fig. R6A). The level of Bax decreased by 37 % after reperfusion only in CNH group (Fig. R6B). The Bax/Bcl-2 ratio significantly decreased (by 54%) after reperfusion in CNH, while no effect of I/R occurred in the N group (Fig. R6c).

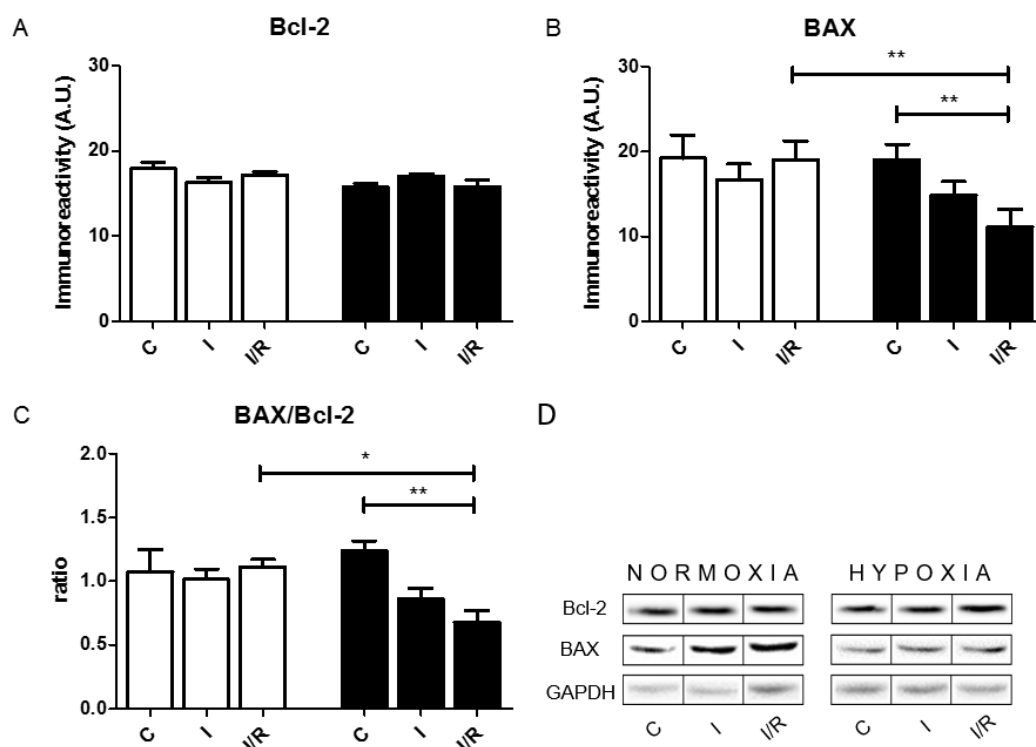


Figure R6: Effect of ischaemia and reperfusion on Bcl-2 and Bax in rat LV. Relative protein levels of Bcl-2 (A) and Bax (B) in the left ventricles of perfused control (C), ischaemic (I), and reperfusion (I/R) hearts from normoxic (white columns) and CNH rats (black columns) were determined by Western blotting (D). The Bax/Bcl-2 ratio was also estimated (C). Values are mean $\pm$ S.E.M. (n = 6), * p < 0.05, ** p < 0.01.

STUDY B

7.7. Protein kinase B/Akt expression is SHR and SHR-mtBN

Surprisingly, while CNH had no effect on Akt1 expression in LV of both strains neither on Akt2 protein level in SHR, it resulted in a significant increase (by 43%) in SHR-mt BN (Fig. R7A, B, C, D).

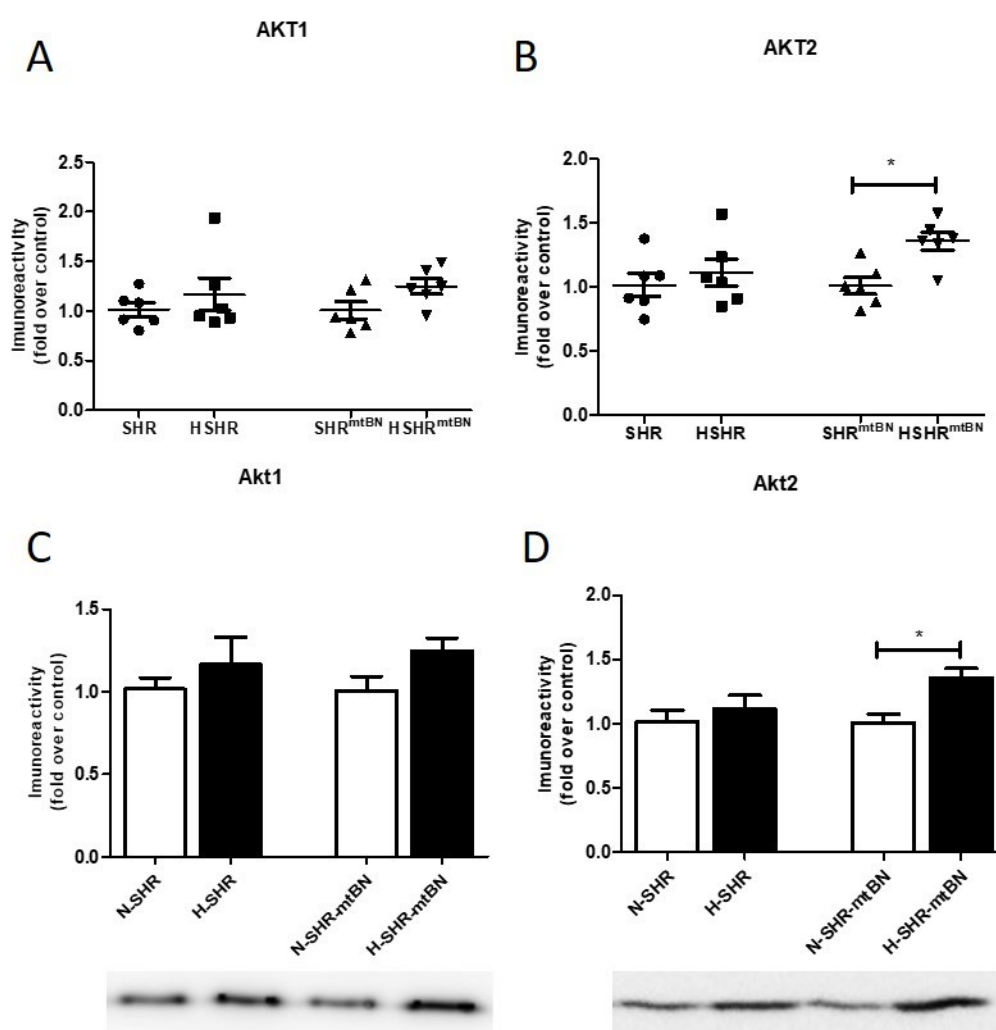


Figure R7: Effect of CNH (black columns) on the expression of cardiac Akt major isoforms in SHR (dots) and SHR-mtBN (triangles). The upper part is (A, B) is showing individual data of Akt 1 (A) and Akt 2 (B) expression. The lower part (C, D) is showing the relative protein level of Akt1 (C) and Akt 2 (D). The representative blot pictures are shown underneath each graph. Values are mean $\pm$ S.E.M., * $p < 0.05$

7.8. Effect of CNH on HK activity in SHR and SHR-mtBN

Due the fact that HK was shown as a component regulating mPTP opening, we first determined the specific enzyme activity of HK in LV tissue homogenates. The specific HK activity was significantly higher in LV homogenates of normoxic SHR-mtBN compared to with normoxic SHR (by ~40 %). Adaptation to CNH increased HK activity by 19 % and 17 %, respectively in SHR and SHR-mtBN (Fig. R8A). Due the differences between HK activities in tissue homogenates, we analysed the mitochondrial HK activity. Any differences between the strains could be determined in control groups, although the adaptation to CNH upregulated HK activity in SHR (by 50 %) and in SHR-mtBN (by 43 %). Both adapted CNH groups did not differ between each other (Fig. R8B).

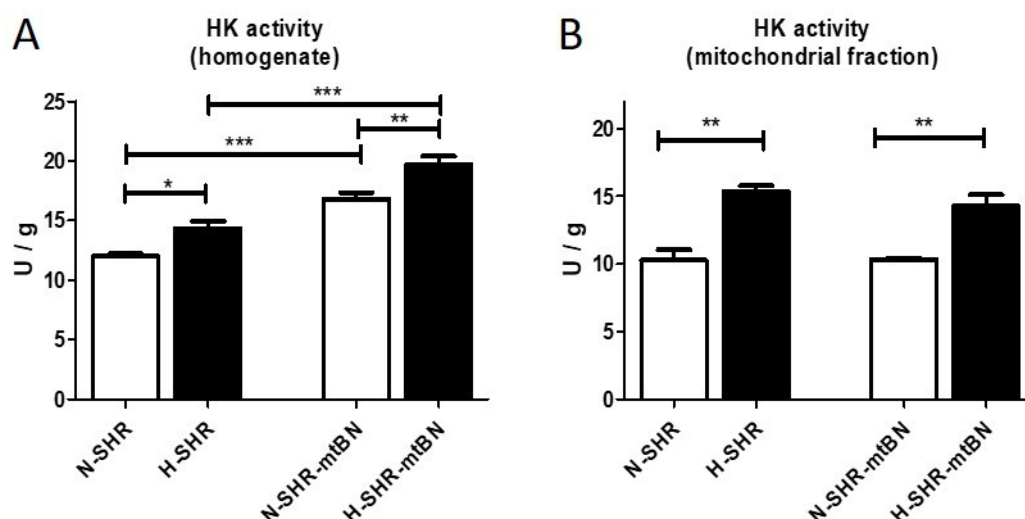


Figure R8: Specific HK activity. The effect of CNH (black columns) on the specific HK activity in LV tissue homogenates (A) and mitochondrial fraction (B) of SHR and SHR-mtBN animals. Values are mean $\pm$ S.E.M., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

STUDY C

7.9. The effect of IHH on mRNA expression of selected genes:

The Biomark-Chip analysis revealed an increased mRNA expression of genes related to glucose metabolism e.g. both major glucose transporters (GLUT1 and GLUT4), hexokinases (HK1, HK2), and phosphofructokinase 2 (PFKFB2), LDHA isoform as well as monocarboxylate transporter 4 (MCT4). Contrary to that, relative LDHB mRNA expression dropped together with mitochondrial mtCKs and muscle-type of sarcoplasmic creatine kinase (CKM). Nevertheless, the brain-type isoform, CKB, was upregulated. Pyruvate dehydrogenase (E1 α -subunit) and pyruvate dehydrogenase kinase 3 mRNA levels were both elevated by IHH. IHH-afforded changes in relative mRNA expression of the analysed genes are summarized in Table D.

Table D: The effect of IHH on mRNA expression of metabolic genes

Gene name:	Difference (N/IHH); p value	Gene name:	Difference (N/IHH); p value
CAT	+50 %; 0.1811 (n.s.)	HK2	+46 %; 0.0249 (*)
CKB	+37 %; 0.0265 (*)	HPRT	no change
CKM	-34 %; 0.0092 (**)	LDHA	+81 %; 0.0007 (***)
mtCKS	-26 %; 0.0001 (***)	LDHB	-28 %; 0.0018 (**)
CS	+22 %; 0.3329 (n.s.)	MCT1	no change
GLUT1	+64 %; 0.0008 (***)	MCT2	no change
GLUT4	+47 %; 0.0107 (*)	MCT4	+124 %; 0.027 (*)
HIF1 α	+108 %; 0.0003 (***)	PDHA1	+48 %; 0.0379 (*)
HIF2 α	+16 %; 0.1842 (n.s.)	PDK3	+64 %; 0.0491 (*)
HK1	+35 %; 0.0264 (*)	PFKFB2	+54 %; 0.0290 (*)

Abbreviations: Normoxia (N), Intermittent hypobaric hypoxia (IHH), Hypoxanthine-guanine phosphoribosyl transferase (HPRT, housekeeping gene), Catalase (CAT), Creatine kinase brain-type (CKB), muscle-type (CKM), mitochondrial sarcomeric-type (mtCKs), Citrate synthase (CS), Glucose transporter 1/4 (GLUT1/4), Hypoxia-inducible factors (HIF1 α , HIF2 α), Hexokinase 1/2(HK 1/2), Lactate dehydrogenase muscle-type (LDHA), heart-type (LDHB), Monocarboxylate transporters (MCT1/2/4), E1 α -subunit of Pyruvate dehydrogenase (PDHA1), Pyruvate dehydrogenase kinase 3 (PDK3), Phosphofructokinase/fructose-bisphosphatase 2 (PFKFB2). + IHH-induced increase, - IHH-induced decrease, (N=7) * p < 0.05, ** p < 0.01, *** p < 0.001

7.10. The effect of IHH on β -oxidation in mitochondrial and peroxisomal fractions of LV

The results presented demonstrate a decrease in mitochondrial β -oxidation rates followed by an increase in peroxisomal β -oxidation capacity in LV adapted to IHH. Palmitoyl-carnitine (PC)-stimulated O_2 consumption rates were affected by IHH in both mitochondrial fractions. In the fraction of subsarcolemmal mitochondria (SSM), PC-stimulated respiration rates fell by 16% and by 51% in interfibrillar mitochondria (IFM). In the case of other respiratory substrates (malate and/or malate+ADP), IHH did not affect SSM, whereas IFM consumption rates were reduced by 54% just when malate was used as sole substrate (Fig. R9A).

In terms of peroxisomes, IHH adaptation resulted in the upregulation of the rate-limiting enzyme of peroxisomal β -oxidation, ACOX1, by 12%. Accordingly, peroxisomal NAD(H)-redox shuttle, essential for this pathway and represented by peroxisomal lactate dehydrogenase B (LDHBx), was upregulated after IHH by $\sim 12\%$. The expression of neither 70 kDa peroxisomal membrane protein (PMP70) nor peroxisomal catalase was affected (Fig. R9B). Higher co-localization of LDHB with peroxisomal PMP70 in IHH (by $\sim 10\%$) was confirmed by quantitative immunofluorescent microscopy using Pearson's correlation coefficient (Fig. R9C). On the other hand, we were not able to detect any significant change in LDHA abundance in peroxisomes between the groups (data not shown).

7.11. The effect of IHH on respiratory rates and ETS

Figure R10 demonstrates the suppressing effect of IHH on the capacity of aerobic metabolism in mitochondria. The individual ETS complexes protein expression in tissue homogenate was affected differently by the adaptation. Whereas IHH downregulated the protein expression of complex I (C_I) and complex IV (by 21 % and 55 %, respectively), it had no effect on the expression of complex II, III and V (Fig. R10A).

As for the comparison between both mitochondrial fractions (SSM and IFM), specific O_2 consumption rates of SSM dropped in IHH compared to N by 34 % when malate+glutamate+ADP+succinate were used as substrates. The difference was also apparent (28%) when IFM isolated from both groups were analysed. Moreover, IFM from IHH LV samples showed significantly lower (by 33 %) consumption rates when only malate+glutamate were used (Fig. R10B).

Figure R10C shows higher activity of citrate synthase (CS) and complex I (NADH:ubiquinone oxidoreductase; C_I) in IFM isolated from control (N) compared to those

observed in SSM (by 90 % and 52 %, respectively). No significant difference was found in complex II (Succinate dehydrogenase; CII) activity. IHH decreased the activity of CI in IFM by 56 %. IHH also reduced the activity of CS in IFM by 38 % and in SSM by 40 %.

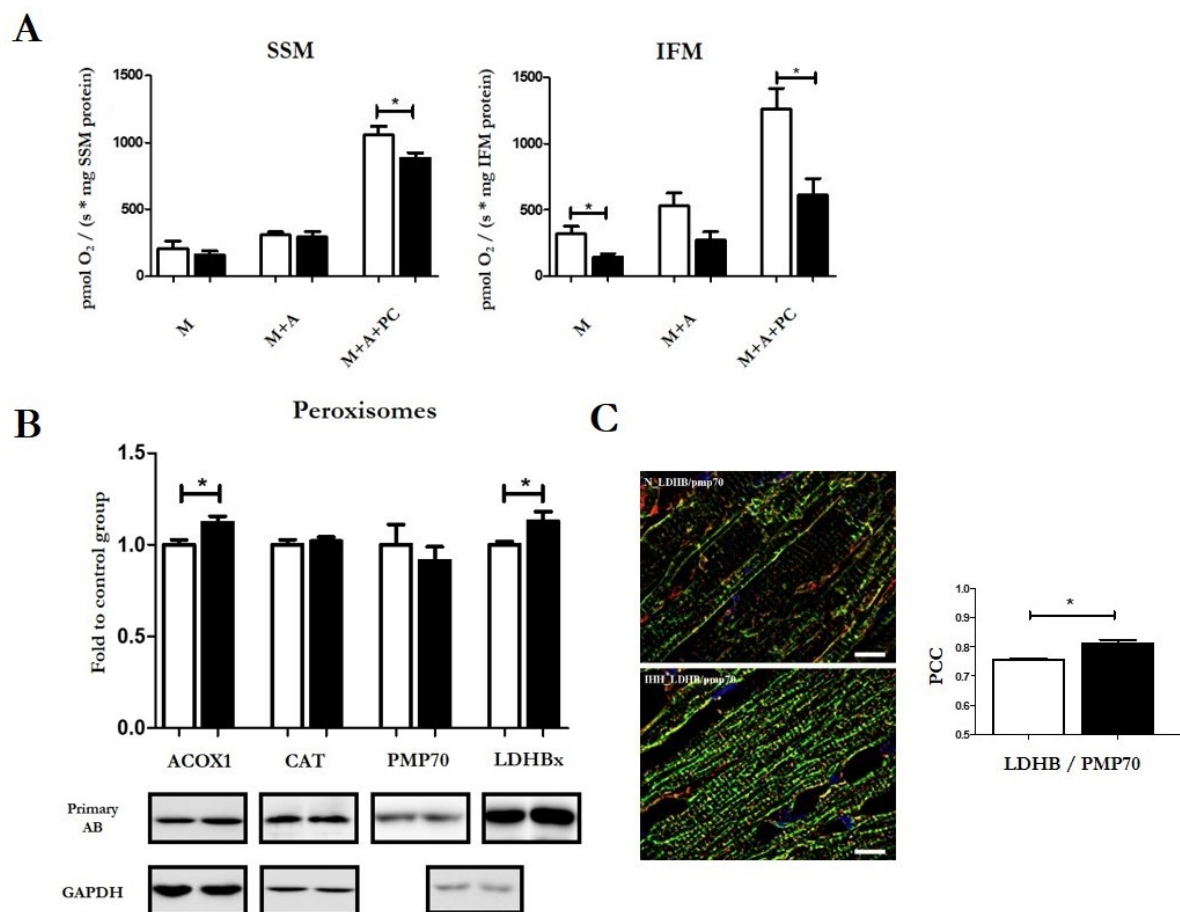


Figure R9: The effect of intermittent hypobaric hypoxia (IHH) on β -oxidation pathways in the heart. IHH represented by black columns. **A:** Specific β -oxidation rates in subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria. Malate (M), Adenosine 5'- diphosphate (A), palmitoyl-carnitine (PC). **B:** The effect of IHH on the expression of peroxisomal proteins; acyl-CoA oxidase 1 (ACOX1), catalase (CAT), lactate dehydrogenase B (LDHBx) and PMP70. **C:** Co-localization of LDHB isoform with PMP70. Representative micrographs show the distribution of LDHB in longitudinal cryo-sections of the left ventricles from normoxic (N) and IHH rats. LDH staining (green), PMP70 (red), nuclei DAPI (blue). Scale bar equals to 10 μ m. The co-localization was evaluated by Pearson's correlation coefficient. Values are mean $\pm$ SEM, (N=5), * $p < 0.05$.

7.12. The effect of IHH on glycolytic compartment

Fig R11 presents the empowered glycolytic apparatus by adaptation to IHH. Enzyme activities of phosphofructokinase (PFK) and LDH in IHH samples rose by 18% and 26%, respectively, whereas the overall activity of malate dehydrogenase (MDH) dropped by 17%. Although we were able to detect a significant increase in LDHA protein expression in cardiac tissue homogenates (by 107%), no changes in LDHB protein expression were observed after IHH. The IHH-induced alterations of glycolytic compartment are also apparent from relative mRNA expression (Table D).

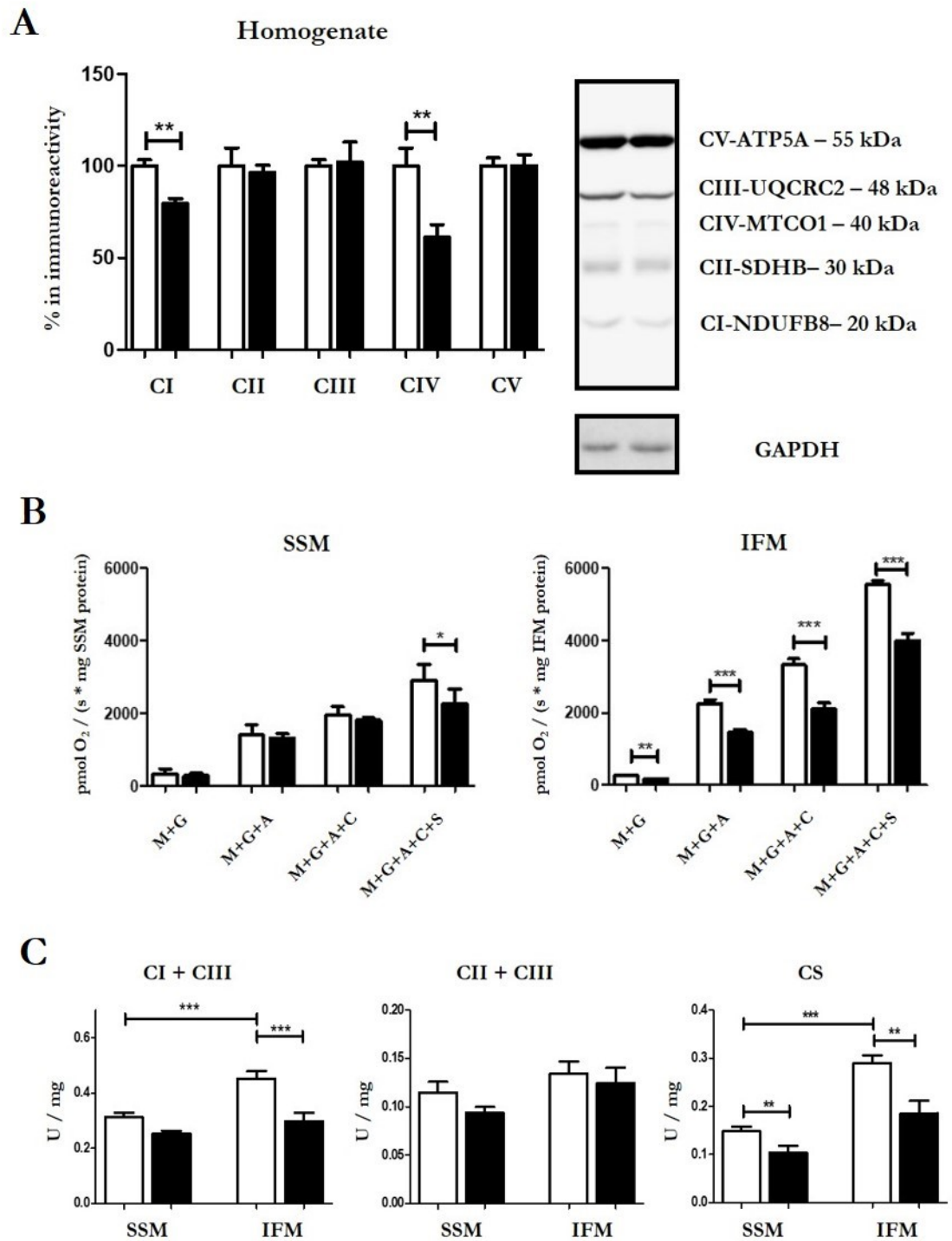
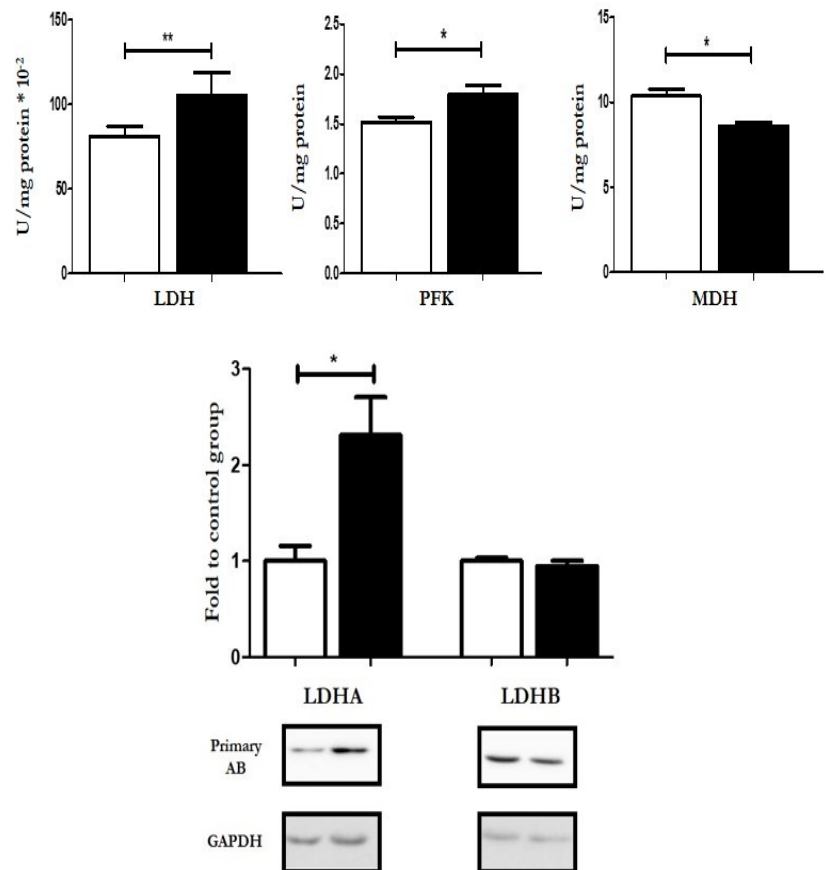


Figure R10: The effect of intermittent hypobaric hypoxia (IHH) on aerobic metabolism parameters in mitochondria. Normoxic group (N, white columns), IHH (black columns). **A:** protein expression of electron transport system (ETS) complexes. Complex I/NADH dehydrogenase (C_I), Complex II/succinate dehydrogenase (C_{II}), complex III/coenzyme Q:cytochrome c-oxidoreductase (C_{III}), Complex IV/cytochrome c oxidase (C_{IV}), Complex V/ ATP synthase (C_V) **B:** specific respiration rates of subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria. Malate (M), Glutamate (G), Adenosine 5'-diphosphate (A), Cytochrome c (C), Succinate (S). **C:** specific enzyme activities of NADH dehydrogenase – cytochrome c oxidoreductase (Complex I+III), succinate dehydrogenase – cytochrome c oxidoreductase (Complex II+III), and citrate synthase (CS) in mitochondrial subpopulations. Values are mean $\pm$ SEM, (N=5), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure R11: The effect of intermittent hypobaric hypoxia (IHH) on glycolytic apparatus. Normoxic group (N, white columns) and IHH (black columns). Enzyme activities in LV homogenates: Lactate dehydrogenase (LDH), phosphofructokinase (PFK), malate dehydrogenase (MDH). Protein abundance and representative western blotting images: Lactate dehydrogenase muscle-type (LDHA), heart-type (LDHB). Values are mean $\pm$ S.E.M. (N= 5), * $p < 0.05$, ** $p < 0.01$.

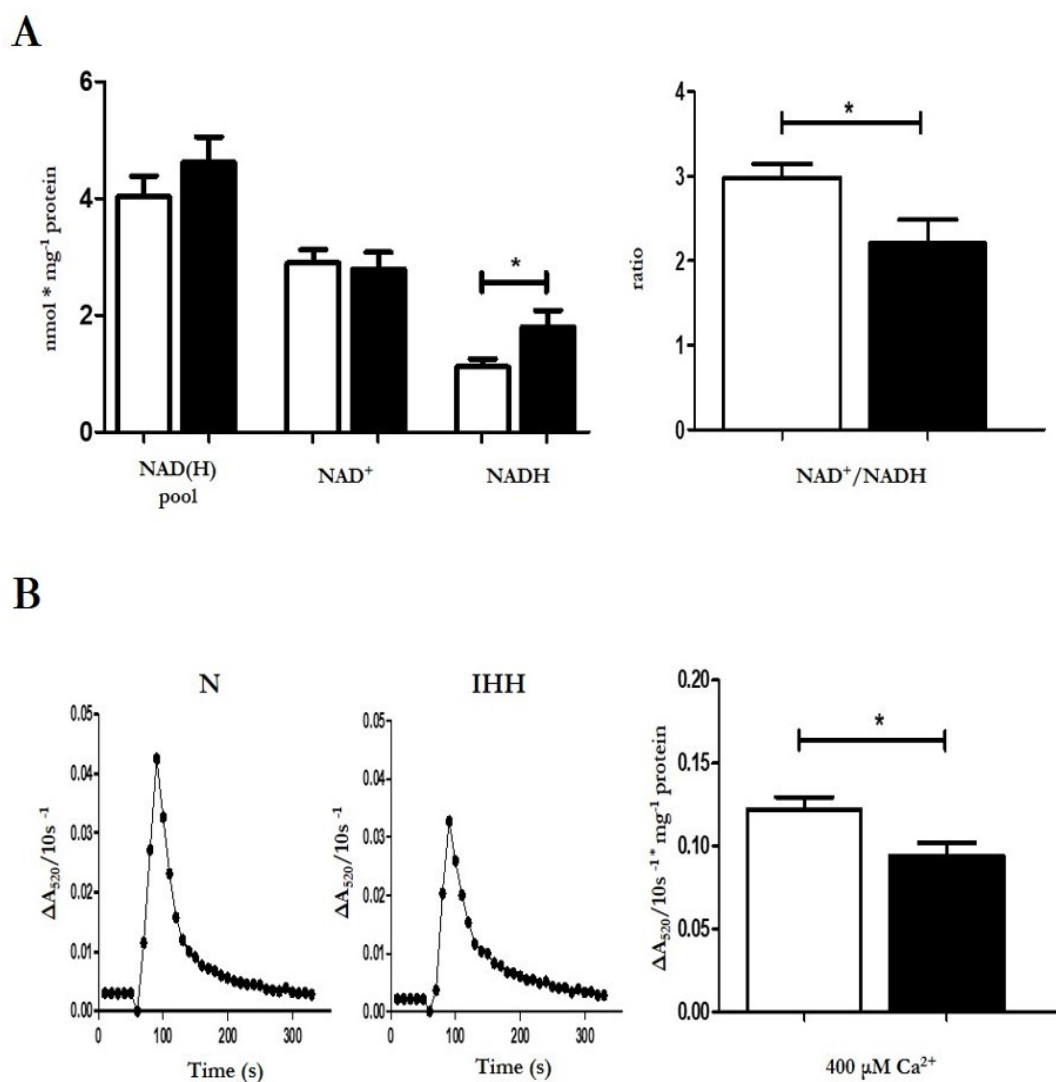


7.13. NAD(H) pools and probability of mitochondrial transition pore opening in IHH

Fig R12A shows that IHH affected neither the total NAD(H) pool nor NAD⁺ specific concentration in LV samples, yet it led to a higher specific concentration of NADH by 61%. Thus, the overall NAD⁺/NADH ratio dropped by 21% after the adaptation to IHH.

Fig 12B presents IHH-afforded reduction of the maximal swelling rate in heavy mitochondrial fraction (see peroxisomal isolation) induced by 400 μ M Ca²⁺ by 23%.

Figure R12: Cardiac NAD(H) pool and swelling rates. **A:** The effect of intermittent hypobaric hypoxia (IHH) on total NAD(H), NAD⁺, NADH pools, and NAD/NADH ratio. Normoxic group (N, white columns), IHH (black columns). Values are mean $\pm$ SEM (N=5), **p* < 0.05. **B:** actual record of heavy mitochondria swelling induced by 400 μ M Ca²⁺ at t = 60s. Values are mean $\pm$ SEM (N=3), **p* < 0.05.

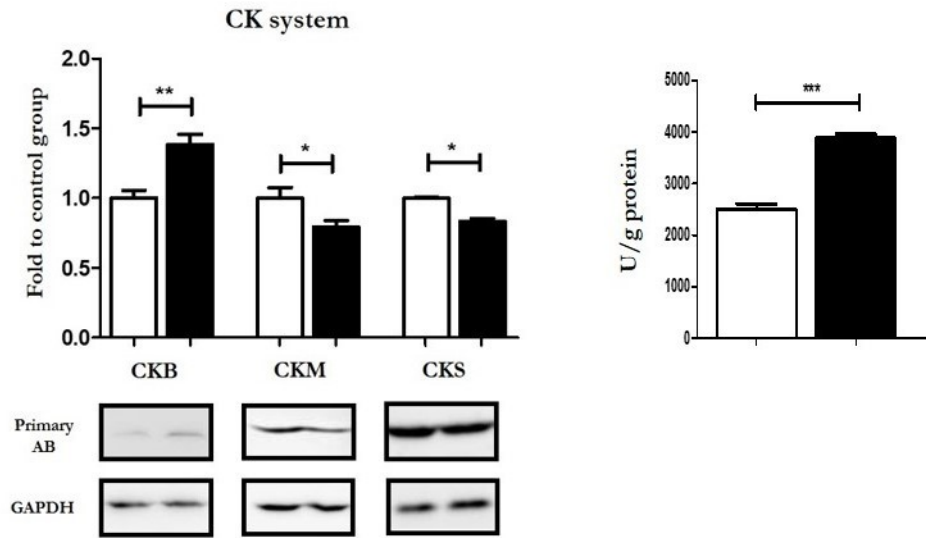


7.14. IHH-afforded changes of CK system

Fig R13A shows that IHH led to an increase in CKB expression by 42% whereas the expression of CKM and mitochondrial (mtCKs) isoforms decreased by 37% and 17%, respectively. All CK isoforms analysed thus followed the representative mRNA expression (Table 1). We have also observed an increase in overall CK activity by 55% after IHH adaptation.

The localization of cytosolic CKM and CKB within a sarcomeric M-band is depicted in Fig R13B. CKM abundance in M-band decreased (by 24%) after IHH, whereas CKB localization therein increased (by 34%).

A



B

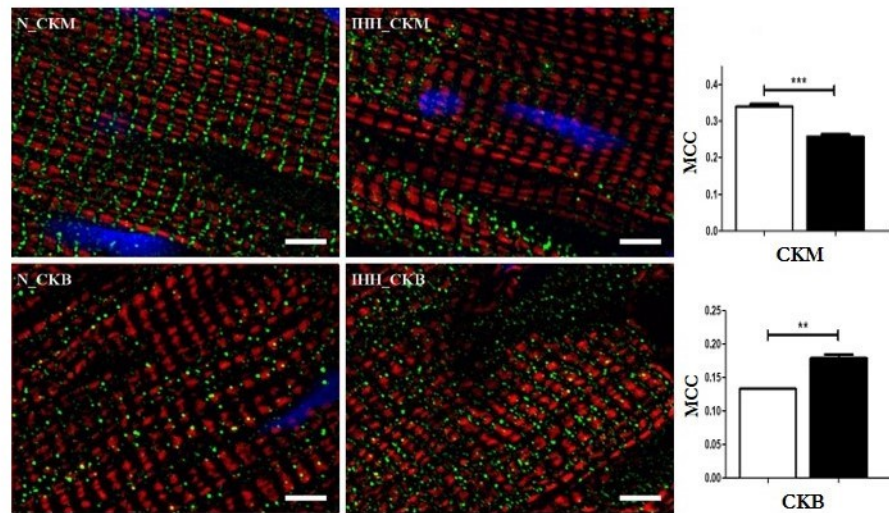


Figure R13: Cardiac CK system after IHH in LV. **A:** The effect of intermittent hypobaric hypoxia (IHH; black columns, N; white columns) on the protein expression of cytosolic muscle-type (CKM), brain-type (CKB), and mitochondrial sarcomeric-type creatine kinase (CKS), and total CK activity. **B:** Localization of CKM and CKB isoform with sarcomeric M-band. Representative micrographs show the distribution of CKM and CKB within cardiac sarcomeric M-band evaluated by Mander's correlation coefficient (MCC). Staining CKM and CKB (green), Phalloidin (red), DAPI (blue). Scale bar equals to 5 μ m. Values are mean $\pm$ SEM, (N=5), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

8. DISCUSSION

In our journey, my colleagues and I have been fully focused on the adaptation to chronic hypoxia in different regimens. At first, we investigated the role of Akt salvage (RISK) pathway affecting HK expression and activity with respect to HK subcellular localization in the model of CNH. Therefore, we randomly separated animals into two groups, control group and CNH group, and subjected them to a brief (10 min) I/R protocol *ex vivo*. Beside Akt/HK pathway, we also analysed the expression of mtCKS, the level of phosphorylated AMPK, and the level of Bax and Bcl-2 (pro- and anti-apoptotic proteins of Bcl-2 family of proteins) in each phase of I/R protocol (i.e. 20 min perfusion, 10 min ischaemia, 10 min reperfusion). Our investigation provided a novel insight in the cardioprotective effect of CNH represented by the activation of Akt/HK2 signalling, particularly in ischaemic conditions. The hypoxic intervention has resulted in many beneficial aspects and these can be summarized as: (i) increased phosphorylation of Akt (Ser⁴⁷³), (ii) increased expression of HK1 and HK2 isoforms together with increased enzyme activity, (iii) prevention of HK2 detachment from mitochondria, (iv) preservation of the mtCK-mitochondria interaction, (v) decreased Bax/Bcl-2 ratio, and vi) decreased amount of activated AMPK in ischaemic conditions of CNH hearts during a brief non-lethal I/R insult (See Figure R1-R6 for more details).

Although IHH adaptation was shown to induce a higher co-localization of HK2 with OMM [305], the CNH itself has not shown any significant alterations in HK2 localization. Nevertheless, CNH increases the expression and activity of HK which also contribute to cytoprotecting against I/R [330]. Akt activation, which was seen in ischaemic CNH-adapted hearts, may exhibit many beneficial functions related not only to energetics, but also to cell-survival (e.g. by glycogen synthase kinase 3 β inhibition), proliferation (as PI3K signalling is necessary in context to cardiac growth) but also in the antioxidant system [331], see [198] for more information. Akt activity may be modified post-translationally *via* acetylation, SUMOylation, glycosylation, and by phosphorylation [332]. The Akt activity is mainly controlled by phosphorylation which can be enhanced in the heart under various physiological and pathological conditions [333]. In our study, we observed an increased phosphorylation of Ser⁴⁷³ but not of Thr³⁰⁸ (Figure R1). These data are in line with studies showing independent phosphorylation of both residues. Alessi *et al.* showed that a mutant expressing Akt-T308A in HEK-293 cells was independently phosphorylated at Ser⁴⁷³ and *vice versa* – the mutant Akt-S473A was phosphorylated at Thr³⁰⁸ [334]. While in mouse tissue that was lacking PDK1, Akt remained phosphorylated at Ser⁴⁷³ but not on Thr³⁰⁸, in cells lacking mTORC2 activity, Akt was still phosphorylated on Thr³⁰⁸ residue [335–337]. Regardless the other mechanisms, it may be assumed that the Akt activity in our settings was strongly affected after being phosphorylated at

Ser⁴⁷³ residue during ischaemic period (See Figure R1). Besides other effects, Akt was shown to phosphorylate HK2 at Thr⁴⁷³ residue after which the HK2-mitochondrial association increases [300,301]. The capability of HK1 and HK2 to OMM is due their N-terminal motif [82] and both HK are believed to bind OMM specifically in interaction with VDACs [83]. Interestingly, GSK3 β , which can be inhibited by activated Akt, was shown to inhibit HK-OMM association via phosphorylation of VDAC Thr⁵¹ residue [83]. In any case, this association provides both the anti-apoptotic/necrotic effects of HK2 [303,304] and its metabolic benefits [338] during I/R insult. The anti-apoptotic effect can be mediated by the following mechanisms: First, the increased HK2 association increases preferential supply of ADP to mitochondria stimulating Complex V (i.e. F₀F₁-ATP-synthase) activity, thus maintaining mitochondrial membrane potential at suitably low level and preventing ROS overproduction in mitochondrial compartment [339]. Second, HK2 associated with VDAC competes with pro-apoptotic protein Bax for the binding site, which may prevent apoptosis triggered by MPTP opening. As for both isoforms, HK2 is believed to be more sensitive to glucose-6-phosphate (G-6-P) inhibition than HK1 [340] and the phosphorylation of HK2 at Thr⁴⁷³ diminishes the inhibitory effect by its product, G-6-P [300]. Studies depicting HK association with OMM under ischaemic conditions lead to controversy results. First, a study of Southworth *et al.* showed a progressive increase of HK2 associated with OMM after 30 minutes of normothermic ischaemia in insulin stimulated Langendorff perfused hearts [338]. Next, Gürel *et al.* reported a moderate and progressive loss of HK2 association after 35 min of ischaemia [341]. Finally, a study of Pasdois *et al.* [342] found a significant drop in mitochondria-bound HK after 30 min of ischaemia, which was proven to correlate with cytochrome c release, ROS generation, and the infarct size on reperfusion. This effect did not differ between oleate and glucose as metabolic substrates, suggesting that our perfusion medium (glucose-only) should not affect HK association. We have, therefore, chosen a brief ischaemic period to see a possible time course of activation of the Akt/HK pathway during ischaemia. The second reason why a brief ischaemic period has been used is that we aimed to analyse a reversible ischaemic injury. This was important for the sample data not to be affected by death cells. However, prolonged ischaemia was shown to attenuate the positive stimulation of the Akt/HK pathway. Activated Akt requires glucose to preserve mitochondrial integrity and function and due the fact that prolonged ischaemia withdraws cardiac glucose and glycogen levels, Akt activity during prolonged ischaemia may get limited [343].

In our study, increased activity of HK (See Fig. R2) and an apparent trend of increased association of HK2 with mitochondria was observed after 10-min ischaemia in both N and CNH groups (See Figure R3). As mentioned above, this brief ischaemic period did not cause any irreversible cellular damage, indicating that our data were not affected by the presence of dead cells

during ischaemia and reperfusion. The increased activity of HK in ischaemic period may be ascribed to its association with the OMM [344]. Moreover, further microscopy analyses revealed that the increasing trend in co-localization of HK2 with mitochondria in both normoxic and CNH hearts subjected to ischaemia, as well as the significant drop of co-localization in normoxic hearts after reperfusion, occurred in all subsarcolemmal, intermyofibrillar, and perinuclear mitochondrial sub-populations (Fig. R3E, F, G). Astonishingly, these results correlate with the increase of pAkt Ser⁴⁷³ in both ischaemic groups and with its drop after reperfusion in normoxic hearts. Moreover, higher expression of both HK1 and HK2 and their total activity were found in CNH ischaemic hearts when compared to control. Higher expression of both HK isoforms and elevated total activity of HK under ischaemia observed in the present study imply its vital role played during early ischaemia (See Fig. R2). The HK-OMM interaction makes HK more resistant to degradation [345], which may explain its accumulation during brief ischaemic period in both groups. I/R alone has been shown to inhibit proteasome function (as the process of protein degradation is ATP-dependent) [346,347] indicating that the accumulation may be also caused by impaired function of protein degradation system. Moreover, it was reported that mitochondrial HK is also ubiquitinated by Parkin [348] which makes mitochondrially bound HK2 expression regulated independently from sarcoplasmic proteasome system. However, the effect of adaptation to CNH on proteasome activity has not been studied and needs a further elucidation. It is also important to mention that elevated HK plays an indisputable role in pentose phosphate pathway flux in the sarcoplasm [86]. Pentose phosphate pathway generates NADPH thus increasing the NADPH/NADP ratio that is crucial for heart anabolic pathways. Moreover, NADPH is essential for a proper function of the heart antioxidant system, particularly glutathione reductase. This is important at the onset of reperfusion when a burst of ROS molecules occur thanks to the re-establishment of the blood flow. The enzyme activity of HK was reported to be modified post-translationally by AMPK-mediated phosphorylation [349]. Indeed, a marked increase in the amount of pAMPK α (Thr¹⁷²) has been observed in both ischaemic groups in the present study (See Figure R5) and correlates with HK maximal activity (See Figure R2). Besides that, AMPK modulates the glucose and fatty acid metabolism, mitochondrial function, stress of endoplasmic reticulum, autophagy, and apoptosis. Additionally, pharmacological activation of AMPK can be beneficial by reducing myocardial necrosis and contractile dysfunction during I/R injury [350,351]. It is important to add that CNH adapted hearts expressed significantly less pAMPK α (Thr¹⁷²) than the hearts of normoxic littermates which proves that the energy deprivation of CNH hearts was not as high as in the control heart. This is in accordance with a study of [200] who showed an indirect correlation between Akt and AMPK activity. This inter-regulation may be also mediated by mTOR including Ser²⁴⁴⁸ and Thr²⁴⁴⁶

sites. While Ser²⁴⁴⁸ is the Akt phosphorylation site [352], AMPK is capable of phosphorylating Thr²⁴⁴⁶ residue of mTOR as demonstrated by AICAR or dinitrophenol treatment [353]. Moreover, Thr²⁴⁴⁶ phosphorylation (negative regulation of mTOR activity) limits phosphorylation at Ser²⁴⁴⁸ (positive regulation) and *vice versa* [353]. In ischaemic conditions, it may be tempting to think that only elevated ROS levels are responsible for AMPK activation. Yet, one of reactive nitrogen species (RNS), peroxynitrite, was shown to activate PKC ζ , which phosphorylates LKB1 at Ser⁴²⁸ residue [354]. This may indicate a certain improvement of the redox balance in ischaemic conditions of CNH adapted hearts.

Previously, we showed the upregulation of mtCKS in CNH hearts [177]. The mtCKS located in the intermembrane space preferentially supplies ADP for complex V of respiratory chain and prevents ROS production [355], similarly as HK bound to mitochondria. Thus, both mtCKS and HK associated with OMM contribute to the coupled state of mitochondrial respiration, important for maintaining high ATP/ADP ratio and energetic homeostasis in the cardiomyocytes, which may indirectly inhibit mPTP opening [304,356]. In the present study, we have found a decreased expression of mtCKS in the normoxic but not in CNH hearts under ischaemic conditions. It is known that mtCKS is very susceptible to oxidative stress, which causes dissociation of mtCK octamers into dimers [357]. However, these changes are not detectable by western blot method. In addition, mtCKS binds with high affinity to cardiolipin [166], which represents a primary target of ROS attack [358] during ischaemia. Depletion of cardiolipin has been observed in rabbit heart SSM during 30-min ischaemia [359]. This finding may partially explain a drop in mtCKS expression in the normoxic group during ischaemia. On the other hand, neither ischaemia nor reperfusion changed the mtCKS expression in CNH hearts, suggesting that CNH protects the mtCKS-mitochondria interaction (See Fig. R4).

In addition, our study shows the decreased Bax/Bcl-2 ratio in the ischaemic and in reperfused CNH hearts compared to the corresponding normoxic controls. This provides strong evidence that the probability of apoptosis during brief myocardial I/R insult is lower in hearts adapted to CNH than in normoxic controls (See Fig. R6). This may, at least partially, be ascribed to the cardioprotective effect of Akt, which is activated during the ischaemic period and its activation persists during reperfusion in CNH hearts. But also, the activated Akt possibly promotes better energy turnover in ischaemic conditions of CNH hearts as the activated AMPK expression was lower therein.

Several pathological conditions that increase the probability of I/R injury is hypertension and hypertrophy [360]. Therefore, in 1963, a rodent model of SHR was developed by Okamoto and Aoki [361]. The combination of hypertension and hypertrophy results in continuing adaptation

of cardiac metabolism – from alterations of glycolytic intermediates through loss of FAT/CD36 function, reduction in the inducible PDK4 mRNA and protein expression to reduced lactate/pyruvate ratio and high-energy phosphate levels, insulin resistance, and increasingly compromised cardiac energetics [362]. As the ischaemic preconditioning was shown to improve the post-ischaemic recovery of SHR LV function (e.g. left ventricular diastolic pressure recovery, reduction of ventricular tachycardia and infarct size) [363] we strived to look whether the CNH adaptation would have a similar effect. Moreover, in parallel to SHR CNH adaptation, we also used a newly developed conplastic strain SHR-mtBN.

In the study of Neckář *et al.* [291], we showed that the combination of mitochondrial genome replacement with the adaptation to CNH significantly decreases the I/R injury compared to adapted SHR. The infarct size correlated with the susceptibility to mPTP opening which, interestingly, correlated with the HK activity in LV homogenates (See Fig. R8). The adaptation to CNH upregulated HK activity in both strains, however, a significant difference between adapted SHR and SHR-mtBN was observed. Because Akt is one of the major regulator of HK expression and activity [314], we determined the major cardiac Akt isoform mRNA and protein expression. There are 3 Akt homologues: Akt 1 (or α), Akt 2 (or β), and Akt 3 (or γ) that all share a common domain structure although they are products of distinct genes [364]. The cardiac major isoforms are represented by Akt1 and Akt2 as Akt3 is the major isoform of the brain [365–367]. Akt3 expression in cardiac tissue is relatively low and its overexpression in mice hearts led to maladaptive hypertrophy [368]. In our study, neither the adaptation, nor the replacement of mitochondrial genome had any effect on Akt1 and Akt2 mRNA expression [75]. The same can be concluded about Akt1 protein expression (See Fig. R7), however, the protein expression of Akt2 was upregulated in SHR-mtBN strain. Akt2 is generally accepted as a regulator of glucose metabolism in cardiomyocytes [369], where it contributes to the phosphorylation of Akt target proteins stimulated by insulin and IGF1 [370]. Beside that general and well accepted role, recent studies have revealed its pleiotropic effects influencing many other signalling pathways. Reinartz *et al.* [371] reported that Akt2 significantly affects development of cardiac muscle tissue, regulation of contractility, and last but not least Akt2 was observed to play a fundamental role in the intracellular Ca^{2+} handling. Furthermore, transfection of HL-1 cardiac cells with constitutively active Akt changed mitochondrial morphology [372]. Importantly, translocation of Akt into mitochondria suppressed mitochondrial apoptotic signalling in cardiac muscle cells [373]. Based on our recent study [291], the present data suggest an involvement of Akt pleiotropic action in mitochondrial genome-dependent amplification of infarct size-limiting effect of chronic hypoxia and point to Akt2 as a potential target of cardioprotective therapies (See Fig. R7).

Subsequently, we analysed the HK activity in the mitochondrial fraction. We observed an upregulation of HK enzyme activity in both strains. Surprisingly, there was no difference in the HK enzyme activity in both adapted mitochondrial fractions (See Figure R8). These data pointed to two corollaries – i) that another signalling pathway, besides Akt, may be responsible for the HK activity differences in homogenate and mitochondrial fraction ii) the upregulated HK enzyme activity reflects other than mitochondrial compartment. An important point of the organismal adaptation to CNH is the stabilization of HIF and subsequent potentiation of its transcriptional activity, which preserves energy homeostasis under hypoxic conditions beside other effects [374]. In the present study, we detected markedly increased expression of HIF1a mRNA in SHR-mtBN, while HIF2a declined in both strains after CNH. The subsequent immunofluorescence and western blot analyses revealed that the HK-association is increased in sarcolemmal and SR-enriched fraction in SHR-mtBN. The SR-HK coupling may provide a better functional coupling between mitochondrial and SR *via* mitochondria-associated membranes. For a complete set of results, please see [75].

These 3 studies [75,272,291] have proved that CNH, in context to cardiac energy metabolism, provides an upregulation of glucose metabolism in rat LV. However, not much has been shown in context to lipid metabolism. Data are suggesting that the cardiac FA metabolism is compromised in hypoxic conditions [375] as β -oxidation rate likely to be the limiting step in cardiac FA metabolism [376]. Therefore, we have aimed to analyse IHH-afforded changes in β -oxidation rates of rat left ventricular (LV) myocardium in mitochondrial and peroxisomal compartments and how these changes affect other energy domains of the cardiomyocytes. we conducted experiments deploying severe IHH regimen (7000 m) of adaptation. The major finding of this study is that the downregulated mitochondrial β -oxidation rates are partially compensated by the upregulation of β -oxidation pathway within cardiac peroxisomes. The shift may represent a novel oxygen saving mechanism of the heart in fatty acid utilization under oxygen deprived conditions (as discussed later). The concomitant changes in energy fluxes of other cellular compartments are further discussed.

It was described in Chapter 2 that under physiological conditions, the continuous contractile activity of the heart is met primarily by the β -oxidation of long chain fatty acids (LCFAs). Almost 80% of supplied LCFAs is rapidly utilized and appears in form of CO₂ in coronary venous blood. The rest of LCFAs enters intra-myocardial triacylglycerol (TAG) pool. Endogenous TAG represent 36% of the energy expenditure in hearts perfused with glucose as the sole substrate, decreasing to 11% when palmitate is added to the perfusate (reviewed in [11]). Although mitochondria represent the major site of LCFA utilization, peroxisomal compartment

may represent up to 30% of total cardiac β -oxidation capacity [377]. It was shown earlier by Morash *et al.* that adaptation to hypoxia (7 days, 5000 m, continuous) decreases the amount of CO₂ produced by mitochondrial β -oxidation when stimulated by palmitoyl-carnitine [315]. A study of Bass *et al.* observed a reduced ability of both rat ventricles to break down fatty acids after IHH adaptation [295]. In agreement with these studies, we observed a decrease in O₂ consumption rates stimulated by palmitoyl-carnitine in both mitochondrial subpopulations (SSM, IFM) after IHH (See Fig. R9A). In contrast, the expression of peroxisomal ACOX1 increased after the adaptation (Fig. R9B). This enzyme is responsible for the initial and rate-limiting step in peroxisomal β -oxidation pathway which may indicate that the FA utilization is partially redeemed in the peroxisomes after IHH. In contrast to *Saccharomyces cerevisiae*, where malate/oxaloacetate-based redox shuttle for re-oxidation of peroxisomal NADH exists, in higher eukaryotes, including human, a lactate/pyruvate-based redox shuttle has been proposed and explained [47,63,378]. In the present study, we have detected an increase in peroxisomal LDHBx abundance after IHH by multiple methods (Fig. R9B, C). These data support our current notion about IHH-afforded upregulation of peroxisomal LCFAs utilization.

In line with reduced mitochondrial β -oxidation rates, we have detected downregulation of ETS protein expression in LV tissue after IHH (Fig. R10A). Additional analyses to determine the IHH effect on individual mitochondrial subpopulations have revealed that IFM are influenced more than SSM with respect to normoxic settings (Fig. R10B). Episodes of reduced oxygen tension during the adaptation decreases the respiration rate, due to non-saturating substrate for cytochrome c oxidase (COX; complex IV) but also to its allosteric modulation [298]. The observed decrease in COX expression may thus be an answer to repetitive oxygen deprived conditions and this finding is in agreement with our study [291] where both COX4.1 and COX4.2 subunits were downregulated in heart by chronic exposure to different regimes of hypoxia. As shown in guinea pig offspring hearts (90 days of age) exposed to hypoxia in the last 14 days of pregnancy, hypoxic conditions reduced mitochondrial protein level and COX activity [379]. A study done on mouse C2C12 myocytes revealed that except mRNA level of COX1, the COX2, COX4, and COX5b mRNA transcripts as well as mitochondrial transcription rates, were downregulated by hypoxia [380]. Except the reduced expression of COX, we were also able to detect a drop in CI expression and activity which is in accordance with another study of Tello *et al.* [297]. The CI is a multi-subunit integral membrane complex of ETS catalysing electron transfer from NADH to ubiquinone which is coupled to proton translocation. Interestingly, an interrelationship between COX and NADH dehydrogenase assembly has been shown [381]. Furthermore, the reduction of mitochondrial phospholipid cardiolipin content, seen in our IHH model, may partially explain the reduced

expression in both, CI and COX [382]. Indeed, cardiolipin is required for optimal function of all respiratory complexes, except complex II (i.e. CI, CIII, COX, and CV) [383]. This phospholipid is not only a passive component of COX, but it was shown to be essential in normal electron transport and proton translocation activity. As well as COX, the association of CI with cardiolipin was shown [384]. The stabilization of mitochondrial super-complexes was dependent on the presence of cardiolipin. Likewise, the activity of the pyruvate carrier and the carnitine acylcarnitine translocase stimulating mitochondrial β -oxidation have been shown to be the most efficient in cardiolipin presence [383]. The reduced activity of CI detected in SSM and IFM subpopulations can be ascribed to reduced CI protein expression observed in LV of IHH-adapted animals in conjunction with reduced expression of COX (Fig. R10C). Basic evidence for physical association of mitochondrial fatty acid oxidation enzymes and ETS complexes and super-complexes has been found [385,386]. Such association possibly promotes advanced metabolic channelling, thus the reduction in CI and cardiolipin abundance may partially contribute to diminished β -oxidation rates seen in IHH mitochondria.

CI is also able to catalyse NADH oxidation generating either superoxide or hydrogen peroxide molecules. Two situations result in significant $O_2^{\cdot-}$ production by CI (a) when mitochondria are not producing ATP while the protonmotive force is high together with reduced coenzyme Q pool and (b) when there is an elevated NADH/NAD⁺ ratio in the mitochondrial matrix [387]. Excessive superoxide production by CI in acute hypoxia enhances CI transition from the active to “deactive” form triggering reactive oxygen species (ROS) production activity in endothelial cells and brain tissue [388]. IHH leads to the overproduction of ROS in the heart [256]. As it was suggested, ROS molecules do not only contribute to ROS-induced I/R damage but are involved in ROS-triggered cytoprotective signalling seen in IHH [252]. Reduction in CI expression and activity could thus represent a self-defence mechanism avoiding exaggerated production of CI-derived ROS in IHH LV myocardium particularly in severe hypoxic and/or anoxic conditions.

The combination of reduced aerobic metabolism with protective signalling deploying hypoxia inducible factor 1 α (HIF1 α) is responsible for the upregulation of glucose utilization and for higher glycolytic flux. IHH upregulated mRNA transcripts of both major cardiac glucose transporters, GLUT1 and GLUT4, but also mRNA transcripts of HK1, HK2, PFK2, LDHA whereas it downregulated mRNA of LDHB (See Table D). These alternations may be ascribed to the activity of HIF1 α [289] and are in accordance with recently published studies [305,316]. Additionally, PFK and LDH activities rose after the IHH adaptation (See Fig. R11). Lactate dehydrogenase is a tetramer made up of two different subunits encoded by two individual genes. Heart-subunit (H) is encoded by LDHB gene, whereas muscle-subunit (M) is encoded by LDHA

one. The M-subunit exhibits a higher K_m for pyruvate and a higher V_{max} for pyruvate reduction than the H-subunit so the increase in LDH activity observed in this study and in rats exposed to hypobaric hypoxia continuously [389] may be ascribed to upregulation of LDHA gene. Notably, a higher LDHBx abundance observed in IHH should also be considered.

Reduction of aerobic capacity and the drop in CI activity are likely responsible for the altered $NAD^+/NADH$ ratio. Neither the $NAD(H)$ pool nor NAD^+ have been affected, however the specific concentration of NADH has risen, so the final $NAD^+/NADH$ ratio has been reduced by IHH (See Figure R12A). The CAC is regulated primarily by the concentration of ATP and NADH, principally by ATP/ADP and $NAD^+/NADH$ ratios. High NADH was reported to strongly inhibit the activity of mitochondrial 3-hydroxyacyl-CoA dehydrogenase and thus β -oxidation [390]. IDH, OGDC, and PDH are also inhibited by high NADH level [391], with the latter being inactivated by PDK. The shift in $NAD^+/NADH$ ratio thus could be the reason of reduced CS activities seen in SSM and IFM after IHH and reduced amounts of citrate would further stimulate PFK [392] and LDH activities (Compare Fig. R10, R11, and R12A). Besides changes in energy metabolism and other effects reviewed in [391], NADH was shown to decrease the activity of cardiac ryanodine receptors (RyRs) as opposite to skeletal muscle RyRs [288] affecting cardiac Ca^{2+} levels. Accordingly, higher glycolytic rates appeared to have vital function in Ca^{2+} homeostasis restoration after I/R [393]. In our model, we have observed a lower sensitivity to Ca^{2+} -induced opening of mitochondrial transition pore in heavy mitochondria (Fig. R12B). This non-purified mitochondrial fraction includes reticular cisternae and as such is unsuitable for mitochondrial functional analyses. On the other hand, it reflects the Ca^{2+} shuttle between mitochondria and sarcoplasmic reticulum more appropriately, so our data suggest a certain improvement in Ca^{2+} handling in IHH adapted myocardium.

CK system of the heart performs as a temporal and spatial energy buffer. Due to the low phosphocreatine (PCr) storages within the heart, cardiac CK system strongly depends on the ATP supplying sources [212]. Two cytosolic (muscle-type, M and brain-type, B; forming -MM-, -MB, and -BB dimers) and one mitochondrial (sarcomeric, mtCKs; forming octamers) are the major isoforms expressed in the cardiomyocytes and are regulated by many stimuli as discussed in one of our previous study [177]. In the present study, the increase in total CK activity in IHH setting is due to elevated expression of CKB which importantly possess higher affinity to PCr than that of CKM [167]. In contrast, CKM and mtCKs protein expressions decreased following their representative mRNA transcripts which differ from the effect of less severe normobaric hypoxia [177]. MtCKs activity can make up to 25% of total CK activity and is inactivated by high levels of ROS due to its sensitivity and localization [175]. Accordingly, a negative regulation between CKM and changes in

cellular redox state [394] may explain the decrease in CKM expression. Moreover, CKM interaction with sarcomeric M-band was shown to be pH- and substrate dependent [173,174], however CKB presence in M-band has not been shown till now. The proportional decline of CKM and elevation of CKB in M-band in this study suggests the presence of CKMB dimers in the M-band as CKB itself does not possess the desirable N-terminal binding domain [160]. On the other hand, it is interesting to mention that insulin was shown to increase both CK-MB and -BB activity in rat LV [172] which could predict that glucose metabolism is likely coupled rather to CKB than to CKM. Additionally, hypoxia was shown to induce a foetal-pattern of gene expression relying more on glucose metabolism in comparison with the adult myocardium with CKB being upregulated as a HIF-targeted gene. Hypoxia also affects the contractile apparatus with the overexpression of myosin heavy chains β in the adult LV [395] which was shown to be more economic in terms of ATP utilization [396]. Its alteration is in line with the results of IHH-induced switch in energy metabolism including the moderate changes of CK system in sarcomeric M-band (See Fig. R13). When compared to CNH adaptation, it is of note that in contrast to CNH, IHH downregulated mtCKs expression in rat LV (Compare Fig. R4 and R13) suggesting that ROS levels are elevated, particularly in IHH settings.

In the light of the results from IHH study, it seems tempting to suggest that the upregulation of peroxisomal β -oxidation helps to utilize mitochondrially-unprocessed FAs but in certain conditions may also represent a very sophisticated oxygen saving mechanism. As it was mentioned, the activity of ACOX and other peroxisomal oxidases are coupled with hydrogen peroxide generation which is rapidly eliminated by the activity of catalase (CAT). Several studies have documented the activity and/or the expression of CAT to be upregulated by hypoxia [256,397]. We were unable to detect any significant increase in CAT expression (Fig. R9B). Nevertheless, CAT is an enzyme with dual enzyme activity. Whereas it processes H_2O_2 molecules and further peroxidises proteins or lipids when ROS levels are low, it becomes catalytically active when ROS levels are high generating oxygen molecules [398]. Upregulation of ACOX in IHH modulates peroxisomal redox state possibly keeping CAT in its catalytically-active state thanks to the fact that peroxisomes are small and membranous organelles. In ischaemia, the heart switches the metabolism towards glucose utilization, nevertheless fatty acid oxidation continues to be the predominant source of energy (Stanley, 2001). Overall mitochondrial oxidative metabolism decreases in proportion to the decrease in cardiac oxygen supply and the intracellular FA levels rise. The co-ordinated activity of peroxisomal ACOX and CAT may alleviate higher sarcoplasmic FA levels, especially in the case of LCFAs. Moreover, the activity of CAT switched to O_2 generation in IHH may be hereafter stimulated by FA flux in ischaemia enabling the myocardium to continue

with oxidative processes. A portion of glycolytic flux may thus exclusively serve to stimulate peroxisomal FA oxidation in form of the pyruvate molecules. This mechanism would allow the long-chain acyl-CoA molecules to be processed without such needs of oxygen. Although not wholly, this phenomenon could also explain the abolishing effect of N-acetylcysteine (NAC) on IHH-induced cardioprotection (Kolar et al., 2007). CAT has been shown to participate in the protection of peroxisomal β -oxidation machinery from the effect of H_2O_2 [399]. The antioxidant effect of NAC may reverse CAT catalytic activity in ischaemic conditions, promoting CAT peroxidase activity affecting peroxisomal function, thus abolishing the observed cardioprotective effect of IHH itself.

9.CONCLUSION

In conclusion, our data suggest that the cardiac resistance to I/R injury induced by adaptation to chronic hypoxia is manifested mainly by alteration of cardiac energy metabolism. Both regimens of adaptation (i.e. CNH and IHH) promoted upregulation of glucose metabolism. This phenomenon was exhibited by upregulation of GLUT and HK, PFK and LDH and as whole may be ascribed to the combined effect of Akt and HIF-1 signalling. Both adaptations lead to the re-structure of CK system, illustrated by upregulation of CKB and downregulation of CKM. Contrary, the centre of lipid metabolism represented by mitochondrial subpopulations was subdued in IHH. However, this adaptation led to the increased proportion of peroxisomal β -oxidation capacity, possibly as consequence of decreased mitochondrial aerobic rates. This shift likely represents cardiac oxygen saving mechanism in FA utilization and is partly stimulated by elevated glycolytic rates and altered NAD^+/NADH ratio serving as a basis of IHH-induced protection against Ca^{2+} -induced mitochondrial transition pore opening. Nevertheless, all of these energetic effects may provide the basis for cardioprotection against I/R by downregulation of ROS burst observed at the onset of reperfusion period, augmentation of Ca^{2+} handling, lowering the probability of mPTP opening, re-establishment of post-ischaemic cardiac functions, mitochondrial, and cellular protection.

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