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Odolnost myokardu k ischemicko/reperfuznímu poškození - možné ochranné mechanizmy

Myocardial tolerance to ischemia/reperfusion injury - possible protective mechanisms

Ph.D. thesis

Supervisor: RNDr. Jan Neckář, Ph.D.

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Declaration

I hereby declare that I completed this Ph.D. thesis independently, except where explicitly indicated otherwise. It documents my own work, carried out under the supervision of RNDr. Jan Neckář, Ph.D. Throughout, I have properly acknowledged and cited all sources used. Neither this thesis nor its substantial part under my authorship has been submitted to obtain any other academic degree.

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Mgr. Petra Alánová

Declaration of co-authorship

On behalf of all co-authors, I hereby declare that Mgr. Petra Alánová has substantially contributed to the formation of the articles which represent an integral part of this Ph.D. thesis. She performed most of the experiments, especially in the papers where she is the first author and she actively participated in the set-up of the experiments, in the interpretation of the results and in the preparation of the manuscripts.

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RNDr. Jan Neckář, Ph.D.

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Abstract

Ischemic heart disease is the leading cause of death and disability worldwide. The effects of ischemic heart disease are usually attributable to the detrimental effects of acute myocardial ischemia/reperfusion (I/R) injury. The aim of the thesis was to contribute to current effort to clarify the basis of mechanisms that could save the heart from I/R injury.

The whole thesis is based on four studies; while the first three are published, the fourth one has been under revision. In the first study, we proved the involvement of nitric oxide (NO) in the cardioprotective mechanism of chronic hypoxia (CH). We described that exogenously increased availability of NO as well as inhibition of phosphodiesterase type 5 led to increased myocardial tolerance of normoxic and chronically hypoxic rats. The effects of both interventions were not additive, suggesting that NO is included in cardioprotective signaling of CH. Second study continued in investigating molecular mechanisms underlying cardioprotection induced by CH. We showed that infarct size-limiting effect of adaptation to CH was accompanied by increased myocardial concentration of tumor-necrosis factor alpha (TNF-α) and TNF-α receptor R2. In the third study, we examined the effect of dexrazoxane (DEX), the only clinically approved drug against anthracycline-induced cardiotoxicity, on I/R injury. We found a narrow dose range that could supress ischemic and reperfusion arrhythmias in isolated perfused hearts, while only the highest dose of DEX reduced infarct size in open-chest rats. Surprisingly, DEX-mediated cardioprotection was not associated with the decrease in oxidative stress, which had been believed as a major cause of anthracycline-induced cardiotoxicity as well as I/R injury. In the last study, epoxyeicosatrienoic acid analog exhibited neither cardioprotective nor blood pressure-lowering effect in two-kidney, one-clip Goldblatt hypertensive rats, a model resembling human renovascular hypertension. Unexpectedly, we found an infarct size-limiting effect in untreated hypertensive rats.

In conclusion, this thesis provided new findings in the field of experimental cardiology. We examined components of molecular signaling pathways leading to cardioprotection provided by CH and described the effects of exogenous drugs with possible beneficial impact on the ischemic myocardium. All these findings could be useful for development of new strategies for protecting the heart against acute I/R injury.

Contents

LIST OF ABBREVIATIONS

2K1C	two-kidney, one-clip
3-NT	3-nitrotyrosine
ATP	adenosine triphosphate
BH_2	dihydrobiopterin
BH_4	tetrahydrobiopterin
BK_{Ca}	Ca^{2+}-activated K^+ channels
BW	body weight
cGMP	3,5-cyclic guanosine monophosphate
CH	chronic hypoxia
CYP	cytochrome P-450
DEX	dexrazoxane
DHETs	dihydroxyeicosatrienoic acids
EET-A	epoxyeicosatrienoic acids analog
EETs	epoxyeicosatrienoic acids
ECG	electrocardiogram
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPx	glutathione peroxidase
GRed	glutathione reductase
GSH	reduced glutathione
GSK-3β	glycogen synthase kinase-3β
GSSG	oxidized glutathione
HanSD	Hannover Sprague-Dawley
HETEs	hydroxyeicosatetraenoic acids
H_2O_2	hydrogen peroxide
HPLC	high-performance liquid chromatography
i.p.	intraperitoneally
I/R	ischemia/reperfusion
i.v.	intravenously
K_{ATP}	ATP-sensitive K^+ channels
mK_{ATP}	mitochondrial ATP-sensitive K^+ channels
sK_{ATP}	sarcolemmal ATP-sensitive K^+ channels
LV	left ventricle
MAP	mean arterial pressure

MDA	malondialdehyde
mPTP	mitochondrial permeability transition pore
NAC	N-acetylcysteine
NADPH	nicotinamide-adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
eNOS, NOS3	endothelial nitric oxide synthase
iNOS, NOS2	inducible nitric oxide synthase
mtNOS	mitochondrial nitric oxide synthase
nNOS, NOS1	neuronal nitric oxide synthase
O_2^-	superoxide anion
$OH^·$	hydroxyl radical
PC	preconditioning
PDE-5	phosphodiesterase type 5
PDK	phospholipid-dependent kinase
PH	pulmonary hypertension
PKC	protein kinase C
PKG	protein kinase G
pO_2	partial pressure of oxygen
PostC	postconditioning
PVCs	premature ventricular complexes
RNS	reactive nitrogen species
ROS	reactive oxygen species
RV	right ventricle
RVSP	right ventricular systolic pressure
sEH	soluble epoxide hydrolase
SEM	standard error of the mean
sGC	soluble guanylate cyclase
SOD	superoxide dismutase
MnSOD	manganese superoxide dismutase
TNF-α	tumor necrosis factor alpha
TNFR1	tumor necrosis factor alpha receptor R1
TNFR2	tumor necrosis factor alpha receptor R2
XO	xanthine oxidase

1. INTRODUCTION

According to the World Health Organization, ischemic heart disease is the leading cause of death and disability worldwide. The effects of ischemic heart disease are usually attributable to the detrimental effects of acute myocardial ischemia/reperfusion (I/R) injury. Myocardial I/R injury includes a series of events, such as ischemic and reperfusion arrhythmias, myocardial stunning, microvascular damage and cell death (Dhalla et al., 2000; Perrelli et al., 2011).

During ischemia, severely reduced or interrupted blood flow to the heart causes an imbalance between oxygen demand and supply, resulting in damage of the cardiac tissue. Early and fast restoration of blood supply seems to be essential for the salvage of ischemic myocardium. Indeed, the use of thrombolytic therapy, primary percutaneous coronary intervention or coronary bypass surgery is the most effective strategy for reducing the myocardial ischemia and improving the clinical outcome.

It has been observed that reperfusion of the ischemic myocardium can also induce injury. During the crucial moments of reperfusion, significant reversible and irreversible organ damage is initiated, and is referred to as I/R injury, firstly described by Jennings et al. (1960). They observed an acceleration of necrosis development during reperfusion in canine heart with coronary ligation. They showed that left ventricular (LV) myocardium could sustain up to 15-20 minutes of coronary occlusion followed by reperfusion with no cell death and no permanent changes in myocyte ultrastructure. This was defined as reversible reperfusion injury. However, when the duration of the ischemic episode was extended up to 60 minutes, restoration of arterial blood flow causes additional cell injury known as lethal reperfusion injury.

It is, therefore, obvious that clinical and experimental cardiologists would like to understand the underlying molecular mechanism of myocardial I/R injury to design therapeutic strategies ultimately reducing the final extent of damage.

1.1. MYOCARDIAL ISCHEMIA

Reduced blood flow to the myocardium causes deprivation of oxygen and nutrient supply. These consequences result in a series of metabolic, functional and morphological changes. The absence of oxygen halts oxidative phosphorylation, leading to mitochondrial membrane depolarization, adenosine triphosphate (ATP) depletion and inhibition of myocardial contractile function (reviewed in Hausenloy and Yellon, 2013). Fifteen-twenty seconds after the occlusion of coronary vessels, cellular metabolism switches to anaerobic

glycolysis, resulting in the accumulation of lactate, which reduces intracellular pH. This is sufficient to meet the most basic energy demand of cardiomyocytes, however within 60-90 minutes of ischemia the affected area of the heart develops contracture-rigor (Jennings and Reimer, 1991). If the anaerobic glycolysis was inhibited, in less than five minutes the reserve supplies of energy phosphates would be totally depleted and heart would undergo contracture-rigor (Frank et al., 2012). The intracellular accumulation of protons activates the Na^+/H^+ exchanger, which extrudes protons from the cell in exchange for Na^+ entry. In response, the reverse activation of the Na^+/Ca^{2+} ion exchanger results in intracellular Ca^{2+} overloading and cell death (reviewed in Avkiran and Marber, 2002). Cardiomyocytes can undergo cell death by two different mechanisms: necrosis and apoptosis (reviewed in Majno and Joris, 1995). While apoptosis is a highly regulated process that is activated via death receptors in the plasma membrane or via permeabilization of the mitochondria, necrosis is generally viewed as an uncontrolled process that leads to mitochondrial swelling, cell rupture, and subsequent inflammation (reviewed in Orogo and Gustafsson, 2013).

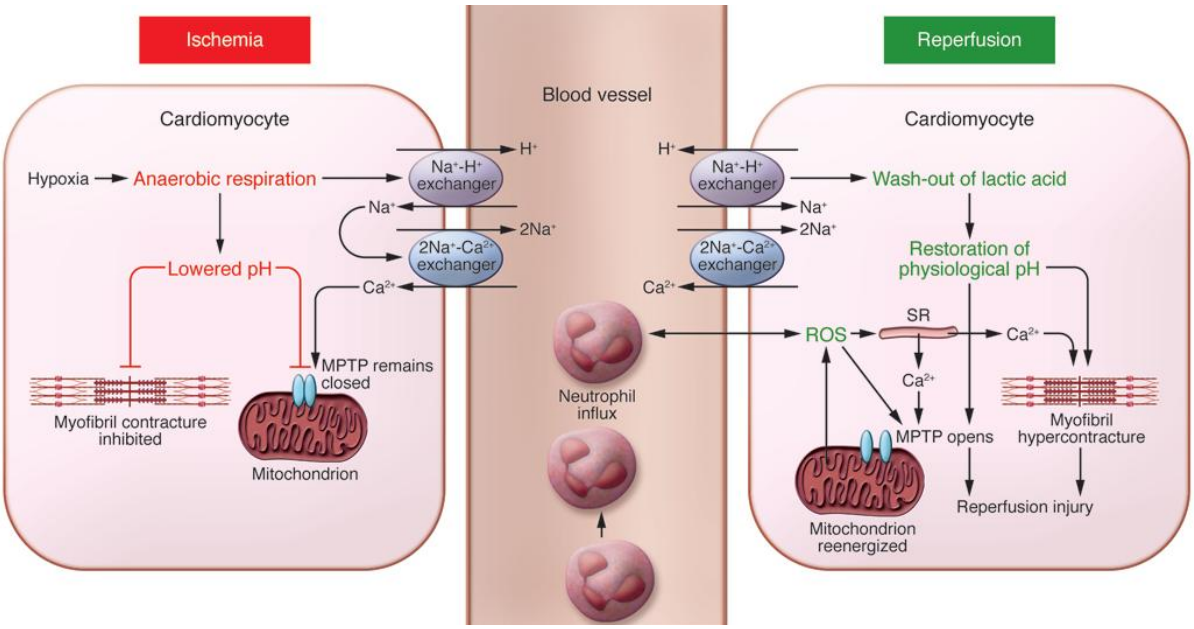

Figure 1 *Main components of myocardial I/R injury. Hausenloy and Yellon, 2013.*

1.2. MYOCARDIAL REPERFUSION

Early coronary reperfusion is essential to salvage viable myocardium, limit myocardial infarct size, preserve LV systolic function and prevent the onset of heart failure. Nowadays, myocardial reperfusion is the only way of treatment of evolving myocardial infarction in

clinical practice. However, as mentioned earlier, reperfusion may cause further tissue damage (Piper et al., 1998). This phenomenon, including reversible (reperfusion-induced arrhythmias, myocardial stunning) and irreversible (microvascular obstruction, cardiomyocytes death) changes, is known as reperfusion injury.

In the first few minutes of myocardial reperfusion, a burst of oxidative stress (Eefting et al., 2004) is produced by a variety of sources. This detrimental reactive oxygen species (ROS) production has been widely accepted as the main mediator of reperfusion injury. Intracellular and mitochondrial Ca^{2+} overload begins during acute myocardial ischemia and is exacerbated at the time of reperfusion due to disruption of plasma membrane, oxidative stress-induced damage to the sarcoplasmic reticulum and mitochondrial re-energization. Mitochondrial re-energization allows the recovery of the mitochondrial membrane potential that drives the entry of Ca^{2+} into mitochondria and subsequently induces the opening of the mitochondrial permeability transition pore (mPTP; Di Lisa et al., 2001).

Experimental studies have shown that pharmacological antagonists of the sarcolemmal Ca^{2+} channel administered at the onset of myocardial reperfusion, reduce infarct size by up to 50% (Herzog et al., 1997). However, clinical studies with Ca^{2+} channel blockers administered at the onset of myocardial reperfusion have not exhibited beneficial results (Bär et al., 2006). During acute myocardial ischemia, the intracellular pH decreases to less than 7.0, whereas at reperfusion, physiological pH is rapidly restored by the washout of lactate and the activation of the Na^+/H^+ exchanger. This pH shift contributes to the lethal myocardial reperfusion injury by permitting mPTP opening. Therefore, a potential treatment strategy for preventing lethal myocardial reperfusion injury would be to slow the normalization of physiological pH at the time of myocardial reperfusion by slowing the process of myocardial reperfusion, as in case of ischemic postconditioning (Fujita et al., 2007).

The mPTP, a voltage-dependent, nonselective channel of the inner mitochondrial membrane, became a critical determinant of lethal reperfusion injury (Halestrap et al., 2004). Fate of the cell is determined by the extent of mitochondrial permeabilization. If minimal, the cell may recover; if severe, the cell may die from necrosis. Opening the channel results in mitochondrial membrane depolarization and uncoupling of oxidative phosphorylation, leading to mitochondrial membrane potential collapse, ATP depletion and cell death (Hausenloy and Yellon, 2003; Heusch et al., 2010). In the setting of acute myocardial I/R injury, the mPTP has been shown to remain closed during ischemia and only open at reperfusion in response to mitochondrial Ca^{2+}, oxidative stress and rapid pH correction (Halestrap et al., 2004).

Preventing mPTP opening at the time of reperfusion provides an important therapeutic target for preventing lethal myocardial reperfusion injury (Fancelli et al., 2014; Gomez et al., 2008).

1.3. CARDIAC PROTECTION

Cardioprotective strategies for ameliorating reversible and irreversible injuries associated with I/R are highly desirable. Research on cardiac protection has a long history in the discovery of new principles of protection, replete with triumphs but also broken dreams with respect to their clinical application. Over the last 40 years, hundreds of experimental interventions have been reported to protect the ischemic myocardium in experimental animals. However, with the exception of early reperfusion, none has been translated into clinical practice, although a limited number appear to be quite promising in initial clinical studies (reviewed in Ošťádal, 2009).

The extent of ischemic injury depends not only on the intensity and duration of ischemic insult, but also on the degree of myocardial tolerance to oxygen deprivation. Therefore, it is not surprising that the interest of many experimental and clinical cardiologists during the past 50 years has been focused on the question of how cardiac tolerance to ischemia might be increased. More recently, many studies are focused on finding novel signal transduction complexes of cardioprotection and understanding the underlying intracellular mechanisms. Following chapters will be devoted to the effective cardioprotective phenomena such as adaptation to chronic hypoxia (CH) and different types of conditioning.

1.3.1. Chronic hypoxia

Over 140 million people live at high altitude, defined as living at an altitude of 2400 m or more above sea level (Hurtado et al., 2012). The characteristic aspect of these high-altitude regions is the low oxygen levels due to the low barometric pressure. For this reason, native dwellers have developed mechanisms to survive in a chronic hypoxic environment. Defence mechanisms include increased erythropoiesis and angiogenesis in order to augment red blood cell mass and oxygen delivery, and metabolic remodeling that increases utilization of oxygen-efficient fuel substrates such as carbohydrates (reviewed in Essop, 2007). Exposure to chronically hypoxic conditions is associated with increased protection against various disease states. Besides smaller incidence of diabetes and obesity, it is mainly a low prevalence of ischemic heart disease. In the late 1950s, the first observations appeared showing that the incidence of myocardial infarction was lower among Andean populations living at high

altitude compared with people living at the sea level (Hurtado, 1960). The epidemiological observations were repeatedly confirmed also in experimental studies using simulated hypoxia. Kopecký and Daum were the first to demonstrate experimentally in Prague in 1958 that adaptation to CH increases tolerance of the heart against injury caused by acute oxygen deprivation. They found that cardiac muscle isolated from rats exposed every other day for 6 weeks to an altitude of 7000 m recovered its contractile function during reoxygenation following a period of acute anoxia to a higher level than that of control animals (Kopecký and Daum, 1958). These results were later confirmed by Poupa et al. (1966) and Widimský et al. (1973).

Beside subjects living permanently at high altitudes, there are two physiological situations when the heart is significantly more tolerant to ischemia: the fetal myocardium adapted to hypoxia corresponding to an altitude of 8000 m and the female heart prior to menopause. It is necessary to point out that clinical relevance of adaptation to CH can be found in common cardiopulmonary diseases, such as chronic ischemic heart disease, chronic obstructive lung disease, sleep apnea and cyanosis due to a hypoxemic congenital heart disease (reviewed in Ošťádal and Kolář, 2007). We are aware that introduction of CH has limitations resulting from the complicated clinical accessibility of the simulated hypoxic environment. However, adaptation to CH may serve as a useful tool for studying molecular identity underlying its cardioprotective pathways. Future basic research in the field of cardiology should be able to provide the possibility of translation of new discoveries obtained from experiments into the clinical setting.

1.3.1.1. Experimental models of chronic hypoxia

Hypoxia is the result of disproportion between oxygen supply and the demand at the tissue level. The most common forms are ischemic hypoxia, induced by the reduction or complete interruption of the coronary blood flow; systemic hypoxia, characterized by a drop in partial pressure of oxygen (pO_2) in the arterial blood; and anemic hypoxia caused by the decreased ability of blood to transport oxygen (reviewed in Ošťádal and Kolář, 2007).

The most common experimental model in research of CH is either the natural mountain environment or hypoxia simulated under laboratory conditions in a normobaric (controlled gases exchange) or hypobaric chamber (pO_2 reduction by partial air depletion). These models allow studying both beneficial and adverse adaptive changes, process of desadaptation and pharmacological protection against the unfavorable manifestations.

Adaptation to CH does not need to be only permanent; it is often of intermittent nature, occuring during ascends to high altitude or sleep apnea. Current experimental protocols of intermittent hypoxia vary greatly in cycle length, severity and number of hypoxic episodes per day and number of exposure days. Experimental data comparing the effects of permanent and intermittent or normobaric and hypobaric hypoxia on cardiac ischemic tolerance are not conclusive.

1.3.1.2. Cardioprotective effect of chronic hypoxia

The improved tolerance of chronically hypoxic hearts to I/R injury manifests itself as a limitation of myocardial infarct size (Meerson et al., 1973; Turek et al., 1980), increased postischemic recovery of cardiac contractile function (Tajima et al., 1994), and reduced incidence and severity of both ischemic and reperfusion ventricular arrhythmias (Meerson et al., 1987; Asemu et al., 2000).

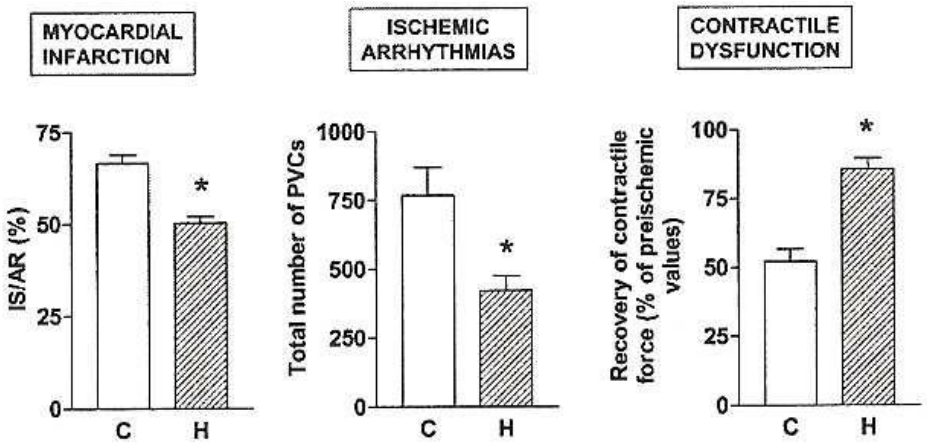

Figure 2 *Effect of CH on three main manifestations of I/R injury. Ošťádal and Kolář, 2007.*

The most important feature of this cardioprotective phenomenon is that the changes induced by CH persist much longer that any form of conditioning. It is well known that the majority of CH-induced cardiopulmonary structural, functional and biochemical alterations persists for a relatively long period after removal of animals from the hypoxic atmosphere (Ošťádal and Widimský, 1985; Faltová et al., 1987). Neckář et al. (2004) showed that residual protective effect on infarct size was detected 7 and 35 days after termination of the adaptation period; it was absent after 90 days of normoxic recovery. In contrast, the antiarrhythmic

protection by CH disappeared already during the first week after restoration of normoxic conditions.

The cardioprotective effect of adaptation to CH seems to be influenced by age. La Padula and Costa (2005) examined the effect of aging. They submitted 7 weeks-old rats to sustained CH for their entire lifetime. They found an increased cardiac tolerance to acute hypoxia up to 18 months, which was lost in 25 months-old rats. Baker et al. (1995) demonstrated that adaptation to CH increased tolerance of the developing rabbit heart from day 7 to day 28. Experiments by Ošťádalová et al. (2002) showed that the protective effect of CH is absent in newborn rats. Prenatal exposure to simulated CH fails to further increase ischemic tolerance in 1 day-old rat hearts; the protective phenomenon develops only during the first postnatal week.

Moreover, a significant sex difference was demonstrated in the resistance of isolated cardiac muscle to oxygen deficiency; the myocardium of female control rats proved to be more tolerant to hypoxia. CH resulted in enhanced resistance in both sexes, yet the sex difference was maintained (Ošťádal et al., 1984). Sensitivity to hypoxia is characterized also by interspecies differences. Cattle and pigs are among the most sensitive animals, sheep and dogs seem less liable to develop hypoxic pulmonary hypertension and right vetricular (RV) hypertrophy, while rats fall between these two groups (Tucker et al., 1975; Waughty et al., 2004). Variations in hypoxic response have been partially related to differences in collateral ventilation. The concept is that collateral ventilation is an efficient protection against local alveolar hypoxia. Dogs have good collateral ventilation, experience less local hypoxia. Pigs have no collateral ventilation, experience more local hypoxia (Kuriyama and Wagner, 1981).

1.3.1.3. Molecular mechanisms of protection by chronic hypoxia

Despite the fact that CH-induced cardioprotection has been known for many decades, the complex mechanism underlying this form of a sustained protective phenotype is still a matter of debate. CH changes the distribution and expression of many cytoprotective proteins including protein kinase C (PKC, Holzerová et al., 2014), mitogen-activated protein kinase (Raffiee et al., 2002, Ravingerová et al., 2007) or antioxidant systems (Guo et al., 2009).

According to our latest study by Chytilová et al. (2015), tumor necrosis factor alpha (TNF-α), a key pro-inflammatory cytokine, does not exert only deleterious effect on the heart, but also activates intracellular signaling pathways that improve cardiac ischemic tolerance. In our experiments, adaptation to CH was associated with increased levels of TNF-α , however,

when chronically hypoxic rats were treated with the inhibitor of TNF-α, the infarct size-limiting effect was blunted.

Large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are another important component localized on inner mitochondrial membrane (Xu et al., 2002). They are opened by hypoxia and contribute to the myocardial protection. The protective effect of BK_{Ca} opening has been attributed to increased matrix K^+ uptake and volume, improved respiratory control, inhibition of mitochondrial Ca^{2+} overload, and prevention of mPTP opening. Borchert et al. (2011) proved that cardiomyocytes isolated from chronically hypoxic rats were more resistant to I/R injury; this effect was attenuated by the BK_{Ca} channel blocker paxilline, while the BK_{Ca} channel opener NS-1619 protected only cells isolated from control normoxic animals.

CH has been shown to protect the heart by a mechanism involving the activation of ATP-sensitive K^+ (K_{ATP}) channels (Neckář et al., 2002; Kolář et al., 2005). K_{ATP} channels are localized in sarcolemma (sK_{ATP}) and in the inner mitochondrial membrane (mK_{ATP}). K_{ATP} channels were found to be activated by hypoxia in the heart (Eells et al., 2000). Adaptation to CH causes an increased current of K^+ ions through the channels (Baker et al., 2001). mK_{ATP} are supposed to be essential for cardioprotection (Costa et al., 2007). Opening of mK_{ATP} promotes K^+ entry into mitochondria with consequent alkalinization of the mitochondrial matrix and generation of ROS with a protective signaling role. The activation of mK_{ATP} channels reduces the action potential duration, thus decreasing contractility during ischemia. Opening of mK_{ATP} and subsequent ROS generation is considered to be a pivotal step in the mechanism of cardioprotection (Perelli et al., 2011). Activators of mK_{ATP} channels increase infarct size, whereas their inhibitors are decreasing it (Baker et al., 1999).

The role of ROS as well as reactive nitrogen species (RNS) in the heart is not simple. Many studies have proposed increased generation of ROS and RNS as a major cause of myocardial I/R injury and toxicity, respectively. On the other hand, both ROS and RNS are essential in signaling pathways leading to cardioprotection. As we have already discussed their unfavorable part in the myocardium, next chapters of the thesis will be dedicated to their positive role in cardioprotective signaling of CH.

1.3.1.3.1. Reactive oxygen species

ROS include both one-electron oxidants such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) and two-electron oxidants such as hydrogen peroxide (H_2O_2). Donation of a single electron to molecular oxygen results in the formation of $O_2^{\cdot-}$. Donation of a second

electron yields peroxide, which then undergoes protonation to yield H_2O_2. Donation of a third electron, such as occurs in the Fenton reaction, results in production of highly reactive $OH^{\cdot}$. Finally, donation of the fourth electron yields water (reviewed in Giordano, 2005).

1.3.1.3.1.1. Reactive oxygen species generation and degradation

ROS can be formed in the heart, and other tissues, by various sources among which the most important are electron transport and leakage during oxidative phosphorylation in the mitochondria, nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases of the Nox family, xanthine oxidase (XO) and uncoupled nitric oxide synthases (NOS).

There are several cellular mechanisms that counterbalance the production of ROS, including enzymatic and non-enzymatic pathways. These include enzymes such as superoxide dismutase (SOD), which facilitates the formation of H_2O_2 from O_2^-. Manganese SOD (MnSOD), as the only known SOD located in the mitochondria, plays a critical role in the control of mitochondrial O_2^- disposal during oxidative phosphorylation. H_2O_2, a product of SOD, is then handled by catalase and glutathione peroxidase (GPx), which catalyzes the removal of H_2O_2 to water through oxidation of reduced glutathione, which is recycled to oxidized glutathione by glutathione reductase (GRed). There are present also non-enzymatic antioxidants such as vitamins E, C or β carotene. The thioredoxin system, including thioredoxin, thioredoxin reductase, and NADPH, forms an additional integrated antioxidant defense system, which operates as a powerful protein-disulfide oxidoreductase (Sawyer et al., 2002).

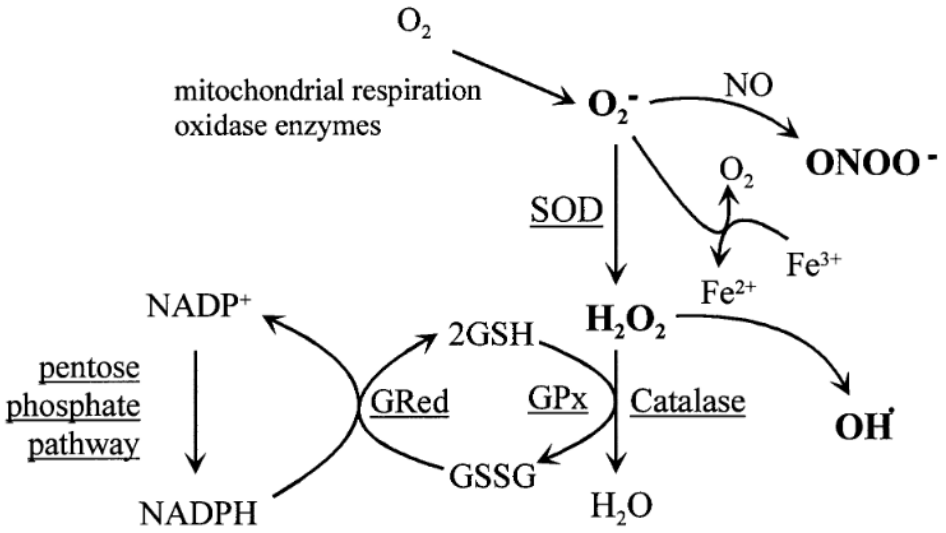

Figure 3 *ROS and the enzymes regulating their levels. Sawyer et al., 2002.*

1.3.1.3.1.2. Role of reactive oxygen species in chronic hypoxia

In the past, ROS were considered exclusively injurious, but now it is generally accepted that they may exert both deleterious and beneficial actions. The balance between ROS production and scavenging is important, because oxidative stress can be either protective or damaging in several diseases. Detrimental processes can result from an imbalance between the excess formation of ROS and limited antioxidant defenses. Excessive generation of ROS during the early phase of reperfusion after myocardial ischemia has been proposed to contribute to reperfusion injury. On the other hand, ROS generated at the same phase may also act as second messengers, modulating cardioprotective pathways, referred to as redox signaling or ROS signaling.

ROS are also thought to have an important role in the protective mechanism of CH. Many cellular responses to hypoxia are known to be mediated by the production of ROS in mitochondria. ROS are produced by the electron transport chain at complexes I, II and III. While complexes I and II produce ROS into the matrix, complex III is capable of producing ROS on both sides of the mitochondrial inner membrane (Muller et al., 2004). Kolář et al. (2007) demonstrated for the first time that antioxidant N-acetylcysteine (NAC) completely prevented the development of cardioprotection in chronically hypoxic rats. Chronic treatment with NAC decreased infarct size in the normoxic animals, but it abolished protection induced by CH. Moreover, CH was associated with tissue oxidative stress, which was prevented by NAC treatment. Similar results were found by Balková et al. (2011), when chronic intermittent hypoxia reduced infarct size and increased the expression/activity of MnSOD. NAC treatment abolished effects of CH on both infarct size and expression/activity of MnSOD. Wang et al. (2011) investigated whether the ROS generated during early reperfusion contribute to the cardioprotection induced by adaptation to CH. They demonstrated that intermittent hypobaric hypoxia confers protection of the heart through elevation of ROS production during early reperfusion, which is associated with the activation of Akt kinase and PKCε pathways. It indicates that CH is associated with oxidative stress and increased ROS generation may be implicated in the induction of cardioprotective mechanism against I/R injury.

1.3.1.3.2. Reactive nitrogen species

Nitric oxide (NO) as well as peroxynitrite, nitrogen dioxide and dinitrogen trioxide belongs to the group of RNS. NO is a unique, endogenous regulatory molecule involved in a

variety of physiological processes. These include regulation of vascular tone in the circulatory system, neurotransmission and immune defense. More specifically, its role in the heart relates to the control of coronary tone, regulation of myocardial contractility, effect on platelet aggregation and free radical production. Dysregulation of its mediated effects have been implicated in the pathology of hypertension, myocardial infarction and cardiomyopathy (Nava et al., 1995). While NO is highly diffusible, the distances that this free radical effectively travels are short due to its high reactivity with molecules such as hemoglobin, myoglobin and other radicals.

1.3.1.3.2.1. Nitric oxide synthesis

There are three NOS widely distributed through most cells and tissues, which can produce NO by converting L-arginine to L-citruline in the presence of NADPH, O_2 and other cofactors. The neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3) are constitutive and Ca^{2+}-dependent, whereas the expression of inducible NOS (iNOS or NOS2) is Ca^{2+}-independent and stress-induced. There is growing evidence supporting the existence of mitochondrial NOS (mtNOS) and the involvement of mitochondrial NO in the regulation of cellular functions. However, the short mitochondrial genome encodes only a few proteins, none of which resembles cytoplasmic NOS. Therefore, if mtNOS is one of the cytoplasmic NOS isoforms, one of the nuclear-encoded NOS isoforms should be transported to the mitochondria after the protein is synthesized in the cytosol (reviewed in Zaobornyj and Ghafourifar, 2012). In the heart, the most convincing data implicate nNOS as the primary candidate for the cytoplasmic NOS isoform targeted into mitochondria (Dedkova and Blatter, 2009).

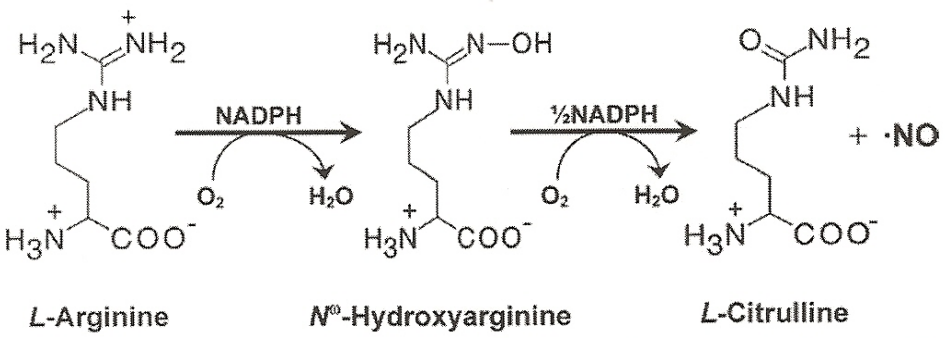

Figure 4 *NO synthesis from L-arginine. Manukhina et al., 2006.*

NO can be stabilized by oxidation to nitrite and nitrate, which can be considered as endocrine molecules that are transported in blood, accumulate in tissue and have the potential to be converted back to NO. This physiological NO recycling is called NOS-independent NO generation (Lundberg et al., 2008).

1.3.1.3.2.2. Nitric oxide regulation

NO generation by nitrate - nitrite - NO pathway is greatly enhanced during hypoxia, thereby ensuring NO production in situations for which the oxygen-dependent NOS enzyme activities are compromised. Interestingly, L-arginine - NOS pathway is oxygen-dependent, whereas the nitrate - nitrite - NO pathway is activated as oxygen tension falls. NO bioavailability is also reduced early in vascular disease states, such as hypertension. This is a result of both reduced NO synthesis and increased NO consumption by ROS.

eNOS enzymatic activity appears to be determined by the availability of its cofactor tetrahydrobiopterin (BH_4). When BH_4 levels are adequate, eNOS produces NO, when BH_4 levels are limiting, eNOS becomes enzymatically uncoupled and generates O_2^-, contributing to the vascular oxidative stress (reviewed in Schmidt and Alp, 2007). By contrast, dihydrobiopterin (BH_2) an oxidized form of BH_4, can cause uncoupling between L-arginine and eNOS. Under this condition, eNOS utilizes molecular oxygen as the substrate to generate O_2^- instead of NO. eNOS uncoupling may induce NO insufficiency, but also contribute to the oxidative stress under various pathological conditions, such as I/R (Chen et al., 2010). Similarly, Sumeray et al. (2000) found that mice lacking eNOS demonstrated significantly greater infarct size after I/R injury than wild type mice. According to these data showing the positive effect of NO on I/R injury, it has been suggested that nitric oxide may play a role also in the cardioprotective effect of CH. Nowadays, it is generally accepted that NO is an important mediator of adaptation to CH.

It is well known that NO activates soluble guanylate cyclase (sGC). This activation leads to the production of 3,5-cyclic guanosine monophosphate (cGMP) that stimulates protein kinase G (PKG) and cGMP-regulated phosphodiesterase activities. Phosphorylated PKG inactivates sarcolemmal Ca^{2+} channels, thus decreasing intracellular Ca^{2+} concentration. PKG also contributes to vasodilatation by reducing Ca^{2+} sensitivity of troponin C (Rastaldo et al., 2007). On the other hand, cGMP-independent pathways include nitrosylation of proteins, when NO modifies free thiol group of cysteines to produce nitrosothiols. This posttranslational modification is proposed to be a widespread mediator of signaling. Because NO is highly reactive, transport of NO signal can be facilitated through reaction with

glutathione and movement of S-nitrosoglutathione, which can transduce the signal by modifying thiol groups on target protein by transnitrosylation (Lipton et al., 2001).

1.3.1.3.2.3. Role of nitric oxide in chronic hypoxia

Chronically hypoxic hearts exhibit increased coronary vasodilatation, generate more nitrite plus nitrate and more cGMP than normoxic hearts (Baker et al., 1999). According to Manukhina et al. (2000), plasma levels of nitrite/nitrate are increased by adaptation to CH. The accumulation of nitrite/nitrate indicates enhanced NO synthesis and release of additional NO from the NO stores. These results were confirmed by Shi et al. (2000), who also showed increased NOS3 protein as well as its increased activity in chronically hypoxic hearts. Therefore, it seems that increased ischemic resistance induced by CH is associated with increased NO production from the NOS3 isoform.

As already mentioned, K_{ATP} channels also serve as important mediators of adaptation to CH (Baker et al., 1997). This group, working with isolated hearts of infant rabbits exposed to hypoxia from birth, later showed that CH from birth increases current through the sK_{ATP} channels and results in increased NO production from NOS3 (Shi et al., 2000). NO from increased NOS activity activates the sK_{ATP} channel, in chronically hypoxic rabbit hearts. Moreover, activation of sK_{ATP} channel by NO in both normoxic and chronically hypoxic hearts occurs by a cGMP-dependent mechanism (Baker et al., 2001). Fitzpatrick et al. (2005) demonstrated a memory of increased resistance against ischemia. This memory of increased cardioprotection persisted at least 20 days following removal from the stimulus of CH. The mechanism underlying the memory appears to involve activation of both NOS and enhanced current through K_{ATP} channels.

1.3.1.4. Adverse effects of adaptation to chronic hypoxia

Besides the positive adaptive changes (increased ischemic tolerance), CH exhibits also adverse impact on cardiopulmonary system. It was Rotta et al. (1956) who reported for the first time that healthy men and women living at high altitude in the Peruvian Andes have some degree of pulmonary hypertension (PH) and RV hypertrophy. This observation was confirmed by Penaloza et al. (1962) for the same geographical region as well as by Vogel et al. (1962) for residents of high altitude in the United States. The critical altitude for the development of PH and RV hypertrophy was set to be 3000 m (Hurtado, 1960).

CH is considered a critical factor causing sustained PH followed by the development of RV hypertrophy. PH is a severe disease characterised by vasoconstriction and remodeling

of precapillary pulmonary arteries without a change in systemic circulation. Remodeling affects endothelial cells and smooth muscle cells, which proliferate. These changes cause medial hypertrophy, adventitial thickening and reduction in vascular lumen diameter (Herget et al., 1999).

Nevertheless, PH is an important physiological mechanism that optimizes ventilation-perfusion matching by diverting blood flow from poorly ventilated areas of the lung (reviewed in Ward and McMurtry, 2009). Associated RV hypertrophy is also a beneficial adaptation since it allows RV to cope with the increased afterload to maintain a normal cardiac output. However, it leads to progressive right heart failure and death, if left untreated. The general treatment for PH includes oxygen supplementation, calcium channel blockers and diuretics. In addition to primary care, therapeutics such as endothelin receptors antagonists, phosphodiesterase type 5 (PDE-5) inhibitors and prostacyclin analogues were introduced to improve the quality of life for patients (D´Alonzo et al., 2001).

Many factors like vasoconstrictors, such as endothelin-1, angiotensin II, and vasodilatators, such as NO and prostacyclin, can regulate pulmonary vasoconstriction during CH (Aoshima et al., 2009). The primary locus of PH is the pulmonary artery smooth muscle cell, which undergoes enhanced proliferation and a slowly developing vasoconstriction that is sustained as long as hypoxia is present. A growing body of evidence indicates that oxidative stress contributes to both acute hypoxic vasoconstriction (Waypa et al., 2001) and to PH associated with CH (Hoshikawa et al., 2001). Administration of antioxidants, such as tempol (Elmedal et al., 2004) or NAC (Hoshikawa et al., 2001), has been demonstrated to significantly attenuate the effect of CH on right ventricular systolic pressure (RVSP) and RV hypertrophy. Also research of nitric oxide pathway modulators, such as NO donor molsidomine, seems beneficial. Andersen et al. (2005) proved that treatment with molsidomine or sildenafil, a PDE-5 inhibitor, reduced RVSP and RV weight.

1.3.2. Conditioning

‘Conditioning’ the heart to tolerate the effects of acute I/R injury can be initiated through the application of several different mechanical and pharmacological strategies. Inducing brief non-lethal episodes of ischemia and reperfusion to the heart either prior to, during, or after an episode of sustained lethal myocardial ischemia has the ability to reduce myocardial injury. These phenomena are called preconditioning (PC), perconditioning and postconditioning (PostC), respectively. Similarly, brief episodes of non-lethal ischemia and reperfusion applied to the organ or tissue distal to the heart reduce myocardial infarct size,

which is known as remote ischemic conditioning. Transient limb ischemia is a simple noninvasive stimulus with important potential clinical application. This procedure can be applied before and during sustained ischemia and at the onset of reperfusion.

The identification of the signaling pathways which underlie the effects of conditioning, has provided novel targets for pharmacological agents to mimic these cardioprotective phenomena resulting in pharmacological PC and PostC.

1.3.2.1. Preconditioning

In 1986, Murry et al. reported that a series of four 5-min occlusions, each separated by 5-min reperfusion, followed by a sustained 40-min occlusion in the dog heart dramatically attenuated I/R injury (Murry et al., 1986). This phenomenon was named ischemic PC. Thus ischemic PC is an endogenous protective mechanism activated by a mild ischemic stress that makes the heart better able to cope with another similar or greater stress. It can reduce infarct size, lethal arrhythmias and contractile dysfunction.

The effect of PC consists of two distinct windows of cardioprotection: the first window (classical PC), which develops within a few minutes from the exposure to the stimulus and lasts only 1-2 hours, and a second window, which appears more slowly after 12-24 h but lasts much longer 2-3 days. A major difference in the cardioprotective mechanisms of the first and second window is that the first one results in rapid modification of existing proteins, whereas the second window is exerted by newly synthesized cardioprotective proteins. The range of protection is also different. The early phase is very effective in limiting lethal I/R injury (infarction), but does not protect against reversible postischemic contractile dysfunction (myocardial stunning). The late phase protects against both infarction and stunning, but it is less powerful (reviewed in Bolli, 2007).

Unfortunately, possibility of clinical application of PC is very limited due to unpredictable onset of cardiac ischemia in clinical practice. The other disadvantages are direct stress to the target organ and mechanical trauma to major vascular structures. On the other hand, remote ischemic PC is a novel method where ischemia followed by reperfusion of one organ is believed to protect remote organs either due to release of biochemical messengers in the circulation or activation of nerve pathways, resulting in the release of messengers that have a protective effect (Tapuria et al., 2008). Moreover, studying of PC conducted the researchers to the discovery of PostC, a clinically more relevant tool of cardioprotection.

1.3.2.2. Postconditioning

Although it has been shown that PC can make ischemic myocardium resistant to I/R injury, the need for the pretreatment – as it has been mentioned above - could limit its clinical application. In 2003, Zhao et al. reported that three episodes of 30-s reperfusion followed by 30-s ischemia performed immediately after 60-min ischemia in the dog heart attenuated reperfusion injury (Zhao et al., 2003). This phenomenon was named ischemic PostC. PostC can be defined as intermittent interruption of coronary flow in the very early phase of reperfusion. Unlike PC, this cardioprotective mechanism is more clinically relevant. Protection provided by PostC was shown to be as potent as that provided by PC and has been described in differet animal species (Ferdinandy et al., 2007; Zhao et al., 2003). Importantly, evidence for the existence of the protective effect of PostC has also been obtained in patients experiencing an acute myocardial infarction (Laskey, 2005; Staat et al., 2005).

PostC provides an intervention which can be applied after the onset of myocardial ischemia and at the time of reperfusion, facilitating its application to patients suffering from acute myocardial infarction. PostC is a clinically relevant cardioprotective invasive strategy that can be applied only to patients undergoing cardiac surgery. However, PostC stimulus to an organ remote from the heart offers an innovative non-invasive approach. Clinical studies are underway to determine whether remote PostC is capable of reducing myocardial injury in patients with acute myocardial infarction (reviewed in Hausenloy and Yellon, 2009).

1.3.2.3. Molecular mechanisms of conditioning

The question whether cardioprotection from PC and PostC use different mechanisms is under discussion. According to Hausenloy and Yellon (2009) signal transduction pathway underlying PostC is similar to that recruited by PC. ROS-induced PKCε activation followed by mPTP inhibition is supposed to be the primary means by which PC as well as PostC prevents cardiac cell death (Costa and Garlid, 2008).

Cardioprotection by ischemic PC and PostC is triggered by autacoids such as adenosine, bradykinin and opioids produced as a response to the cycles of brief I/R. Their G-protein coupled receptors mediate signal transduction resulting in activation of phosphatidylinositol 3-kinase and series of phospholipid-dependent kinase (PDK). PDK causes phosphorylation and activation of Akt kinase, which induces NOS phosphorylation and NO generation. After that, sGC is activated to produce cGMP, which finally activate PKG. In the last step of cytosolic signaling, PKG reacts on mitochondria, resulting in the opening of mK_{ATP} channels. Once it is opened, the increase in K^+ uptake leads to matrix

alkalinization, which in turn inhibits complex I, leading to increased production of O_2^- (Andrukhiv et al., 2006). The increase in ROS activates PKCε that then inhibits the mitochondrial mPTP in a phosphorylation-dependent manner (Costa and Garlid, 2008). Short-term opening is involved in cardioprotection that involves transient ROS formation. In contrast, long-term mPTP opening, which is facilitated by pH restoration, Ca^{2+} overload and the burst of ROS formation at the onset of reperfusion, results in increased mitochondrial permeability, collapse of the mitochondrial membrane potential, matrix swelling and finally rupture of mitochondrial membrane. It is now generally accepted that ROS and RNS signaling play an important role in ischemic PC and cardioprotection.

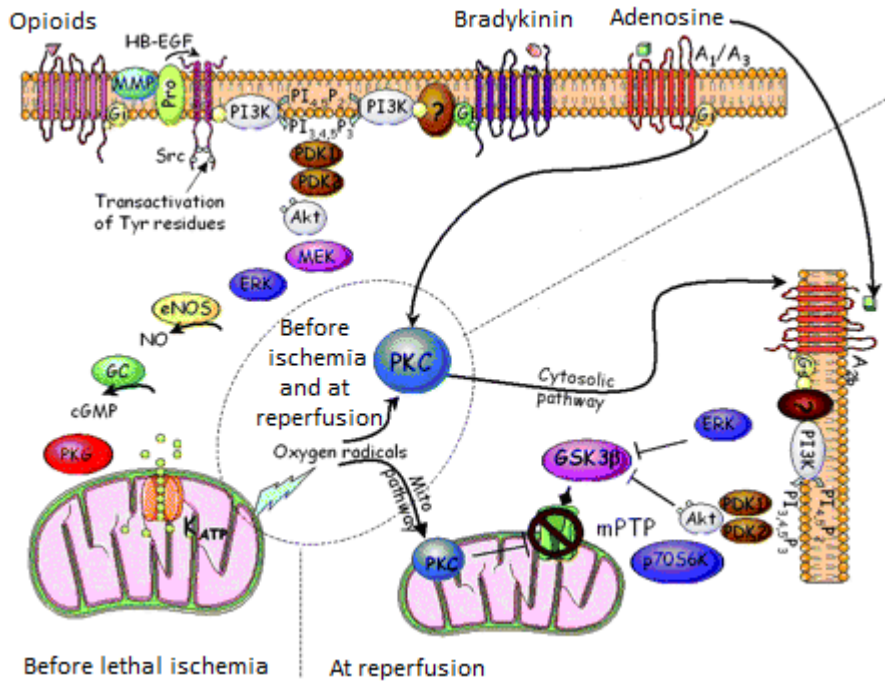

Figure 5 *Signaling pathways of myocardial PC. Costa et al., 2007.*

Although PC was initially described as a response of the myocardium to ischemia, it became apparent soon that a similar phenotype can be elicited by different stimuli, such as pharmacological agents, which have been found to elicit PC-like phenotype. For example, Ockaili et al. (2002) demonstrated powerful PC-like cardioprotection with PDE-5 inhibitor sildenafil against I/R injury. Also agonists of G-protein coupled receptors, such as bradykinin, adenosine, opioids, or NO donors have been found to elicit a PC-like effect. Similarly, PostC enables cardioprotection against I/R injury either by application of short, repetitive periods of ischemia or by pharmacological intervention prior to reperfusion. Pharmacological PostC has

been described for PDE-5 inhibitors. Salloum et al. (2007) showed that intravenous administration of sildenafil or vardenafil at reperfusion induced a significant cardioprotective effect as demonstrated by a reduction in infarct size. The cardioprotective effect of PDE-5 inhibitors was similar to their PC-like effect (Ockaili et al., 2002). The protection was blocked by 5-hydroxydecanoate suggesting that it was mediated by opening of mK_{ATP} channels. Ebner et al. (2013) extended these findings to bolus application, which is more convenient for clinical use.

1.4. DEXRAZOXANE

As mentioned above, myocardial I/R injury is associated with an increased oxidative stress. For many years, it has been believed that ROS are also the primary cause of anthracycline-induced cardiotoxicity. The cardioprotective effects of drugs possessing iron chelating properties on anthracycline-induced cardiotoxicity have been well established; hence the use of iron chelators against myocardial injury seems relevant.

Anthracycline antibiotics, such as doxorubicine, epirubicin or daunorubicine, rank among the most effective anticancer drugs, but their clinical use has been hampered by the risk of cardiotoxicity. The most important forms of anthracycline-induced cardiotoxicity are chronic forms associated with cardiomyopathy with a decrease in LV ejection fraction and heart failure (Jones et al., 2006). The chronic cardiotoxicity is common to all anthrycycline derivatives introduced into the clinical practice; however, molecular determinants of the toxic damage remain to be established. The optimal approach seems to be the effective prevention of anthrycycline-induced cardiotoxicity with effective pharmacological cardioprotectant. As it was proposed that anthracycline cardiotoxicity has been associated with oxidative stress-induced injury with an involvement of free cellular iron (Keizer et al., 1990), the search for new treatments revealed Dexrazoxane (DEX).

DEX has been the only drug with proved effective cardioprotection in both clinical and experimental settings (reviewed in Cvetkovic and Scott, 2005; van Dalen et al., 2011). DEX has been shown to induce effective protection from both anthracycline-induced degenerative changes and apoptotic cell death of cardiomyocytes (Popelová et al., 2009; Sawyer et al., 1999). DEX is a bis-dioxopiperazine compound that is converted intracellularly to its metabolite ADR-925, which was shown to possess iron and other metal ion chelating properties. This mechanism was assumed to be the basis of its protective activity against the anthracycline-induced cardiotoxicity, by decreasing the redox activity of chelated iron (Hasinoff, 1998). However, stronger and more selective iron chelators failed to provide better

or at least the comparable cardioprotection as DEX in chronic anthracycline cardiotoxicity models (Štěrba et al., 2013), which argues against this hypothesis.

Therefore, it seems that DEX mediate its cardioprotective action through different pathways. DEX is also a strong inhibitor of topoisomerase II (Hasinoff et al. 1995), which plays an important role in DNA transcription and replication. However, at least the latter effect seems unlikely to contribute to the cardioprotection observed in this study, because ventricular myocytes are terminally differentiated and myocardium contains only low levels of this enzyme (Hasinoff and Herman 2007). Thus the mechanisms responsible for cardioprotection provided by DEX are still poorly understood.

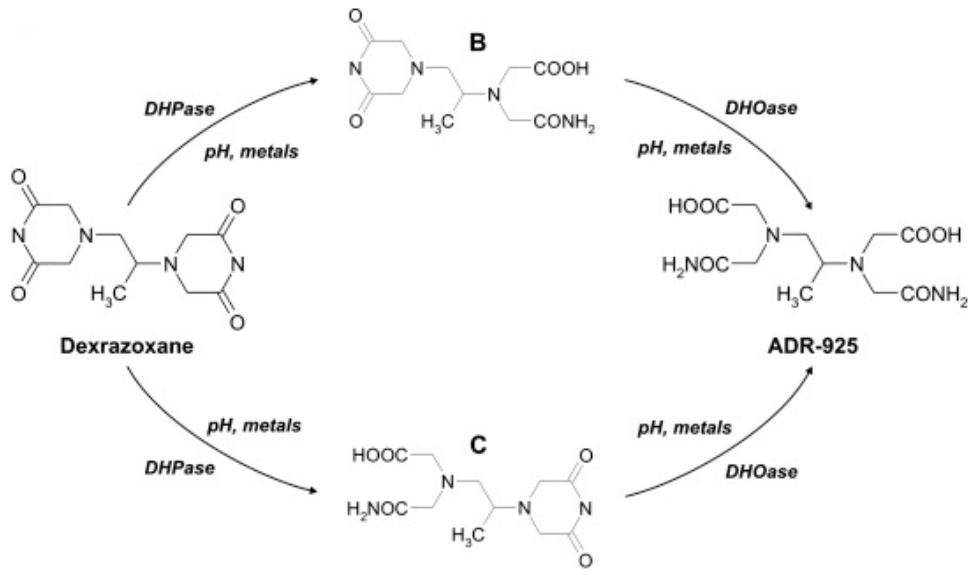

Figure 6 *Stepwise hydrolysis of dexrazoxane to intermediate metabolites B and C and iron-chelating metabolite ADR-925. Štěrba et al., 2013.*

1.5. EPOXYEICOSATRIENOIC ACIDS

Evidence suggests that ischemic heart disease is the most common outcome of hypertension. Hypertension accelerates the development of atherosclerosis, and sustained elevation of blood pressure can destabilize vascular lesions and precipitate acute coronary events. Besides, increased ROS formation is a common feature of hypertension and I/R injury. Therefore, antihypertensive agents may be a unifying option for treatment of both of them.

Arachidonic acid is a major component of cell membranes that resides in the sn-2 position of phospholipids. Once liberated from the cell membrane phospholipids, this 20-

carbon fatty acid is converted by a series of enzymes to biological active metabolites termed eicosanoids. Beside cyclooxygenase and lipoxygenase, the third eicosanoid enzymatic pathway is the cytochrome P-450 (CYP) pathway that contains two distinct enzymatic activities. CYP hydroxylase generates hydroxyeicosatetraenoic acids (HETEs), whereas CYP epoxygenase produces epoxyeicosatrienoic acids (EETs). CYP epoxygenase adds an epoxide across one of four double bonds in arachidonic acid to produce four EETs regioisomers: 5,6-EETs, 8,8-EETs, 11,12-EETs and 14,15-EETs. The main EETs catabolic pathway is conversion to their corresponding dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH; reviewed in Imig, 2012).

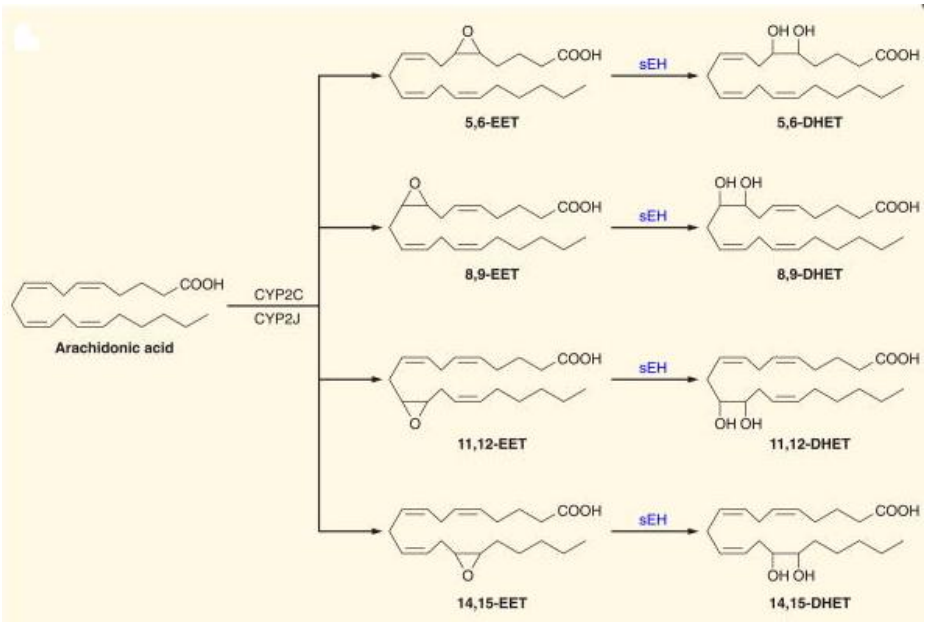

Figure 7 *CYP epoxygenase metabolic pathway. Imig, 2012.*

For investigation of EETs cardiovascular and cell signaling agonists, antagonists and inhibitors have been used in cell cultures as well as in animal models. EETs play an important role in the regulation of renal tubular ion transport and renal and systemic vascular tone (Capdevila et al., 2007; Sarkis et al., 2004). Moreover, EETs have been shown to be potent vasodilators involved in the action of the endothelium-derived hyperpolarizing factor and in the kidney they inhibit tubular reabsorption of sodium and water in the proximal tubule and collecting duct. Both these actions could contribute to potential antihypertensive properties of EETs (Čertíková-Chábová et al., 2007; Campbell et al., 1996; Madhun et al., 1991; Sakairi et al., 1995). It has been also suggested that EETs serve as a compensatory system against

enhanced renin-angiotensin system (Sarkis et al., 2004; Imig, 2010). Kopkan et al. (2012) showed that chronic treatment with sEH inhibitor decreased blood pressure in two-kidney, one-clip (2K1C) Goldblatt hypertensive mice, which was associated with normalization of the reduced availability of EETs in the nonclipped kidney.

Cardioprotection is another area, where therapeutic targeting of the epoxygenase pathway has demonstrated its promise. Recent studies have provided evidence that EETs are cardioprotective in several models of I/R injury (reviewed in Imig, 2010; Nithipatikon and Gross, 2010). Neckář et al. (2012) showed that chronic treatment with sEH inhibitor limited myocardial infarct size induced by I/R in Ren-2 transgenic hypertensive rats and was associated with marked decrease in blood pressure.

EETs seem to have many actions that contribute importantly to cardiac and vascular pharmacology to maintain cardiovascular homeostasis. The future studies of EETs signaling pathways may provide novel EETs targets for developing treatments for cardiovascular diseases.

2. AIMS OF THE THESIS

The specific aims of the thesis were:

1.) To investigate the role of signaling pathways including NO/cGMP and TNF-α in the cardioprotective mechanism of CH.

2.) To find out whether dexrazoxane can reduce myocardial I/R injury in rats.

3.) To evaluate the effects of EET analog on myocardial infarct size in 2K1C Goldblatt hypertensive rats.

3. MATERIAL AND METHODS

This section of the thesis includes chosen methodics, which was completely or at least partly performed by the author.

3.1. ANIMALS

All experiments were performed in rats according to the Guide for the Care and Use of laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.1.1. Model of continuous normobaric hypoxia

Adult male Wistar rats (250 – 300 g body weight) were adapted to continuous normobaric hypoxia (inspired O_2 fraction 0.1) in a normobaric chamber equipped with hypoxic generators (Everest Summit Hypoxico Inc., NY, USA) for 3 - 4 weeks. No reoxygenation occured during this period. The control rats were kept for the same period of time at room air. All animals were kept in a controlled environment (23 °C; 12:12-h light-dark cycle; light from 5:00 AM) with free access to water and standard chow diet.

3.1.2. Model of two-kidney, one-clip Goldblatt hypertension

Male Hannover Sprague-Dawley (HanSD) rats (100 – 120 g body weight) were anesthetized with a combination of tiletamine, zolazepam (Zoletil, 8 mg/kg), and xylasine (4 mg/kg, Rometar, Spofa, Czech Republic) administrated intramuscularly. The right renal artery was isolated through a flank incision, while a silver clip (0.25 mm in internal diameter) was placed on the renal artery. 2K1C Goldblatt hypertensive rats are a model closely resembling human renovascular hypertension. Sham-operated rats underwent the same surgical procedure except placing the arterial clip.

3.2. INFARCT SIZE AND VENTRICULAR ARRHYTHMIAS DETERMINATION IN OPEN-CHEST RATS

Susceptibility to ventricular arrhythmias and myocardial infarction were evaluated in anesthetized (pentobarbital sodium; 60 mg/kg body weight, i.p.; Sigma-Aldrich, USA) open-chest rats, pump-ventilated (rodent ventilator 7026; Ugo Basile, Italy) via tracheal cannula with room air at 68-70 strokes/min (tidal volume of 1.2 ml/100 g body weight). A cannulation of carotid artery (mean arterial pressure recording) was accomplished. The rectal temperature

was maintained between 36.5 and 37.5 °C by a heated table throughout the experiment. Left thoracotomy was performed to expose the heart; myocardial ischemia was induced by occlusion of the left anterior descending coronary artery for 20 min, followed by 3-h reperfusion. A single-lead electrocardiogram (ECG) and blood pressure were continually recorded. The number of premature ventricular complexes during ischemia and at the beginning (3 min) of reperfusion was counted from ECG records according to the Lambeth Conventions (Walker et al., 1988). At the end of 3-h reperfusion, the hearts were excised and washed with 20 ml saline through the aorta. The area at risk and the infarct size were determined by staining with 5% potassium permanganate (Sigma-Aldrich, USA) and 1% 2,3,5-triphenyltetrazolium chloride (Sigma–Aldrich, USA), respectively. The hearts were cut perpendicularly to the LV long axis into slices 1 mm thick and stored overnight in 10% neutral formaldehyde solution. The next day, the RV free wall was separated and both sides of the LV slices were photographed. The size of the infarct area, the size of the area at risk, and the size of the LV were determined by a computerized planimetric method using the software Ellipse (ViDiTo, Slovakia). The infarct area was normalized to the area at risk, and the area at risk was normalized to LV.

3.3. VENTRICULAR ARRHYTHMIAS DETERMINATION IN ISOLATED PERFUSED HEARTS

In this experimental protocol, rats were anesthetized (pentobarbital sodium; 60 mg/kg body weight, i.p.; Sigma-Aldrich, USA) and given heparin (500 IU, i.p.). Hearts were rapidly excised and perfused via aorta at a constant flow and temperature (37 ºC) according to the method of Langendorff. A modified Krebs-Henseleit solution was gassed with 95% O_2 and 5% CO_2 (pH 7.4) and contained (in mmol/l): NaCl, 118.0; KCl, 3.2; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; $CaCl_2$, 1.25; glucose, 7.0. An epicardial ECG was continuously recorded and subsequently analyzed. After a period of stabilization, hearts were subjected to 30 min ischemia followed by reperfusion. Ventricular arrhythmias occuring during 30 min ischemia and first 5 min of reperfusion, respectively, were counted and evaluated according to the Lambeth Conventions (Walker et al., 1988).

3.4. BIOCHEMICAL METHODS

3.4.1. Analysis of oxidized and reduced glutathione

The reduced (GSH) and oxidized (GSSG) glutathione concentrations were determined simultaneously in LV tissue samples using the method of Reed et al. (1980) adapted by Yoshida (1996), with slight modifications. The tissue was homogenized in cold 5% metaphosphoric acid containing 10 mmol/l EDTA (1 ml). The precipitated proteins were removed by centrifugation, and the supernatant (0.4 ml) was reacted with 0.4 mol/l iodoacetic acid (100 μl) to block the thiol group of GSH and then with 1-fluoro-2,4-dinitrobenzene (100 μl) to derivatize amino groups of both GSH and GSSG. The excess reagent was removed by incubation with glycine. The solution was analyzed using an high-performance liquid chromatography (HPLC) system 1100 (Agilent, USA; Zorbax NH2 column; 4.6 mm × 150 mm; 5 μm). The mobile phase for gradient elution was methanol-water 4:1 (v/v; solution A) mixed with 2 mol/l sodium acetate-water-methanol 3:1:2 (v/v/v; solution B). Ultraviolet detection was set at 365 nm.

3.4.2. Analysis of malondialdehyde

The LV tissue samples were pulverized into a powder under liquid nitrogen. After adding 500 μl of the homogenization buffer (25 mmol/l Tris-HCl and 0,1% Triton X-100), the samples were homogenized and centrifuged (1000 g, 10 min, 4 °C). Supernatant (100 μl) was taken for the determination of malondialdehyde (MDA) concentration. After adding 20 μl of NaOH (6 mol/l) and vortexing, the samples were kept at 60 °C for 30 min followed by 5 min cooling at -20 °C, deproteinized by 50 μl of $HClO_4$ (35% v/v) and centrifuged (10 000 g, 5 min, 4 °C). Supernatant (100 μl) was mixed with 10 μl of 2,4-dinitrophenylhydrazine (5 mmol/l), kept in the dark for 10 min, and analyzed by an HPLC system (Shimadzu, Japan; column EC Nucleosil 100-5 C18; 4.6 mm × 125 mm; flow 1.0 ml/min; sampling volume 30-100 μl) with the UV detection set on 310 nm. Concentration of MDA was normalized to total protein determined by the method by Bradford (1976).

3.4.3. Electrophoresis and western blot analysis

Proteins from the LV myocardium were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% gel and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 5%

dry low-fat milk in TRIS-buffered saline for 60 min at room temperature, membranes were washed and incubated with specific monoclonal antibodies overnight at 4 °C. Membranes were washed again and incubated with secondary antibody for 60 min at room temperature. Bands were visualized by enhanced chemiluminescence on the LAS system, and ImageJ software was used for quantification of the relative abundance of the enzymes. All protein data were normalized to the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

3.4.4. Analysis of nitrite and nitrate

Detection of nitrosative stress markers, nitrite and nitrate was assessed. For measurements of total nitrite and nitrate concentrations in plasma samples we used Nitrate/Nitrite Colorimetric Assay kit (Cayman, Czech Republic). This assay was performed on samples from different experimental groups according to the protocols described by the manufacturer.

3.5. STATISTICAL ANALYSIS

Data are presented as mean±standard error of the mean (SEM). GraphPad Prism software was used and statistical evaluations were done using one-way analysis of variance with the Newman-Keuls post test. Differences in number of ventricular arrhythmias between the groups were evaluated by the Kruskal-Wallace nonparametric test followed by Dunn test. Values exceeding the 95% probability limits ($P<0.05$) were considered statistically significant.

4. RESULTS

4.1. ROLE OF NO/cGMP PATHWAY IN THE CARDIOPROTECTIVE EFFECT OF CHRONIC HYPOXIA (PUBLICATION A)

In this study, we tried to uncover the effect of pharmacological increase in acute NO production on cardioprotective effect of CH in rats. Therefore, normoxic and chronically hypoxic male Wistar rats were treated acutely with NO donor molsidomine (10 mg/kg, i.v., 30 min before ischemia) or PDE-5 inhibitor sildenafil (0.7 mg/kg, i.v., 30 min before ischemia). These animals together with the corresponding untreated controls were subjected to acute I/R injury for subsequent infarct size determination.

As shown in ***Figure 8A***, area at risk expressed as percentage of LV size was the same in all experimental groups; this allowed comparing the average values of infarct size. ***Figure 8B*** shows that adaptation to CH induced a significant decrease in infarct size, expressed as the percentage of area at risk (40.6±2.4%) as compared to the normoxic controls (56.3±2.8%). Acute molsidomine or sildenafil administration markedly reduced myocardial infarct size in normoxic rats (32.3±6.3% and 33.7±8.4%, respectively) and significantly enhanced protective effect of CH (26±2.5% and 24.4±3.5%) in comparison to the untreated groups. Myocardial infarct size in CH rats treated with molsidomine or sildenafil did not differ from treated normoxic animals.

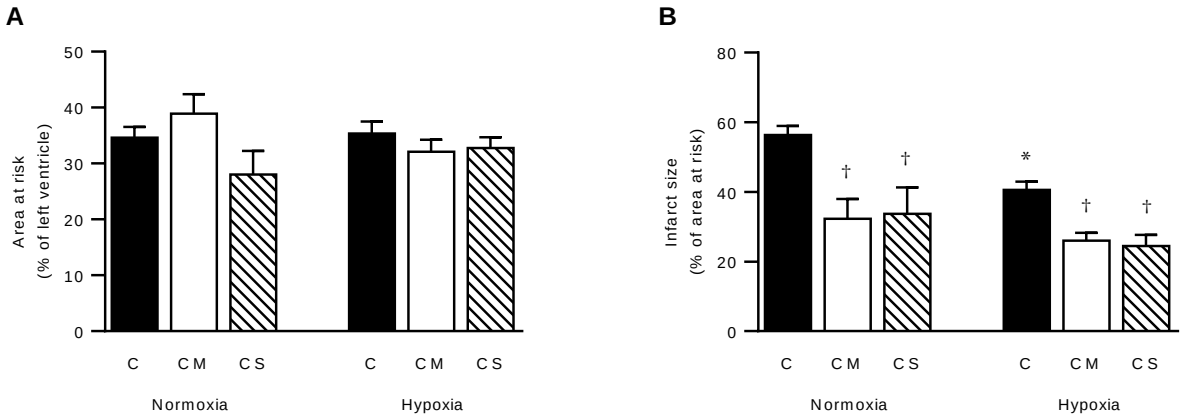

Figure 8 *Area at risk expressed as the percentage of LV size (A) and myocardial infarct size expressed as the percentage of area at risk (B) in normoxic and chronically hypoxic rats untreated (C) or treated acutely with molsidomine (CM) or sildenafil (CS). Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding normoxic group,* † *P<0.05 vs. corresponding untreated group.*

In the second part of the project (not published yet), normoxic and chronically hypoxic rats were treated chronically with sildenafil (1.5 mg/kg/day in drinking water). Sildenafil treatment started 3 days before the hypoxic exposure and lasted during the whole adaptation period. Similarly, we were interested in myocardial ischemic tolerance as well as changes in hemodynamics. Moreover, we examined how CH affects concentration of several markers of oxidative stress.

As shown in ***Figure 9A***, normalized area at risk did not significantly differ among the experimental groups. ***Figure 9B*** shows the effect of CH and sildenafil treatment on the infarct size. Infarct size expressed as percentage of area at risk was markedly decreased from 58.3±2.7% in normoxic controls to 42.7±2.1% in CH rats. Sildenafil treatment reduced infarct size in both normoxic (35.8±3%) and chronically hypoxic (19.8±2.6%) animals.

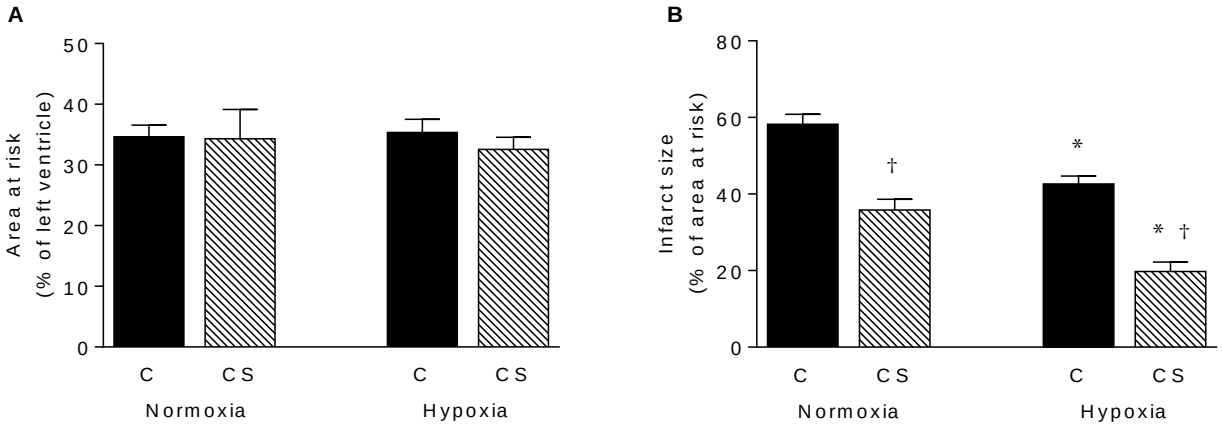

Figure 9 *Area at risk expressed as the percentage of LV size (A) and myocardial infarct size expressed as the percentage of area at risk (B) in normoxic and chronically hypoxic rats untreated (C) or treated chronically with sildenafil (CS). Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding normoxic group,* † *P<0.05 vs. corresponding untreated group.*

Figure 10 demonstrates that exposure of the animals to CH for a period of 4 weeks resulted in a significant increase in RVSP followed by RV hypertrophy. Treatment with sildenafil had no preventive effect against the development of PH. Systemic blood pressure or LV weight were affected neither by CH nor by sildenafil treatment.

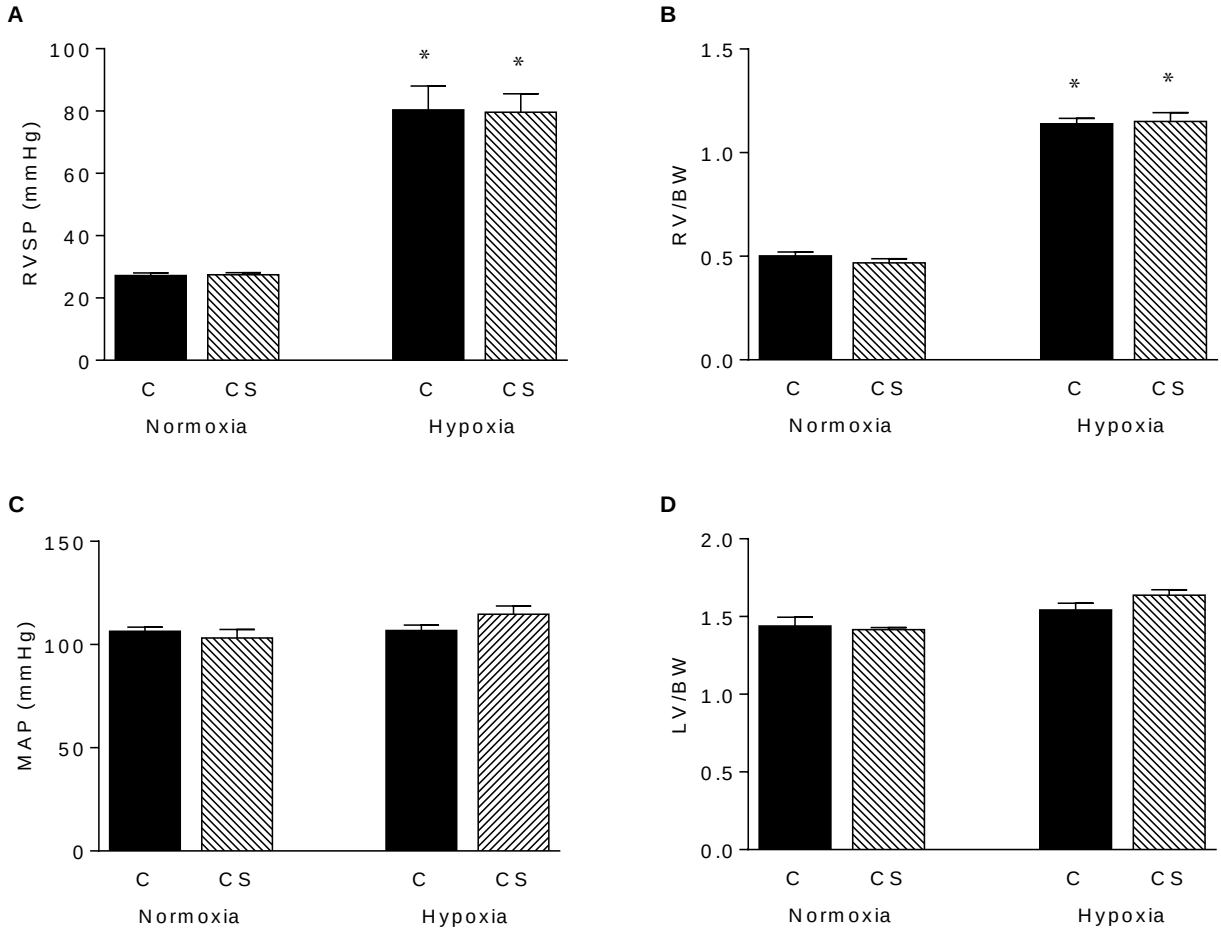

Figure 10 *Right ventricular systolic pressure (RVSP; A), right ventricular weight normalized to body weight (RV/BW; B), mean arterial pressure (MAP; C) and left ventricular weight normalized to body weight (LV/BW; D) in normoxic and chronically hypoxic rats untreated (C) or treated chronically with sildenafil (CS). Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding normoxic group.*

Figure 11 presents changes in myocardial levels of oxidative and nitrosative stress markers. Adaptation to CH increased MDA and 3-nitrotyrosine (3-NT) levels as well as decreased GSH/GSSG and plasma nitrite to nitrate ratio. Sildenafil treatment restored the levels in different ways. It caused a significant increase in 3-NT levels in both normoxic and chronically hypoxic group; it decreased the levels of MDA and nitrite/nitrate in normoxic animals and did not change GSH/GSSG.

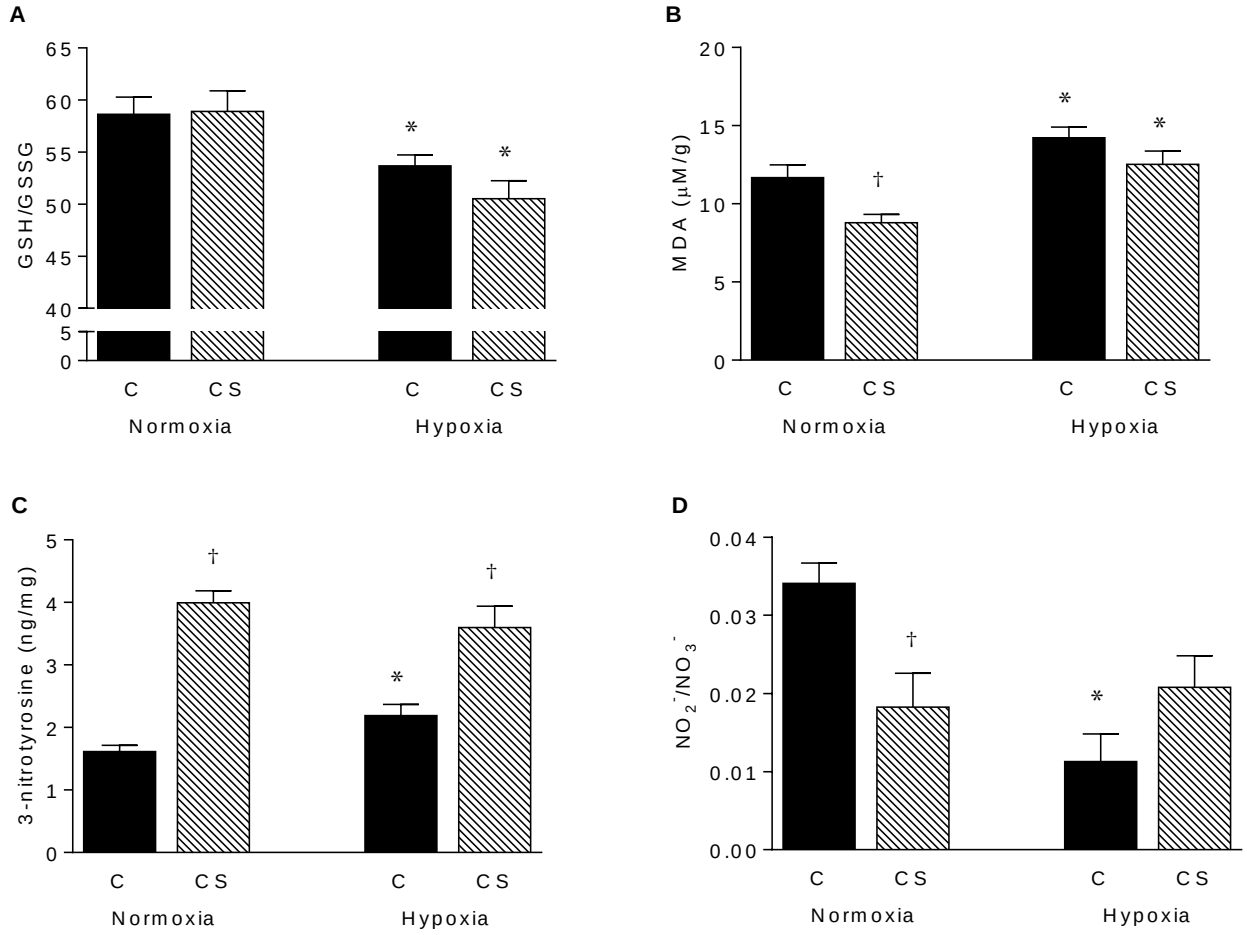

Figure 11 *Myocardial levels of reduced to oxidized glutathione ratio (GSH/GSSG; A), malondialdehyde (MDA; B), 3-nitrotyrosine (C) and plasma nitrite to nitrate ratio (NO_2^-/NO_3^-; D) in normoxic and chronically hypoxic rats untreated (C) or treated chronically with sildenafil (CS). Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding normoxic group,* † *P<0.05 vs. corresponding untreated group.*

The myocardial cGMP level, shown in ***Figure 12***, was increased by CH with respect to normoxia. Sildenafil treatment increased cGMP level in normoxic group; however, it had no effect in CH group.

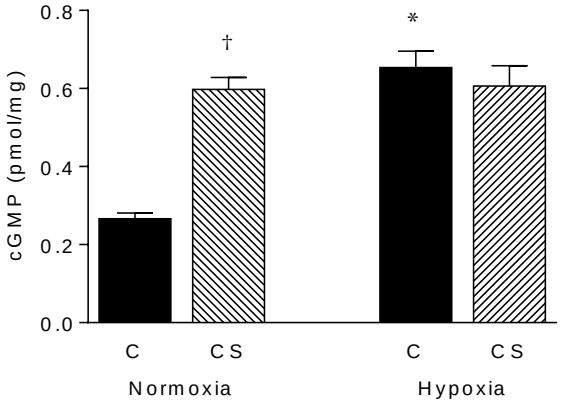

Figure 12 *Myocardial levels of cGMP in normoxic and chronically hypoxic rats untreated (C) or treated chronically with sildenafil (CS). Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding normoxic group,* † *P<0.05 vs. corresponding untreated group.*

The author of the thesis performed and analyzed all experiments on infarct size and ventricular arrhythmias determination, determination of markers of oxidative and nitrosative stress and cGMP concentration.

4.2. THE ROLE OF TNF-α IN THE CARDIOPROTECTIVE EFFECT OF CHRONIC HYPOXIA (PUBLICATION B)

The aim of this study was to characterize the role of TNF-α in myocardium of rats adapted to CH. Normoxic and chronically hypoxic rats were treated weekly with a monoclonal antibody against TNF-α, infliximab (5 mg/kg, i.p.) during the whole adaptation to CH. These animals with corresponding untreated controls were used for infarct size determination. Separated groups of animals were assigned to biochemical analyses.

Figure 13 shows that adaptation to CH improved cardiac ischemic tolerance in chronically hypoxic rats (35.5±2.4%) compared to normoxic controls (50.8±4.3%). Chronic administration of infliximab had no effect on infarct size in normoxic animals (53.0±3.9%), but weakened the infarct size-limiting effect of CH (44.9±2.0%).

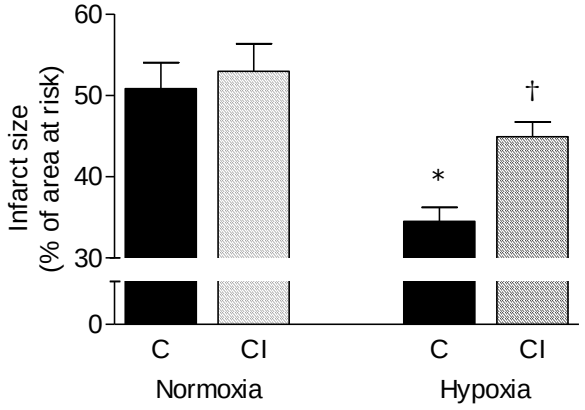

Figure 13 *Myocardial infarct size expressed as the percentage of area at risk in normoxic and chronically hypoxic rats untreated (C) or treated with infliximab (CI). Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding normoxic group,* † *P<0.05 vs. corresponding untreated group.*

Figure 14 demonstrates the increase of TNF-α content in both cytosolic and particulate fractions of LV in chronically hypoxic rats. This increase was completely inhibited by chronic infliximab treatment. This corresponded with the changes in protein level of TNF-α receptor R2 (TNFR2), whose increase caused by CH was completely inhibited by infliximab treatment. We did not observe any change in protein level of TNF-α receptor R1 (TNFR1). Moreover, CH increased myocardial expression of MnSOD and iNOS as well as levels of oxidative stress markers MDA and 3-NT by 64 - 72% compared to the normoxic values. Except for iNOS expression, chronic infliximab treatment completely eliminated these effects of CH without affecting these parameters in normoxic controls (***Figure 15***).

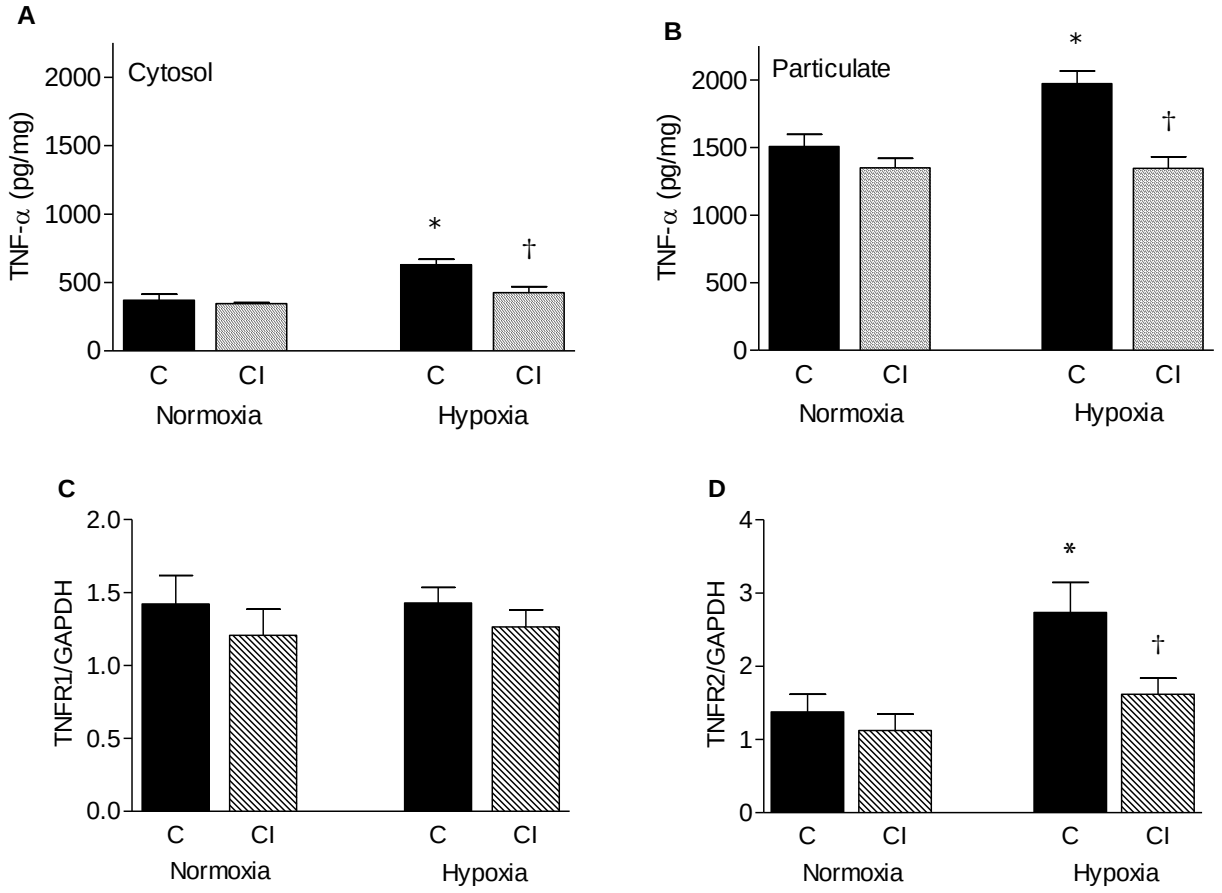

Figure 14 *Myocardial levels of TNF-α in cytosolic (A) and particulate (B) fractions of left ventricle and levels of TNF-α receptor R1 (TNFR1; C) and TNF-α receptor R2 (TNFR2; D) in normoxic and chronically hypoxic rats untreated (C) or treated with infliximab (CI). Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding normoxic group,* † *P<0.05 vs. corresponding untreated group.*

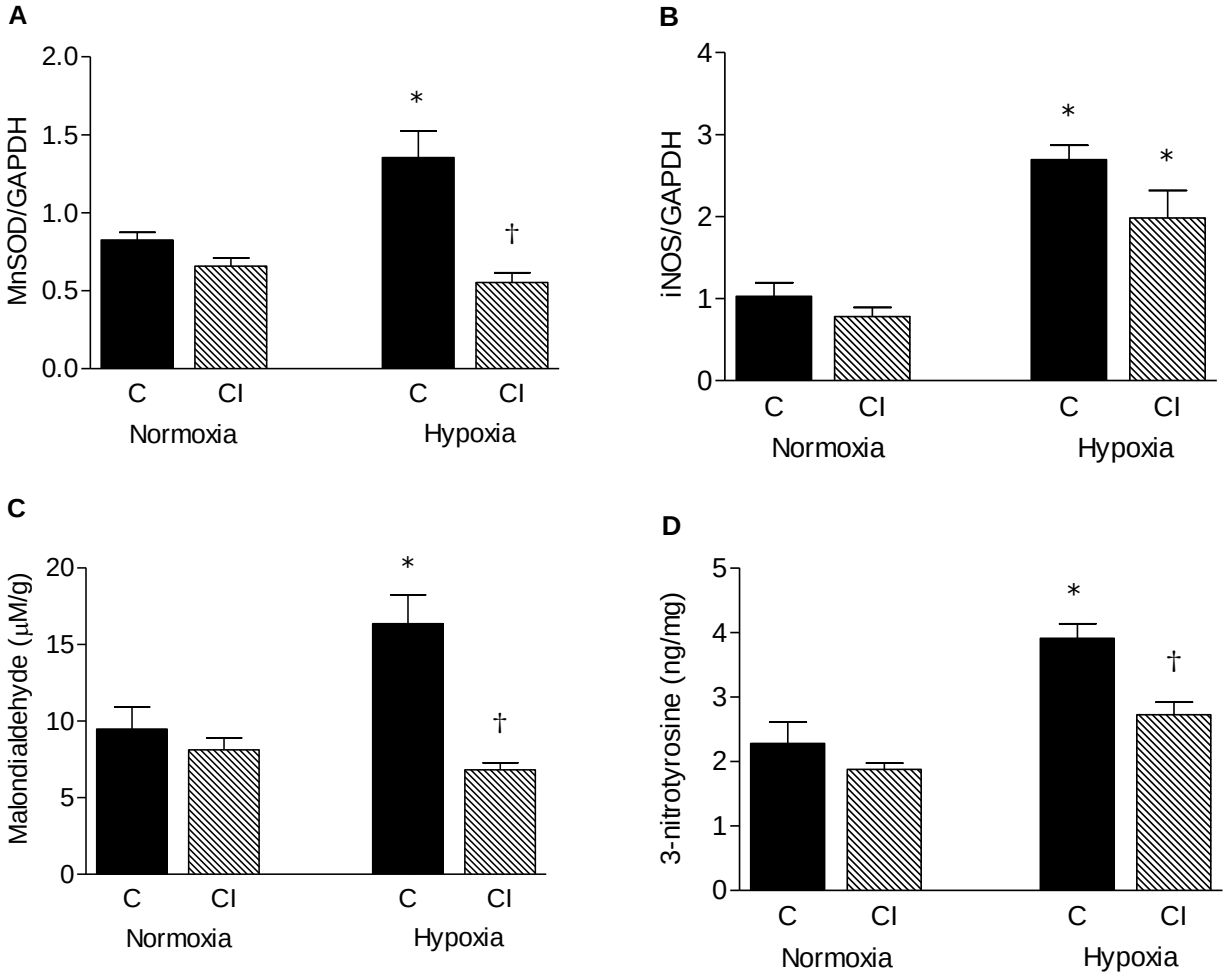

Figure 15 *Myocardial levels of mitochondrial manganese superoxide dismutase (MnSOD; A), inducible nitric oxide synthase (iNOS, B) and concentrations of malondialdehyde (C) and 3-nitrotyrosine (D) in left ventricle of normoxic and chronically hypoxic rats untreated (C) or treated with infliximab (CI). Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding normoxic group,* † *P<0.05 vs. corresponding untreated group.*

The author of the thesis performed and analyzed experiments on infarct size and ventricular arrhythmias determination and determination of markers of oxidative and nitrosative stress.

4.3. THE EFFECT OF DEXRAZOXANE ON ISCHEMIA/REPERFUSION INJURY (PUBLICATION C)

DEX is the only drug approved for the protection of myocardium from the cardiotoxicity induced by anthracycline chemotherapeutics, which is supposed to be based on ROS limitation. The aim of the study was to find out whether DEX exhibits cardioprotective

effect also against I/R injury. Myocardial infarct size and ventricular arrhythmias determination were performed in both isolated perfused hearts and open-chest rats which were administered with DEX in single doses of 50, 150 and 450 mg/kg 60 min before the induction of myocardial ischemia. Biochemical markers were assesed in additional groups of DEX-treated (150 and 450 mg/kg) and vehicle-treated rats subjected to 20 min of ischemia and 60 min of reperfusion.

In open-chest rats, normalized area at risk did not significantly differ among the experimental groups (***Figure 16A***). Only the highest dose of DEX 450 mg/kg significantly reduced the infarct size to 37.5±4.3% of the area at risk compared with the vehicle-treated controls (53.9±4.7%), while the other doses had no effect (***Figure 16B***). None of the tested doses affected the values of ischemic (***Figure 16C***) or reperfusion arrhythmia score (***Figure 16D***).

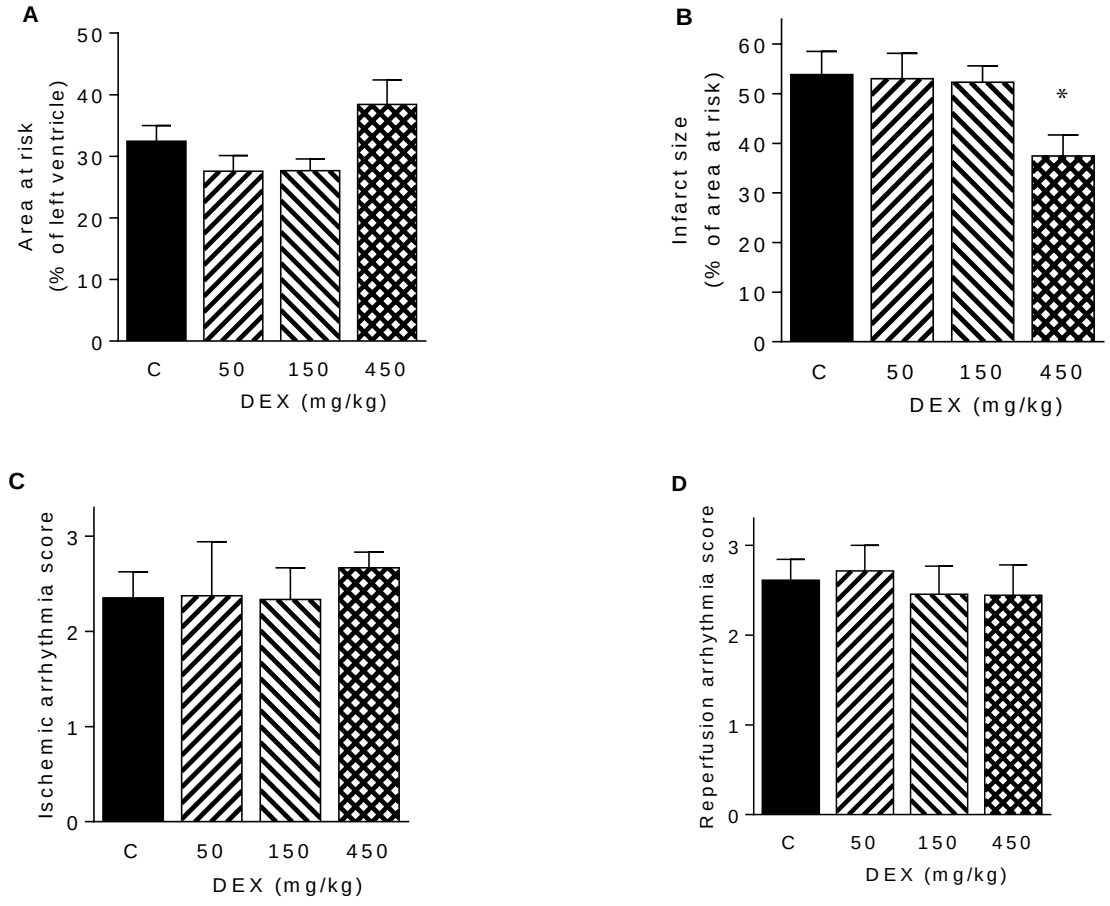

Figure 16 *Area at risk expressed as the percentage of LV size (A), myocardial infarct size expressed as the percentage of area at risk (B), ventricular arrhythmia score during ischemia (C) and ventricular arrhythmia score during early reperfusion (D) in open-chest control (C) and dexrazoxane (DEX)-treated rats. Data are expressed as mean±SEM;* * *P<0.05 vs. C.*

In isolated perfused hearts, arrhythmia score during ischemia did not differ among the groups (***Figure 17A***). Although the dose of 150 mg/kg tended to decrease both the total number of ischemic premature ventricular complexes (***Figure 17B***) and the duration of ischemic tachyarrhythmias (***Figure 17C***), the same dose significantly reduced arrhythmia score during early reperfusion (***Figure 17D***). ***Figure 18*** shows the myocardial GSH/GSSG ratio as well as myocardial level of MDA, expressed as a percentage of vehicle-treated controls. I/R injury decreased GSH/GSSG ratio by 36% and increased the concentration of MDA by 40%. DEX treatment did not significantly affect these markers of oxidative stress.

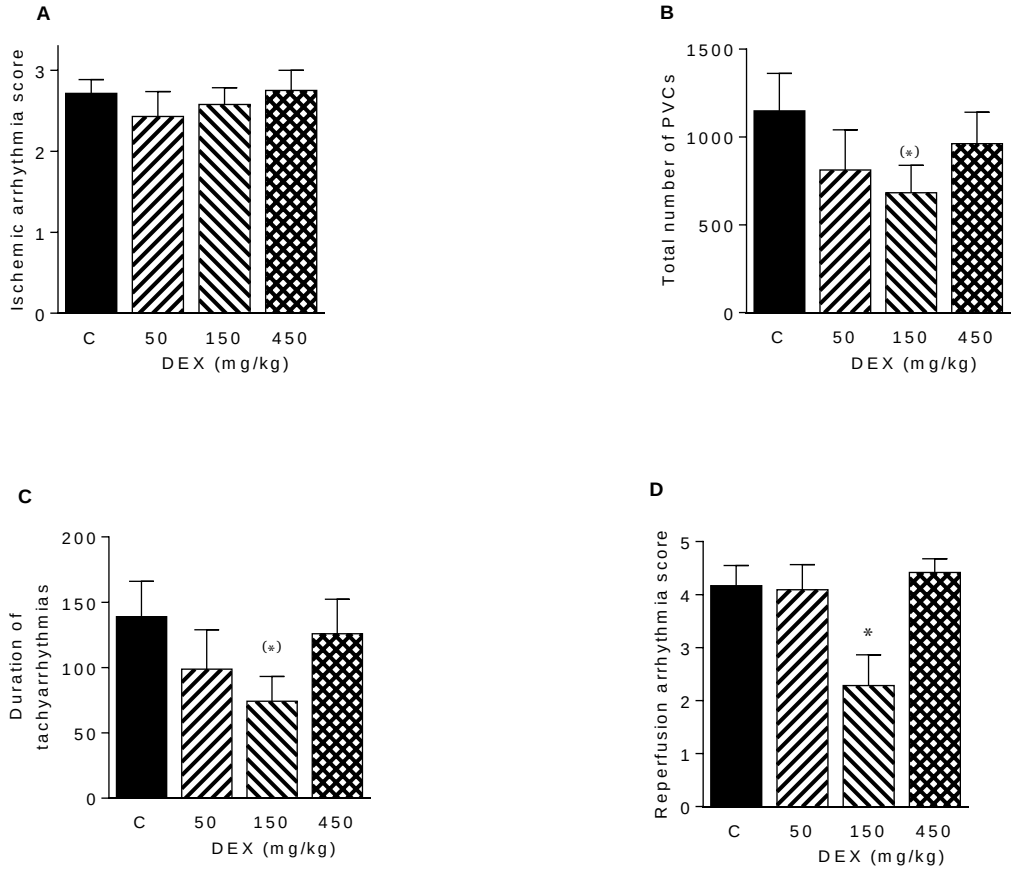

Figure 17 *Ventricular arrhythmia score during ischemia (A), total number of premature ventricular complexes during ischemia (PVCs, B), duration of tachyarrhythmias during ischemia (C), ventricular arrhythmia score during reperfusion (D) in isolated perfused hearts of control (C) and dexrazoxane (DEX)-treated rats. Data are expressed as mean±SEM;* * *P<0.05 vs. C,* $^{(*)}$ *P<0.1 vs. C.*

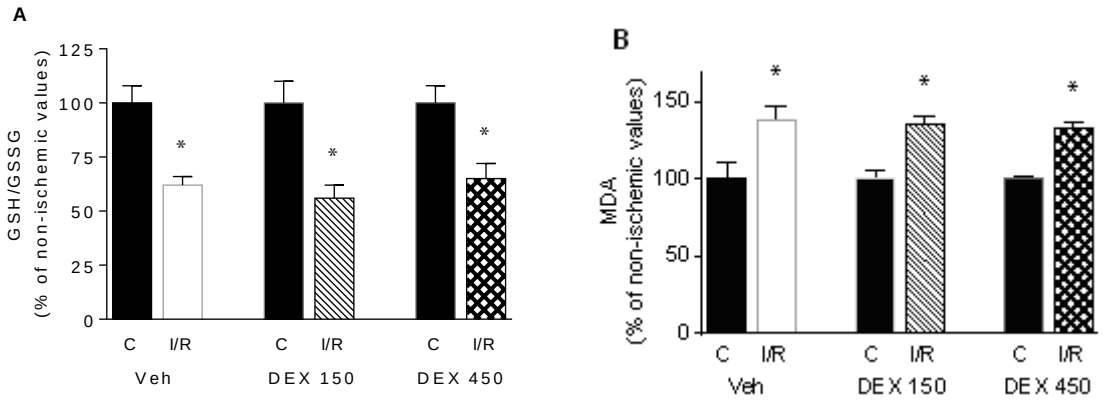

Figure 18 *Myocardial reduced to oxidized glutathione ratio (GSH/GSSG, A) and myocardial concentration of malondialdehyde (MDA, B) in open-chest control (Veh) and dexrazoxane (DEX)-treated rats. Effects of ischemia and reperfusion (I/R) are expressed as a percentage of corresponding non-ischemic (C) values. Data are expressed as mean±SEM;* * *P<0.05 vs. C.*

The author of the thesis performed and analyzed experiments on infarct size and ventricular arrhythmias determination in open-chest animals and determination of markers of oxidative stress.

4.4. THE EFFECT OF EPOXYEICOSATRIENOIC ACID ANALOG ON ISCHEMIA/REPERFUSION INJURY (PUBLICATION D)

The present study was undertaken to evaluate the effects of treatment with EET analog (EET-A) on blood pressure and myocardial infarct size in 2K1C Goldblatt hypertensive rats in sustained phase of hypertension. Adult male HanSD rats underwent a placement of right renal artery clip, while sham-operated animals were used as controls. Treatment with EET-A (10 mg/kg/day in drinking water) started on day 31 after clipping and continued for 4 days. Rats were subjected to I/R injury for infarct size determination. In paralel subgroups, renal EETs/DHETs ratio and myocardial expression of Akt kinase and glycogen synthase-3β (GSK-3β) were measured.

Figure 19 shows that systolic blood pressure in sham-operated rats remained within the normotensive range (from 119±2 to 115±3 mmHg) and the EET-A treatment had no effect. On the contrary, 2K1C rats developed high blood pressure (170±3 mmHg) and EET-A did not alter their systolic blood pressure. As shown in ***Figure 20***, untreated 2K1C rats exhibited severe LV hypertrophy as compared with untreated sham-operated rats (28.92±0.61 vs. 23.08±0.61), which was not altered by the treatment with EET-A.

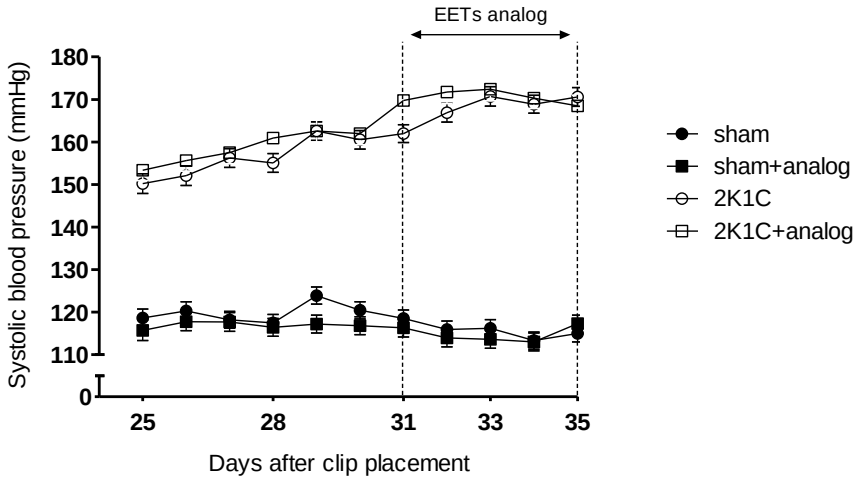

Figure 19 *Systolic blood pressure in sham-operated and 2K1C rats untreated or treated with EET analog. Data are expressed as mean±SEM.*

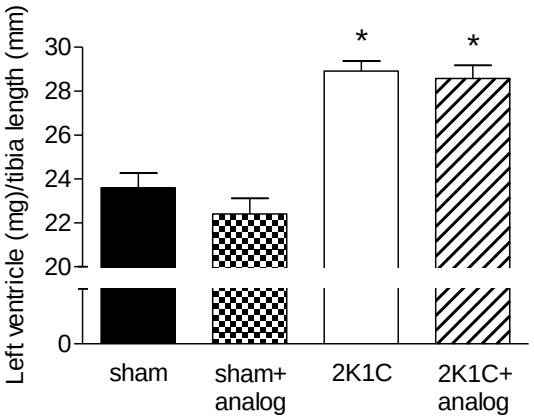

Figure 20 *Left ventricular hypertrophy expressed as left ventricular weight to tibia length ratio in sham-operated and 2K1C rats untreated or treated with EET analog. Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding sham-operated group.*

As shown in ***Figure 21***, the intrarenal availability of biologically active epoxygenase metabolites expressed as the ratio of biologically active EETs to almost inactive DHETs was significantly lower in the nonclipped kidneys of 2K1C rats compared with sham-operated rats, and treatment with EET-A did not alter it in either group. ***Figure 22*** shows that myocardial infarct size normalized to the area at risk was significantly lower in untreated 2K1C rats as compared with untreated sham-operated rats (23.2±3.4 vs. 46.1±3.9%). Treatment with EET-A did not alter the infarct size in 2K1C, but significantly diminished it in sham-operated rats (to 29.9±2.9%). As shown in ***Figure 23***, there were no differences between sham-operated and 2K1C rats when protein levels of Akt kinase and GSK-3β were expressed as the ratio of phosphorylated vs. unphosphorylated fraction, and the treatment with EET-A did not alter protein expression in any experimental group.

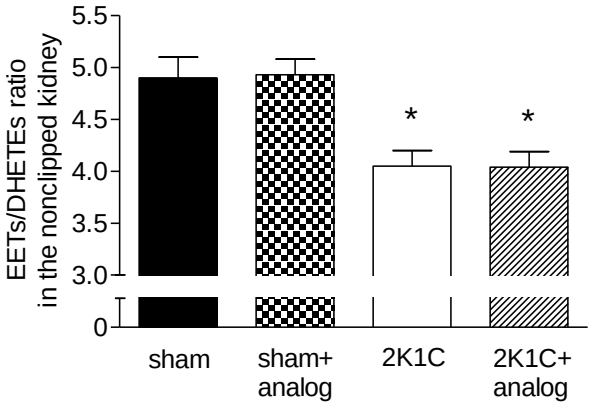

Figure 21 *Nonclipped kidney EETs/DHETs ratio in sham-operated and 2K1C rats untreated or treated with EET analog. Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding sham-operated group.*

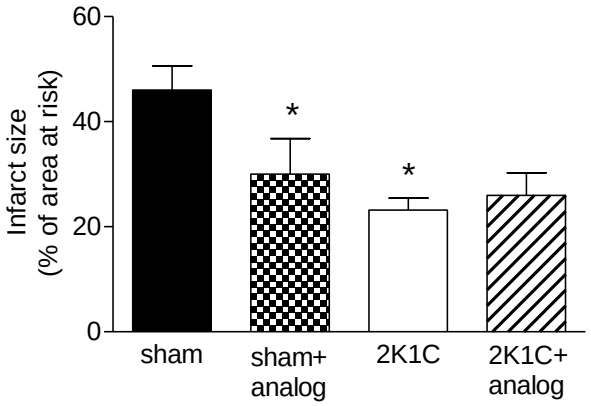

Figure 22 *Myocardial infarct size expressed as a percentage of area at risk in sham-operated and 2K1C rats untreated or treated with EET analog. Data are expressed as mean±SEM;* * *P<0.05 vs. corresponding sham-operated group.*

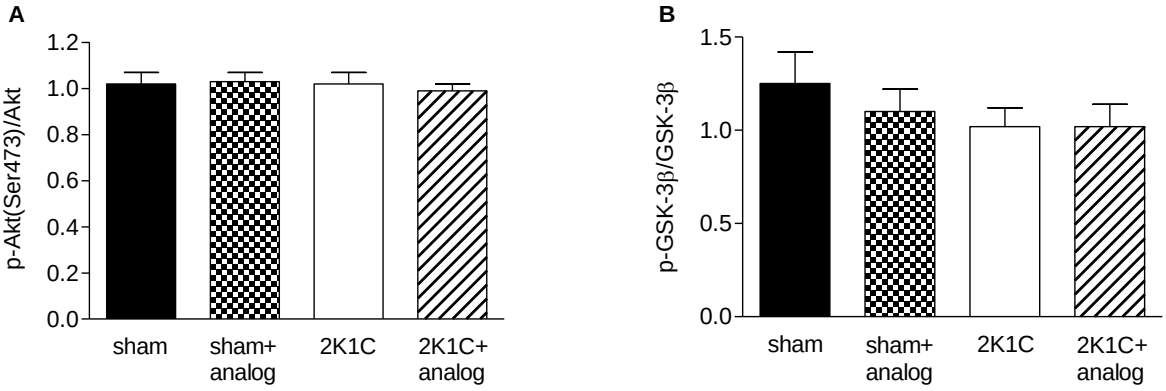

Figure 23 *Left ventricle myocardial protein expression of Akt kinase (A) and glycogen synthase 3β (B) in sham-operated and 2K1C rats untreated or treated with EET analog. Data are expressed as mean±SEM.*

The author of the thesis performed and analyzed all experiments on infarct size and ventricular arrhythmias determination and western blot analyses.

5. DISCUSSION

Our experiments tried to contribute to the clarification of possible mechanisms which could defend the heart against I/R injury. From all above mentioned cardioprotective phenomena, we chose three - adaptation to chronic continuous hypoxia, acute DEX pretreatment before the ischemic insult and chronic treatment with EET-A. The effectiveness of therapeutic strategies to reduce infarct size was investigated in adult male Wistar or HanSD rats.

Role of NO/cGMP pathway in the cardioprotective effect of chronic hypoxia

The first major result was that NO/cGMP pathway is included in the acute phase of cardioprotective mechanism of CH. When studying the role of NO/cGMP pathway in the cardioprotective effect of adaptation to CH, we decided to compare the affection of two different phases of NO/cGMP signaling pathway - increase in NO by exogenous supply and increase in cGMP by PDE-5 inhibition.

We confirmed that adaptation to CH improves cardiac ischemic tolerance (Asemu et al., 2000; Neckář et al., 2004; Wang et al., 2011; Maslov et al., 2014). Acute administration of molsidomine or sildenafil decreased infarct size in both normoxic and chronically hypoxic rats. We showed more profound infarct size-lowering effect in rats adapted to CH without an additive result of CH and drugs administration. Therefore, we assume that NO/cGMP signaling pathway is included in the acute phase of cardioprotection induced by CH.

The second major result was that chronically increased availability of cGMP by sildenafil treatment reduced myocardial infarction in normoxic rats and potentiated cardioprotective effect of CH. Therefore, it seems that signaling via cGMP does not play a key role in the induction of cardioprotection induced by CH.

Adaptation to CH was associated with oxidative and nitrosative stress verified by the increased levels of MDA, 3-NT and decreased GSH/GSSG and nitrite/nitrate ratio. Our data concerning CH-induced oxidative stress are in agreement with the results by Ashmore et al. (2014) or Santos et al. (2011). ROS as well as NO seem to be an integral part of adaptive changes of chronically hypoxic hearts.

CH was associated also with the development of PH. On the contrary to other studies (Andersen et al., 2005; Zhao et al., 2001; Weissmann et al., 2007), chronic treatment with sildenafil was not able to prevent the increase in RVSP followed by RV hypertrophy. Sildenafil is an established therapeutic option, albeit with limited effects. Preston et al. (2004) showed that sildenafil normalized hypoxia-induced increased RVSP without affecting RV

hypertrophy or pulmonary vascular remodeling. The effect of sildenafil on vascular remodeling seems to be dose-dependent. We used dosage according to Milano et al. (2011), which successfully attenuated hypoxia-induced increase in myocardial hypertrophy. This dose was not able to prevent vascular remodeling in our conditions. The discrepancy could be explained by different experimental protocols used. The positive results on PH treated with sildenafil were obtained after two weeks of adaptation in comparison to our results obtained after 4 weeks of adaptation in the period of sustained hypoxia. According to Samillan et al. (2013), PDE-5 inhibitors are able to improve hemodynamic parameters, however, monotherapeutic onsets are still insufficient. In clinical practice, the PDE inhibitors are used most often in combination with other drugs in treatment of PH, in combination with prostacycline analogs (Itoh et al., 2004), in combination with endothelin receptor A inhibitors (Mouchaers et al., 2009). Every used combination has a moderate potentiation effect in decreasing the pulmonary artery pressure compared to the PDE inhibitor alone.

Role of TNF-α in the cardioprotective effect of chronic hypoxia

In our experiments, adaptation to CH was accompanied by the increased myocardial concentration of TNF-α and its receptor TNFR2. This finding is in agreement with the increased expression of TNF-α and proinflammatory genes in hearts of chronically hypoxic adult rats or fetal guinea pigs (Chen et al., 2007; Oh et al., 2008; Klusoňová et al., 2009). TNF-α is generated as a precursor called transmembrane TNF-α, which is cleaved to the secreted form of TNF-α that mediates its action through receptors TNFR1 and TNFR2 (Vandenabeele et al., 1995). The activation of TNF-α receptor-specific response was shown as an important event in cardiac ischemic tolerance. While an excessive TNF-α expression and subsequent TNFR1 activation are deleterious, a lower TNF-α concentration and TNFR2 activation are protective (Flaherty et al., 2008; Lacerda et al., 2009). Our results revealed increased expression of TNFR2 but not TNFR1 in LV of rats adapted to CH. Moreover, chronic treatment with infliximab abolished the increased TNFR2 level and blunted infarct size-limiting effect of CH. These data suggest that CH improved cardiac ischemic tolerance of rat hearts by activation of protective TNFR2 signaling, but had no effect on detrimental signaling mediated by TNFR1. Not only secreted TNF-α but also its transmembrane form exerts biological actions (Horiuchi et al., 2010). Transmembrane TNF-α mediates its biological activities through TNFR2 (Grell et al., 1995) which is the key receptor for beneficial role of TNF-α in cardiac I/R injury. With this background, we analysed the expression of TNF-α in both cytosolic and particulate fractions of LV in normoxic and

chronically hypoxic rats. CH increased TNF-α levels equally in both fractions, therefore, we can not decide whether it is transmembrane or secreted form of TNF-α, which contributes to the cardioprotective phenotype of CH.

It has been suggested that ROS play an important role in the cell survival and death triggered by TNF-α signaling. The main sources of TNF-α-induced ROS generation are mitochondria (Kim et al., 2010), where MnSOD is the dominant antioxidative enzyme. We found that adaptation to CH increased myocardial oxidative stress and induced overexpression of MnSOD that were abolished by infliximab treatment. Therefore, it seems that TNF-α contributes to the improved cardiac ischemic tolerance via activation of protective redox signaling with increased antioxidant defence.

Adaptation to CH was associated also with the increased expression of iNOS. However, chronic infliximab treatment only slightly reduced iNOS expression in chronically hypoxic rats. On contrary to many studies demonstrating the important role of NO in PC (Andrukhiv et al., 2006) or CH (Shi et al., 2000), this result showed that NO signaling does not play a key role in induction of cardioprotective signaling during adaptation to CH, as was metioned above.

The effect of dexrazoxane on ischemia/reperfusion injury

The major result of the study with DEX was that a narrow dose range of DEX could suppress arrhythmias in isolated perfused rat hearts subjected to I/R injury; on the other hand, a higher dose was necessary for a limitation of myocardial infarct size in open-chest rats. To the best of our knowledge, the infarct size and the susceptibility to ischemia and reperfusion arrhythmias in DEX-treated animals were assessed for the first time. We found that a single dose of 150 mg/kg effectively supressed ventricular arrhythmias in isolated perfused hearts, but it was insufficient to reduce arrhythmias in open-chest animals. The reason for the different effect of DEX on arrhythmias in open-chest rats and in isolated perfused hearts is not clear. Asemu et al. (2000) reported also a distinct effect of CH on susceptibility of rat hearts to ischemia-induced arrhythmogenesis assessed under *in vitro* and *in vivo* conditions. The cause is unknown, but the presence of blood components and neurohumoral control mechanisms in open-chest rat may participate on this discrepancy. On the other hand, only the highest dose of DEX significantly reduced infarct size in open-chest rats. Our observation of the infarct-size limiting effect of DEX is in line with results of Ramu et al. (2006), who showed improved postischemic recovery of contractile function in isolated perfused rat hearts subjected to global I/R injury after DEX-pretreatment. Hasinoff (2002) detected lower lactate

dehydrogenase release from DEX-treated neonatal cardiac myocytes exposed to anoxia-reoxygenation.

Based on current knowledge, both anthracycline-induced cardiotoxicity and I/R injury seem to share the involvement of ROS with a supposed catalytic role of free iron. Hence, intracellular iron chelation can be considered as a promising approach. In the present study, we determined two different markers of oxidative stress: GSH/GSSG and MDA. The aim was to test the hypothesis that the protective effects of DEX were associated with the decreased ROS formation during I/R insult. However, none of these markers showed any significant effect of DEX treatment what strongly argues with generally accepted mechanism of action. This result was later confirmed by Štěrba et al. (2013), who demonstrated that stronger and more selective iron chelators failed to provide better or at least the comparable cardioprotection as DEX in chronic anthracycline cardiotoxicity models. It suggests that other protective mechanism than limitation of ROS formation might play a role.

The effect of epoxyeicosatrienoic acid analog on ischemia/reperfusion injury

The first major finding of the study was that the size of the myocardial infarction induced by acute I/R insult was significantly smaller in 2K1C Goldblatt hypertensive rats than in sham-operated normotensive controls. This finding contradicts generally accepted view that the hypertrophic myocardium shows decreased tolerance to I/R injury. However, our findings are not exceptional; it has been observed that hypertensive animals with LV hypertrophy exhibit either comparable or reduced infarct size (Mozaffari and Schaffer, 2003; Saupe et al., 2000; Matsuhisa et al., 2008). Neckář et al. (2012) recently showed that Ren-2 transgenic hypertensive rats exhibited higher myocardial resistance to I/R injury as compared with normotensive rats. It is obvious that the influence of hypertension on cardiac tolerance to I/R injury has not been completely understood until now. It could depend on the phase of hypertension or it may activate a protective mechanism. EET-A treatment significantly reduced infarct size in normotensive sham-operated rats. Chronic treatment with EET-A did not reduce myocardial infarct size in 2K1C Goldblatt hypertensive rats suggesting that 2K1C rats already exhibited the maximal activation of cardioprotective signaling.

The second major result of the study with EETs was that in the sustained phase of 2K1C Goldblatt hypertension, EET-A treatment did not decrease blood pressure and did not attenuate cardiac hypertrophy in 2K1C Goldblatt hypertensive rats. These unexpected findings are not easy to explain, because recent studies have clearly shown that increased availability of EETs in the kidney of animals with angiotensin II-dependent hypertension is

associated with improvement of the impaired renal autoregulatory capacity and blunted pressure-natriuresis relationship (Elmarakby, 2012, Sporková et al., 2011). Moreover, 2K1C hypertensive animals in the sustained phase of hypertension were shown to have a deficit of EETs in the nonclipped kidney as a consequence of enhanced sEH activity and their increased conversion to DHETs (Sporková et al., 2011; Kopkan et al., 2012). Based on these findings, it was proposed that the reduced intrarenal bioavailability of EETs contributes to the angiotensin II-induced derangement of the pressure-natriuresis relationship in the nonclipped kidney of 2K1C hypertensive rats; it plays also a crucial role in the pathophysiology of sustained hypertension. It was reported that in Ren-2 transgenic rats, chronic sEH inhibition lead to the marked elevation of intrarenal EETs levels (Varcabová et al., 2013). Considering the above evidence, it is difficult to explain why chronic EET-A treatment did not lower blood pressure in 2K1C Goldblatt hypertensive rats. This failure can not be ascribed to the low dosage of EET-A, because in a recent study the same dose proved to be effective (Imig et al., 2010). The possible explanation might be that, in contrast to sEH inhibition, EET-A treatment did not normalize the availability of endogenous intrarenal EETs (Sporková et al., 2011). We assumed that the decreased intrarenal EETs bioavailability in 2K1C rats was accompanied by the lack of antihypertensive effect.

In conclusion, all three potential cardioprotective interventions studied were able to defend the myocardium against I/R injury. Adaptation to CH was associated with the oxidative stress as well as cardioprotective effect of DEX pretreatment was not mediated by ROS limitation. It suggests a beneficial role of ROS in signaling leading to cardioprotection. Furthermore, we confirmed involvement of NO/cGMP pathway as well as TNF-α in the CH-induced cardioprotective signaling. While EET-A provided cardioprotective action only in normotensive rats; surprisingly, 2K1C Goldblatt hypertensive rats exhibited increased cardiac tolerance to I/R injury even without EET-A treatment, suggesting already activated cardioprotective signaling.

6. SUMMARY

a) NO/cGMP signaling pathway plays a role in the acute phase of cardioprotection induced by CH. The acute preischemic treatment with NO donor molsidomine and PDE-5 inhibitor sildenafil enhanced cardiac ischemic tolerance not only in normoxic but also in chronically hypoxic rats. Chronic sildenafil treatment was able to provide an additional protection in chronically hypoxic rats.

b) Adaptation to CH exhibited the increased levels of TNF-α in both cytosolic and particulate fractions of LV myocardium. Moreover, an increased expression of receptor TNFR2 without change in TNFR1 suggests that TNF-α contributes to the improved cardiac ischemic tolerance of chronically hypoxic rats via its receptor TNFR2 and increased oxidative stress.

c) DEX exhibited significant protective effect against reperfusion-induced arrhythmias in isolated perfused hearts with the mild dose of DEX. In open-chest animals, the high dose of DEX significantly reduced infarct size. According to the unchanged markers of oxidative stress, we suggest that DEX-induced protective mechanism is not mediated by limitation of ROS formation.

d) 2K1C Goldblatt hypertensive rats during the sustained phase of hypertension exhibited increased cardiac tolerance to I/R injury as compared to the normotensive controls. Treatment with EET-A did not induce any antihypertensive and cardioprotective actions in this animal model of human renovascular hypertension; however, it reduced infarct size in normotensive animals.

7. REFERENCES

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8. SUPPLEMENTS

Thesis is based on the following publications:

Publication A: P. Alánová, F. Kolář, B. Ošťádal, J. Neckář: Role of NO/cGMP pathway in cardiac ischemic tolerance of chronically hypoxic rats. Physiol Res 2015 (in press)

Publication B: A. Chytilová, GH Borchert, P. Mandíková-Alánová, AH Khan, JD Imig, F. Kolář, J. Neckář: TNF-α contributes to improved cardiac ischemic tolerance in rats adapted to chronic continuous hypoxia. Acta Physiol 214(1): 97 - 108, 2015.

Publication C: J. Neckář, A. Boudíková, P. Mandíková, M. Štěrba, O. Popelová, I. Mikšík, L. Dabrowská, J. Mráz, V. Geršl, F. Kolář: Protective effects of dexrazoxane against acute ischaemia/reperfusion injury of rat hearts. Can J Physiol Pharmacol 90: 1303 - 1310, 2012.

Publication D: P. Alánová, Z. Husková, L. Kopkan, A. Sporková, Š. Jíchová, J. Neckář, JD Imig, M. Klevstig, F. Kolář, NR Reddy, JR Falck, J Sadowski, HJ Kramer, L. Červenka: Orally active epoxyeicosatrienoic acid analog does not exhibit antihypertensive and reno- or cardioprotective actions in two-kidney, one-clip Goldblatt hypertensive rats. (under revision in Vascular Pharmacology)

Other publication:

T. Ravingerová, S. Čarnická, V. Ledvényiová, E. Barlaka, E. Galatou, A. Chytilová, P. Mandíková, M. Němčeková, A. Adameová, F. Kolář, A. Lazou: Upregulation of genes involved in cardiac metabolism enhances myocardial resistance to ischemia/reperfusion in the rat heart. Physiol Res 62 (Suppl. 1): S151 - S163, 2013.