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Antioxidant system in hypoxic heart

Antioxidační systém v hypoxickém srdci

Ph.D. Thesis

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Declaration

I hereby declare that the work presented in this thesis is my own, except where explicitly stated otherwise, and was carried out under the supervision of doc. RNDr. Jitka Žurmanová, Ph.D. I declare that I am the author of the thesis text and that neither this work or its substantial parts were previously submitted to obtain any academic degree. I acknowledged and properly cited all used resources.

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Dita Sotáková, MSc.

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Abstract

The cardiovascular disease, particularly acute myocardial infarction, is the most common cause of death worldwide. It is well documented that adaptation to chronic hypoxia increases resistance to ischemia-reperfusion (I/R) injury in heart tissue. Reactive oxygen species (ROS) play an important signalling role by the activation of the protective pathways during I/R, although, the excess of ROS during reperfusion leads to cardiac tissue injury. As the cellular antioxidant system is responsible for the maintenance of redox homeostasis, the main aim of this thesis was to investigate the relationship between myocardial tolerance to I/R injury and regulation of main components of antioxidant systems, related transcription factors and their target genes in protective and non-protective regimens of chronic hypoxia. We found differences in cardioprotective phenotype in rats exposed to three regimens of chronic normobaric hypoxia (Fi_{O2} 0.1, 3 weeks). The adaptation to continual (CNH) and intermittent (CNH-8; 8 h/day) regimen of hypoxia increased myocardial resistance to I/R damage, whereas 1-hour daily interruption of hypoxic adaptation (INH-23) abolished cardioprotective effect and decreased the ratio of reduced and oxidised glutathione (GSH/GSSG). Both cardioprotective regimens significantly increased mRNA expression of mitochondrial antioxidants (manganese superoxide dismutase, *MnSod*; glutathione reductase, *Gsr*; thioredoxin, *Txn2*; thioredoxin reductase, *Txnrd2*) and cytosolic isoform of peroxiredoxin (*Prx2*). Contrary to that, INH-23 increased only *Prx5*, which was not sufficient to induce cardioprotective phenotype. We also analysed cardioprotective regimen of severe intermittent hypobaric hypoxia (IHH-8; 7,000 m, 8h/day, 5 weeks) which surprisingly did not stimulate most of antioxidant enzymes. Only antioxidants related to the metabolism of iron and TXN1 were elevated. Interestingly, we observed activation of pro-inflammatory transcription factors and cytokines.

We can conclude that antioxidants associated with mitochondria contribute to the modulation of the cardioprotective phenotype conferred by adaptation to chronic well tolerable normobaric hypoxia. On the other hand, the antioxidant system is not able to compensate oxidative stress induced by severe hypobaric hypoxia. Therefore, other signalling pathways promoting myocardial protection against I/R injury are activated.

Abstrakt

Kardiovaskulární onemocnění, zejména akutní infarkt myokardu, je celosvětově nejčastější příčinou úmrtí. Je známo, že adaptace na chronickou hypoxii zvyšuje odolnost srdeční tkáně vůči ischemicko-reperfuznímu (I/R) poškození. Reaktivní formy kyslíku (ROS) hrají důležitou signální roli při aktivaci protektivních drah během I/R, ačkoli přebytek ROS během reperfúze vede k poškození srdeční tkáně. Vzhledem k tomu, že buněčný antioxidační systém je zodpovědný za udržování redoxní homeostázy, bylo cílem této práce analyzovat vztah mezi tolerancí myokardu k I/R poškození a regulací hlavních složek antioxidačních systémů, souvisejících transkripčních faktorů a jejich cílových genů v protektivních a neprotektivních režimech chronické hypoxie. Prokázali jsme rozdíly v kardioprotektivním fenotypu u potkanů vystavených třem režimům chronické hypoxie (FiO_2 0,1; 3 týdny). Adaptace na kontinuální (CNH) a intermitentní (CNH-8; 8h/den) režim hypoxie zvýšila rezistenci myokardu k I/R poškození, zatímco 1-hodinové denní přerušení hypoxické adaptace (INH-23) zrušilo kardioprotektivní účinek a snížilo poměr redukovaného a oxidovaného glutathionu (GSH/GSSG). Oba kardioprotektivní režimy významně zvýšily expresi mRNA mitochondriálních antioxidantů (manganová superoxid dismutáza, *MnSod*; glutathion reduktáza, *Gsr*; thioredoxin, *Txn2*; thioredoxin reduktáza, *Txnrd2*) a cytosolické isoformy peroxiredoxinu (*Prx2*). Naproti tomu INH-23 zvýšil pouze *Prx5*, což nebylo dostatečné k indukci kardioprotektivního fenotypu. Analyzovali jsme také kardioprotektivní režim silné intermitentní hypobarické hypoxie (IHH-8; 7 000 m, 8 h/den, 5 týdnů), která překvapivě nestimulovala většinu antioxidačních enzymů. Zvýšené byly pouze antioxidanty související s metabolismem železa a TXN1. Zajímavým zjištěním bylo, že došlo k aktivaci prozánětlivých transkripčních faktorů a cytokinů.

Můžeme konstatovat, že antioxidanty spojené s mitochondriemi přispívají k modulaci kardioprotektivního fenotypu způsobeného adaptací na chronickou dobře tolerovatelnou normobarickou hypoxii. Na druhé straně, antioxidační systém není schopen kompenzovat oxidační stres vyvolaný silnou intermitentní hypobarickou hypoxií. Dochází tedy k aktivaci jiných signálních drah navozujících ochranu myokardu před I/R poškozením.

Contents

1. Introduction

1.1. Ischemia-reperfusion injury

Recent analyses of the World Health Organization (WHO) show that cardiovascular disease (CVD) is the most significant global cause of death (31% of total deaths). Of this, 85% were coronary artery disease. Its acute form, myocardial infarction comprised of 40%, and the rest is related to ischemic stroke (WHO, 2017). It is therefore not surprising that a great attention is being paid on how to increase myocardial resistance to ischemic insult and finding the molecular basis of the mechanisms responsible for protecting cardiac tissue.

Acute occlusion of the coronary artery, or its narrowing, reduces the blood flow in the affected area of the myocardium. Development of the ischemic damage depends primarily on the duration of the ischemia, resulting in reversible or irreversible damage to the affected area (Hearse, 1979).

Restriction of the blood flow during ischemia is associated with accumulation of metabolites beside restricted supply of substrates (reviewed in Kalogeris *et al.*, 2016). At the early stage of ischemia, attenuated mitochondrial ATP production is displaced by stimulated glycolysis, which may partially supplement ATP deficiency and maintain ATP/ADP ratio (reviewed in Li *et al.*, 2016). In the later phase, cellular defence mechanisms responding to the declining levels of oxygen and nutrients are triggered, predominantly by attenuating contractile function via ATP-sensitive K^+ channel (K_{ATP}). Thus, cardiomyocytes may conserve energy and adapt their metabolism to an emergency mode (Depre and Vatner, 2005). More than 50 years ago it was pointed that a sudden reperfusion, e.g. recovery of the oxygenated blood supply by the coronary system, causes increased myocardial necrosis (Jennings *et al.*, 1960). The evidence of an increased tissue damage following reperfusion phase accumulated (McCord, 1985; Zweier *et al.*, 1987) and contributed to the establishment of the term ischemia-reperfusion (I/R) injury. This phenomenon has attracted a great interest of the research community to this day, as evidenced by the rapid growth in publishing activity over the past 20 years focusing on the processes taking a place during the reperfusion phase (reviewed in Granger and Kvietys 2015). The mechanism lay in a complex and multifactorial interplay of metabolic changes within the cell that occur during restored blood flow (Raedschelders *et al.*, 2012).

One of the important principles of I/R injury is increased production of reactive oxygen species (ROS) and the subsequent development of oxidative stress, which attenuates mitochondrial ATP production (reviewed in Di Lisa and Bernardi, 2006). Also, growing sodium and calcium overload and increasing probability of apoptosis activation are targets of a great interest (reviewed in Hausenloy and Yellon, 2013).

In the late 80's, the elevated production of ROS and nitrogen species were measured after ischemia and reperfusion in various animal models (Garlick *et al.*, 1987; Zweier *et al.*, 1987; Bolli *et al.*, 1988). Extreme ROS production based on molecular oxygen supply during reperfusion phase is generally described as an oxygen paradox (reviewed in Raedschelders *et al.*, 2012).

1.2. The role of ROS in myocardial I/R injury and protective signalling

ROS are highly reactive oxygen-related forms produced by different sources. Previously, they have been described as a detrimental by-product of aerobic metabolism leading to macroscopic tissue damage. Recently, ROS have been recognised as an important part of signalling related to redox sensitive transcription factors. Importantly, overproduction of ROS has been accounted for underlying many pathological changes and diseases such as inflammation, cancer, cardiovascular disease and ageing related processes (Valko *et al.*, 2007).

Incomplete reduction of oxygen may occur, when only one electron is used, and it leads to the production of superoxide anion ($O_2^{\bullet -}$). Through subsequent spontaneous or superoxide dismutase (SOD)-catalysed reaction, superoxide anions are converted to H_2O_2, which may diffuse through the membranes and serve as a trigger of the signalling.

During the ischemia, oxygen supply is interrupted and subsequently restored during reperfusion leading to over generation of ROS mediated mainly by enzymatic source such as xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX). Also, ROS can be generated non-enzymatically, predominantly in respiratory chain in mitochondria (reviewed in Granger and Kvietys, 2015).

Xanthine oxidoreductase (XOR) is an enzymatic complex that catalyses the catabolic oxidation of hypoxanthine to xanthine and controls the hydroxylation of xanthine to uric acid. It consists

of two forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). The XDH preferably uses NAD^+ as an acceptor of electrons yielding NADH, while XO uses O_2 as the electron acceptor thereby exhibiting ability to generate ROS in form of cellular $O_2^{\bullet-}$. Importantly, during ischemia, XDH can be converted to XO by the protein modifications (Friedl *et al.*, 1990). The XO catalyses the conversion of hypoxanthine to uric acid, but it uses molecular oxygen as electron acceptor (George and Struthers, 2009). Insufficient oxidative phosphorylation during ischemia causes ATP degradation and accumulation of its metabolic intermediate product, hypoxanthine, which serves as a substrate for XO when O_2 level is restored at reperfusion, thus causing ROS generation (Tsuda *et al.*, 2012). Despite of reports describing a beneficial effect of XO inhibitors in different post-ischemic tissues, it seems that this approach failed in the heart tissue (Downey *et al.*, 1987). However, high level of XOR was detected by immunohistochemistry staining in vascular endothelium (Vickers *et al.*, 1998). Superoxide released during I/R results in the recruitment and activation of neutrophils and their adherence to endothelial cells (Bonaventura *et al.*, 2016). This stimulates the formation of XO in the endothelium, with further $O_2^{\bullet-}$ production (Houston *et al.*, 1999).

The large membrane-bound enzyme, NOX, catalyses production of $O_2^{\bullet-}$ by transferring one electron to the oxygen from NADPH. There are seven known isoforms of the NOX family, from which NOX1, NOX2 and NOX4 are expressed in the cardiomyocytes (Bedard and Krause, 2007). Appropriate regulation of ROS production by NOX, also called oxidative burst, is essential for the elimination of microorganisms in macrophages and neutrophils and thereby ROS serves as an inflammatory mediator and redox-signalling molecules (Franchini *et al.*, 2013). In case of NOX1 and NOX2 isoforms, activation of Rac1 and p47phox subunits is required to induce ROS production, whereas NOX4 generates ROS constitutively (reviewed in Brandes *et al.*, 2010). It is known that I/R leads to the production of diverse mediators, such as angiotensin II (Wu *et al.*, 2013), cytokines (Gao *et al.*, 2008) and hypoxia inducible factor 1α (HIF1α; Cai *et al.*, 2013), which stimulate activity of NOX resulting in overproduction of ROS and I/R injury (Granger and Kvietys, 2015). All three isoforms expressed in cardiomyocytes have been shown to have important impact on development of I/R injury (Braunersreuther *et al.*, 2013; Matsushima *et al.*, 2014). However, certain amount of ROS derived from NOX activity is required to trigger metabolic adaptation to protect heart from I/R injury via activity

of transcriptional factor as HIF1α (Matsushima *et al.*, 2013) and nuclear factor kappa B (NF-κB; Jiang *et al.*, 2014).

One of the most important sources of ROS during I/R are mitochondria. The primary source of ROS are complexes I and III. The complex I is a large protein complex which contains flavin mononucleotide (FMN) and eight iron-sulphur sites (Fe-S). This complex enables accepting electrons from the NADH source, which allows its recycling. Subsequently, the electrons are passed to the coenzyme Q (CoQ). When FMN is at highly reduced state, superoxide can be produced during conventional electron transport (reviewed in Cadenas, 2018). It has been shown that the reverse electron transport causes the production of superoxide at both the FMN and CoQ reduction site during I/R. If CoQ is highly reduced, it forces electrons to flow back to complex I, thus reducing NAD^+ to NADH and superoxide is produced, when electrons from FMN reduces oxygen (Chouchani *et al.*, 2014, 2016).

It has been repeatedly shown that mitochondria play a critical role in I/R injury, and thus mitochondria became an important target for cardioprotective interventions (Garlid *et al.*, 2009). The massive increase in ROS results in direct oxidative damage of the mitochondria, inhibition of the respiratory chain, destruction of the Ca^{2+} channel, thereby increasing Ca^{2+} in mitochondria and decreasing mitochondrial membrane potential. These factors promote the opening of the mitochondrial permeability transitions pore (MPTP) which leads to the necrotic cell death after I/R injury (reviewed in Halestrap, 2010). Griffiths and Halestrap (1993) demonstrated for the first time that inhibition of MPTP opening promotes cardioprotective effect against I/R injury. Therefore, it is widely accepted that mitochondrial dysfunction plays a major role in extension of I/R injury due to excessive oxidative stress and MPTP opening.

Moderated levels of ROS play important signalling role by the activation of the protective pathways during I/R. This involves induction of important transcriptional factors, such as HIF1α (Loor and Schumacker, 2008), NF-κB (Misra *et al.*, 2003) and nuclear factor erythroid 2-related factor 2 (NRF2; Xu *et al.*, 2014), which are involved in protective response to oxidative stress. ROS also activate various pathways, e.g. mitogen-activated protein kinase (MAPK; Ai *et al.*, 2015), phosphoinositide 3-kinase (PI3K; Deng *et al.*, 2015), protein kinase C (PKC; Gopalakrishna *et al.*, 2008) and phospholipase C-γ (PLC- γ; Banan *et al.*, 2001).

Oxidative stress constitutes a unifying mechanism of injury of many types of disease processes and occurs when there is disproportion between ROS production and antioxidant defence capacity (Juránek and Bezek, 2005).

1.3. Endogenous mechanisms of protection against I/R damage

Ischemia-related cellular damage is not solely dependent on the length and extent of ischemic insult, but also on myocardial tolerance to the reduced oxygen levels, and the ability to respond to ischemic stimuli by endogenous processes. In general, one of the important aims of the cardiovascular research is to identify mechanisms that would increase myocardial resistance to I/R injury and thereby substantially improve physiological functions of myocardium after I/R and prevent heart failure. The research is based on recognition of endogenous mechanisms which could be evoked by different approaches. The established methods related to oxygen restriction include ischemic preconditioning (IPC), ischemic postconditioning (iPost), remote ischemic conditioning (RIC) and chronic hypoxia. Additional cardioprotective interventions include hyperthermia, hypothermia, caloric restriction and physical exercise.

The identification of molecular mechanisms ameliorating consequences of ischemic insult and subsequent reperfusion provided a number of possible pharmacological targets potentially alleviating I/R damage. However, the results from pharmacological studies show mixed and ambiguous conclusions on the effect of infarct size after pharmacological treatment (reviewed in Hausenloy and Yellon, 2013, 2016).

1.3.1. Preconditioning

The IPC has become a popular method of protecting myocardium from ischemic damage by stimulation of endogenous mechanisms. In general, ischemic preconditioning is described as short periods of local controlled episodes of ischemia and reperfusion that alleviate cellular damage after the lethal ischemia.

The beneficial effect of preconditioning was first demonstrated by Murry *et al.* (1986), when they observed a significant reduction in heart damage in dogs after induction of repeated 5-minute I/R intervals followed by lethal 40-minute ischemia. Since then, the protective effect of preconditioning was demonstrated in all species, including humans. The main disadvantage

of the IPC method is its necessity of application before the ischemic event itself, which is not possible in the case of myocardial infarction. However, IPC is used primarily in specific surgery during which coronary circulation is slowed down or stopped (e.g. valve surgery, aortocoronary bypass). Yellon *et al.* (1993) observed the beneficial effect of IPC for the first time in humans when the method was used during cardiopulmonary bypass surgery. Since then, many clinical trials were conducted and showed the beneficial effect of IPC primarily by measuring ischemic damage markers in the heart (ATP and phosphocreatine level), cardiac necrotic markers in plasma (creatine kinase, M-type; cardiac troponin), myocardial physiological functions (ventricular arrhythmias, contractile ventricular function) and by measuring the reduction of ST-segment elevation in heart ECG (reviewed in Bousselmi *et al.*, 2014; Hausenloy and Yellon, 2016). The protective effect of IPC appears in "two windows" and the latter persists for 24 hours after preconditioning. This is referred to the late phase effect of preconditioning or delayed preconditioning (Kuzuya *et al.*, 1993; Marber *et al.*, 1993).

1.3.2. Postconditioning

As early as in 1990s there emerged publications pointing the protective effect of the altered reperfusion after a lethal ischemic insult. The gradual or mild reperfusion resulted in a significant reduction of infarct size (Hori *et al.*, 1991; Sato *et al.*, 1997). Later, Zhao *et al.* (2003) described an iPost method as an intermittent ischemic myocardial reperfusion. They demonstrated that is possible to achieve at least 40-50% reduction in infarct size using iPost, which is comparable to IPC. Unlike IPC, the major advantage of iPost is its very high clinical relevance. Alternating I/R episodes are applied after a fatal ischemic insult, therefore, this method is useful in acute ischemic conditions such as myocardial infarction. Over the past 15 years, the iPost method has been extensively tested in clinical practice and brought mixed positive and neutral results (reviewed in Hausenloy and Yellon, 2016). One of the latest large-scale clinical study did not demonstrate the beneficial effect of iPost on the reduction in infarct size or post-operative parameters (Engstrøm *et al.*, 2017), but further studies are needed to identify the mechanism and effect of iPost as stressed by the authors.

1.3.3. Remote ischemic conditioning

The RIC has been discovered later then IPC. The short ischemia and reperfusion episodes applied to the distal tissue or organ shows a significant reduction of the damaged area of myocardial infarction (Whittaker and Przyklenk, 1994). The method was described by Przyklenk *et al.* (1993) and there is a higher clinical relevance then IPC. In clinical practice, RIC was applied non-invasively through simple ligation of the upper arm or leg in a few minutes long ischemia-reperfusion cycles (Kharbanda *et al.*, 2002; Loukogeorgakis *et al.*, 2007). Important application of this method is used in aorto-coronary bypass surgery and in percutaneous coronary intervention. With the development of this method, different modifications were applied like application of short I/R impulses during lethal ischemia (remote ischemic perconditioning – RIPerC) or in the reperfusion phase after lethal ischemia (remote ischemic postconditioning – RIPostC). The original RIC is sometimes referred to as remote ischemic preconditioning – RIPreC. All of these approaches were tested both in animal models with comparable protective effects (reviewed in Bromage *et al.*, 2017) and in clinical practice (reviewed in Heusch, 2015; Hausenloy and Yellon, 2016; Lau *et al.*, 2017).

1.3.4. Physical excercise

A beneficial effect of physical activity on human health, and especially on preventing the development of cardiovascular disease, has been known for decades and it has attracted attention of researchers to this day (Paffenbarger *et al.*, 1986; Blair *et al.*, 1989; Shortreed *et al.*, 2013). Myocardial conditioning by physical activity prior to ischemia is well described in many species. The protection induced by exercise occurs in two phases. The first phase of protection begins immediately after physical activity and lasts for several hours, while the second phase occurs approximately 24 hours after the end of exercise and may persist for several weeks with regard to the selected exercise protocol (Domenech *et al.*, 2002; Lambiase *et al.*, 2003). The exercise may serve as a protection against I/R injury by reducing arrhythmias (Hamilton *et al.*, 2004; Frasier *et al.*, 2011) and myocardial stunning (Lennon *et al.*, 2004; Taylor *et al.*, 2007). Importantly, it also significantly reduces the size of myocardial infarction (French *et al.*, 2008; Frasier *et al.*, 2013). Physical activity also protects the myocardium from the risk of

cardiovascular events by reducing the probability of other pathophysiological comorbidities such as obesity, increased blood pressure and insulin resistance (Shephard and Balady, 1999).

1.3.5. Chronic hypoxia

Cardioprotective effect of adaptation to hypoxia has been recognised more than fifty years ago. The first evidence of a possible protective effect of adaptation to reduced oxygen tension comes from the late 1950s, when Hurtado (1960) observed lower incidence of myocardial infarction in human population living at high altitude for a long period (4,500 metres above sea level). At the same time, this effect was for the first time confirmed under experimental conditions simulating the hypoxic environment (Kopecky and Daum, 1958; Poupa *et al.*, 1966). Subsequently, the cardioprotective effect has been confirmed by many experimental studies on animal models with different duration and intensities of hypoxia (reviewed in Ošťádal and Kolář, 2007).

Myocardial hypoxia is generally defined as a state of insufficient oxygen supply to the tissue. The most common ways in which myocardium can be exposed to hypoxia are as follows (Ošťádal and Kolář, 2007):

a) Hypoxic hypoxia, characterized by reduced partial pressure of oxygen (pO_2) in blood at unchanged coronary flow. This phenomenon naturally occurs in high altitude conditions and in clinical cases of chronic cardiopulmonary disorders like sleep apnoea.
b) Ischemic hypoxia that occurs with limited arterial blood flow through the coronary system while maintaining pO_2.
c) Anemic hypoxia, in which pO_2 remains the same as blood flow through the coronary bed, but the ability of the molecular transport of O_2 is impaired.
d) Histotoxic hypoxia as a consequence of impaired intracellular O_2 processing, e.g. due to disorders of enzyme from mitochondrial electron transport system.

1.3.5.1. Adaptation to chronic hypoxia

As mentioned before, chronic adaptation to high altitude hypoxia has beneficial effect on recovery of heart after I/R insult. Main positive outcomes after I/R is lowering of myocardial infarct size (Neckář *et al.*, 2002a, 2013), decrease of ventricular arrhythmias (Asemu *et al.*, 2000; Neckář *et al.*, 2002a) and better functional recovery of contractile functions during reperfusion (Tajima *et al.*, 1994; Neckář *et al.*, 2004).

In addition to protective effects, adaptation to chronic hypoxia causes some adverse adaptive responses on myocardial structure, such as hypertrophy of right ventricle (RV), structure remodelling of peripheral pulmonary blood vessels and pulmonary hypertension (Herget *et al.*, 1978; Kolář and Ošťádal, 1991). Even severe chronic hypoxia induces loss of body weight and increased haematocrit (Neckář *et al.*, 2004). However, the protective effects are maintained long after the adaptation to chronic hypoxia procedure, although, other signs of adaptation are normalised after a few weeks.

Molecular mechanisms in cardioprotection

Although the beneficial effects of hypoxia exposure on the heart myocardium have been known for decades, the molecular mechanisms underlying the effects had not received major attention for many years and still remain not completely understood. Only a few of possible mechanisms in chronic hypoxia have been tested experimentally. Also, the limitation of performed studies lays in the different types of the experimental animal models, different hypoxic models and thus the end points results vary between laboratories and do not allow to generalize the observed results in a conclusive interpretation. However, various protective phenomena seem to share the same endogenous protective pathways. Chronic hypoxia not only activates these pathways, but it affects also other components and proteins, often via regulation of transcription factors such as HIF1α.

Cardioprotective effect of severe chronic intermittent hypoxia is associated with oxidative stress (Kolář *et al.*, 2007) and increased adrenergic stimulation (Ošťádal *et al.*, 1978). Although these events were considered as harmful, it appears that they might be involved in the origin of protective ischemia-resistant phenotype. In dogs, robust hypoxia elicited cardioprotection, which was abolished by administration of β1-adrenoreceptor antagonist (Mallet *et al.*, 2006).

Increased sympathetic activity results from the elevated carotid chemoreceptor response to hypoxia that is mediated by ROS-dependent signalling and HIF1α (Prabhakar *et al.*, 2007). Accordingly, antioxidant intervention by administration of N-acylcysteine to rats on hypoxia, attenuated the protective effect (Neckář *et al.*, 2003; Kolář *et al.*, 2007). These data suggest that both increased ROS and catecholamines signalling on β1-adrenoreceptors, which are tightly related, contribute to the stimulation of cardioprotective phenotype, although the mechanism remains unknown.

It has been proposed that also NO plays a positive role in the cardioprotection induced by chronic hypoxia (Baker *et al.*, 1999; Oštádalová *et al.*, 2002), although the exact role in the I/R injury and adaptive protective responses is very complex (Manukhina *et al.*, 2006). The origin of NO in hypoxic myocardium is debated and both constitutive NO synthase (eNOS; Baker *et al.*, 1999) and inducible NOS (iNOS; Rouet-Benzineb *et al.*, 1999) are considered as possible source (Zaobornyj *et al.*, 2007). It is important to note that there is an optimal concentration of NO for protection, since too low or too high concentration may be harmful.

All of above mentioned mechanism (adrenergic stimulation, increased production of both ROS and NO) may affect transcriptional signalling of numerous target genes and their downstream targests. PKC has been reported to be upregulated and activated in the chronically hypoxic hearts (Ding *et al.*, 2004). The most often studied isoforms connected with cardioprotection are PKC-α, PKC-ε and PKC-δ. All three isoforms were increased in chronically hypoxic rat myocardium (Hlaváčková *et al.*, 2010; Holzerová *et al.*, 2015; Míčová *et al.*, 2016), while PKC-δ was shown to be dependent on ROS generation, since the elevated abundance was prevented by antioxidant treatment during the hypoxic protocol (Kolář *et al.*, 2007) and treatment with general PKC inhibitor and specific PKC-δ inhibitor, respectively, attenuated cardioprotective effect (Ding *et al.*, 2004; Neckář *et al.*, 2005). Some evidence pointed out the possible role of the other family of kinases such as mitogen-activated protein kinases, p38 MAPK and JNK (Rafiee *et al.*, 2002), PI3K (Crawford *et al.*, 2003; Ravingerová *et al.*, 2007), protein kinase A, Ca^{2+}-calmodulin-dependent protein kinase (Xie *et al.*, 2005), cGMP-dependent protein kinase (Baker *et al.*, 1999) or extracellular signal-regulated kinase (Crawford *et al.*, 2003). These kinases may contribute to the protective mechanism of various types of chronic hypoxia, but their specific roles remain to be fully elucidated.

Activated protein kinases further exert phosphorylation signalling activity on targeted proteins. In chronic hypoxia, these were not conclusively identified. However, the K_{ATP} has been intensively studied as a prospective candidate. It was shown that chronic hypoxia led to activation of K_{ATP} and increased transcription of the channel subunit (Cameron and Baghdady, 1994; Crawford *et al.*, 2003). However, it is still debated which type of the channel is more important in cardioprotection. The K_{ATP} channel located in the inner mitochondria membrane has been suggested to play a crucial role in the protective mechanism of chronically hypoxic hearts (Neckář *et al.*, 2002b), although both mitochondrial and sarcolemma-located K_{ATP} were shown to contribute to improved postischemic recovery (Kong *et al.*, 2001).

It was reported that chronic hypoxia protects heart myocytes against cytoplasmic Ca^{2+} overload by preserving the function of transport and regulatory proteins involved in intracellular Ca^{2+} homeostasis, namely Na^{+}/Ca^{2+} exchanger, sarcoplasmic Ca^{2+} pump and ryanodine receptors (Chen *et al.*, 2006). Furthermore, chronic hypoxia delayed mitochondrial permeability transition and cytochrome c release upon reperfusion (Zhu *et al.*, 2006).

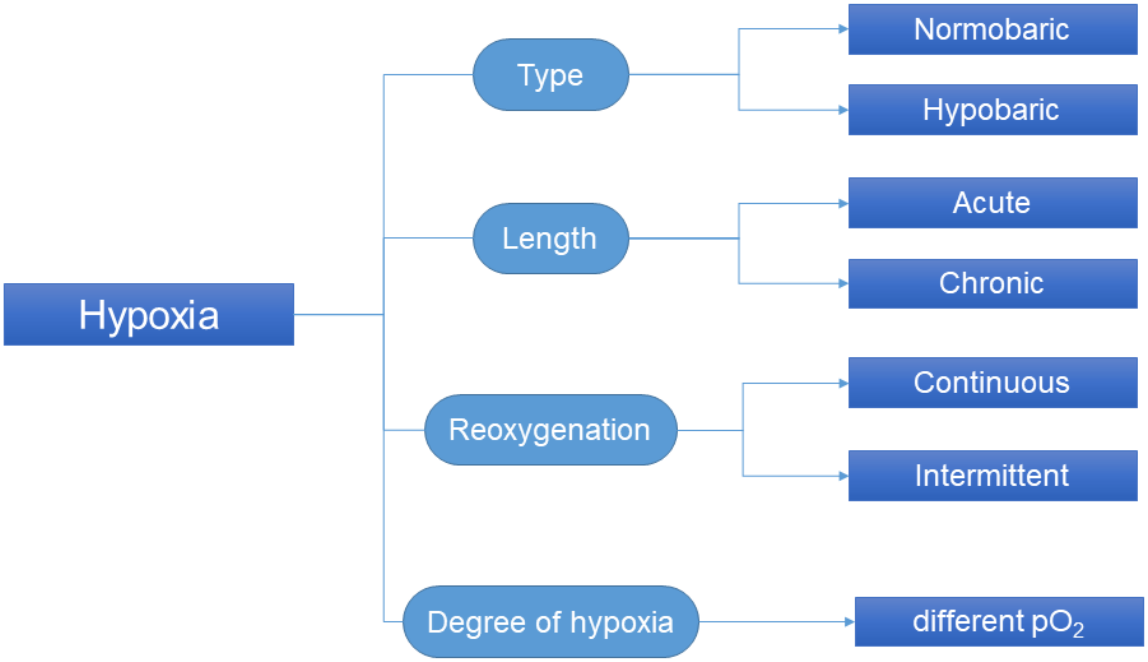
1.3.5.2. Experimental models of chronic hypoxia

There are considerable variations in the experimental data describing the effect of hypoxic adaptation on molecular, biochemical and physiological parameters of cardiac tissue. This is mainly due to the different adaptation protocols, which differ in the intensity of the hypoxic stimulus (O_2 and pO_2), the duration of daily hypoxic adaptation (intermittent and continuous) and essentially in the number of days of total adaptation (acute and chronic). Similarly, the species, sex and age of experimental animal used may be critical.

The most commonly used experimental model to simulate hypoxic environment is adaptation in hypoxic hypobaric or hypoxic normobaric chambers (Scheme 1). During hypobaric adaptation, the hypoxic environment is created by lowering the partial pressure of oxygen to the desired level, whereas in normobaric hypoxia adaptation, the level of oxygen is reduced by increased content of nitrogen without changing the overall air pressure. In the case of extreme hypoxia up to 7,000 m, the animals are usually adapted to hypoxia gradually for several days before the protocol is started at the target pO_2 level (Neckář *et al.*, 2002a). The most commonly used models simulating high mountain environments from 4,000 to 7,000 metres above sea

level. Intermittent adaptation is based on alternating hypoxic stimulus and normoxic condition with varying duration of the phases throughout the day. Model of short intermittent hypoxic insults may mimic sleep apnoea syndrome that is intensively studied in connection with increased cardiovascular risk (Suzuki *et al.*, 2006). Continuous hypoxia is an experimental protocol without reoxygenation phase throughout the whole adaptation period which is predominantly normobaric under experimental conditions.

The duration, frequency and intensity of hypoxia are fundamental parameters that influence whether the effect of hypoxic adaptation will have a beneficial or fatal effect on the investigated target tissue.

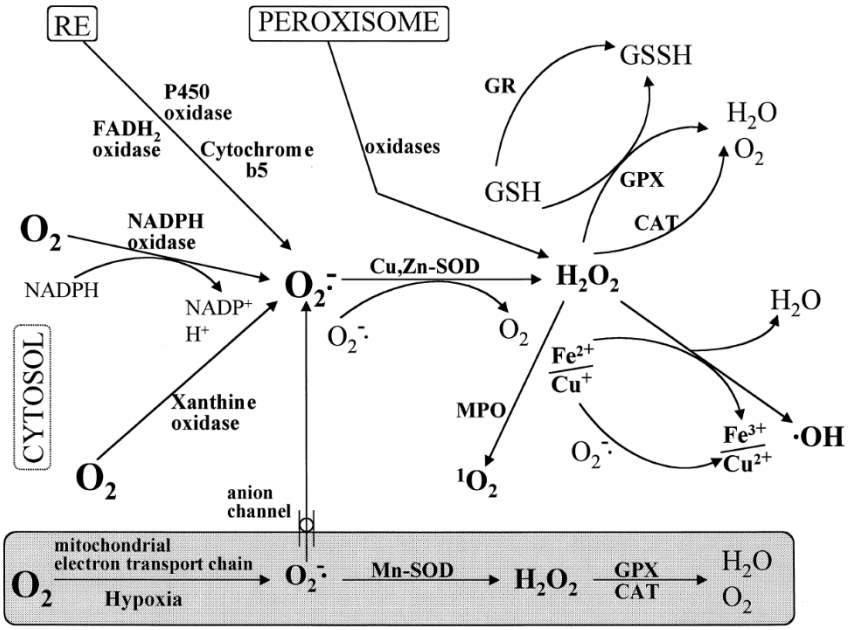


Scheme 1. Models of hypoxia. Modified according to Holzerová (2016).

1.4. Antioxidant system

The term “antioxidant defence system” includes endogenous enzymatic and non-enzymatic antioxidants (Scheme 2) which may prevent oxidative damage caused by free radicals and another ROS. Halliwell and Gutteridge (1988) defined antioxidants as substances being able to compete with substrates, which can be oxidised and thus prevent their oxidation. By this mechanism the level of free radicals is maintained at physiologically appropriate concentration, as they could act as signalling molecules and stimulate transcription factors of antioxidants and pro-inflammatory factors (reviewed in Dröge, 2002). The redox homeostasis is thus result of equilibrium between pro-oxidants and antioxidants (Haddad, 2002).

Enzyme-based system of protection is comprised of SOD, catalase (CAT), glutathione system with glutaredoxines (GRX) and thioredoxin system coupled to peroxiredoxins (PRX). Then, heme oxygenase (HMOX) and aconitase (ACO). Enzymatic system acts simultaneously with non-enzymatic antioxidants as vitamin A, E, and C, glutathione, ubiquinone, urate, lipoic acid, and flavonoids (Giordano, 2005). Under physiological conditions, these mechanisms are balanced and enable the survival of the organism (Valko *et al.*, 2007). Antioxidants can be categorized as first line defence, including mostly enzymatic antioxidant such as SOD, CAT, glutathione peroxidase (GPX) and PRX eliminating hydrogen peroxide, second line defence represented mainly by low molecular-weight antioxidants (ascorbic acid, uric acid, glutathione, alpha tocopherol, ubiquinol, etc.). Latter phase defence antioxidants include a broad range of the molecules and enzymes which repair the damage caused by free radicals to biomolecules and reconstitute the damaged cell membrane (lipase, protease, DNA repair enzymes, transferases, methionine sulphoxide reductase, etc.).



Scheme 2. The cellular defence antioxidant system and reactive oxygen species generation. Cu /Zn-SOD, cooper-zinc superoxide dismutase; Mn-SOD manganese superoxide dismutase; CAT, catalase; GSH, reduced glutathione; GSSH, oxidised glutathione; GPX, glutathione peroxidase; GR, glutathione reductase; $O_2^{\bullet-}$, superoxide radical; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; Vit E, vitamin E; Vit C, vitamin C; $NADP^+$/NADPH, nicotinamide adenine dinucleotide phosphate; $FADH_2$, reduced form of flavin adenine dinucleotide. Adopted from Matés et al., (1999).

As it is well known, that elevated oxidative stress causes wide range of pathological states, including I/R injury, therefore administration of antioxidants or their endogenous activation could be the target to counteract this harmful effect of ROS. The most common non-enzymatic antioxidant, used in dietary supplements to prevent cardiovascular risk, are vitamin A, E and C. There is a wide range of evidence on animal models, that antioxidant enzymes respond to conditions developing oxidative stress (Yamashita *et al.*, 1994; Zhou *et al.*, 1996) and play a role in cardioprotection. Unfortunately, most of the clinical trials did not show any beneficial effect of antioxidant therapy on cardiovascular outcomes of I/R (Sesso *et al.*, 2008; Kang *et al.*, 2009; Bjelakovic *et al.*, 2012).

The studies on patients supplemented with antioxidant enzymes did not show clear effect and possible side effects were discovered (reviewed in Sinning *et al.*, 2017). Therefore, it is not possible to conclude whether supplementation of an antioxidant or antioxidant enzyme is protective.

1.4.1. Main transcription factors of antioxidants

Hypoxia induced factor

The cellular response to hypoxic stimulus is characterized by the stabilization of hypoxia-induced transcription factor (HIF), which is a major signalling molecule promoting an organismal and cellular adaptive mechanism in response to reduced oxygen in the cell. It mainly includes neo-vascularization, increasing of hematocrit and adaptation of the cellular metabolism to limited oxygen delivery.

The HIF is transcriptionally active as a heterodimer consisting of oxygen-sensitive α subunits (HIFα) and constitutively expressed β subunits (HIFβ; Wang *et al.*, 1995). Three tissue-specific isoforms are recognised at the level of HIFα. In a cardiac tissue HIF1α and 2α are the predominant isoforms. The minor isoform HIF3α differs from the other isoforms by its function which is more modulatory, rather than transcription factor like (Duan, 2016). Under normoxic conditions, the α-subunit is the target of the HIF-prolyl hydroxylase (PHD) family, which hydroxylates two of HIFα proline residues using the O_2 molecule. It results in rapid ubiquitinylation and subsequent degradation in proteasome (Huang *et al.*, 1998). Both HIF1α and HIF2α are also negatively regulated by factor inhibiting HIF1 (FIH1), which is also an $O_2^{\bullet-}$ and α-ketoglutarate-dependent dioxygenase (Lando *et al.*, 2002). However, under a hypoxic condition, the HIFα subunit is stabilized and translocated into the nucleus. Subsequent dimerization of HIFα with the HIFβ subunit and interaction with coactivators such as p300 and cAMP response element–binding protein allows HIF association with the hypoxia responsive element (HRE) of the promoter regions of target genes (Jiang *et al.*, 1996). HIF transcriptional activity is responsible for increased expression of more than a hundred of genes involved in adaptive and maladaptive systemic response to reduced oxygen levels, such as activation of angiogenesis and vascular remodelling via vascular endothelial growth factor (VEGF), erythropoiesis via erythropoetin, cell proliferation, glucose transport and energy metabolism by stimulation PI3K/Akt pathway (reviewed in Bernhardt *et al.*, 2007; Prabhakar and Semenza, 2012). The most studied isoforms are HIF1α and HIF2α, which differ mainly in tissue specificity and their target genes (Patel and Simon, 2008), whereas the knowledge about the role of HIF3 is still very poor (Duan, 2016).

Lee *et al.* (2000) demonstrated an increase in HIF and its downstream proteins in animal models, as well as in humans, following acute myocardial infarction which might be stimulated by transient oxygen deprivation. Protective effect was also observed in a mouse model with upregulated HIF signalling, where the size of the infarct was reduced, contractile dysfunction decreased and long-term myocardial remodelling improved (Kido *et al.*, 2005; Natarajan *et al.*, 2006).

The protective role of HIF1α transcription factor on the heart during hypoxia was demonstrated experimentally several times. Pharmacological inhibition or RNA interference of prolyl-hydroxylases has been shown to reduce I/R injury and improve post-ischemic functions of the heart (Cai *et al.*, 2003; Natarajan *et al.*, 2006; Eckle *et al.*, 2008; Wang and Si, 2013).

In a mouse *Hif1α* knock-out model it was demonstrated that HIF1α plays an essential role in the protection of ischemic preconditioning and distant ischemic conditioning from I/R injury (Cai *et al.*, 2008, 2013). Sarkar *et al.* (2012) showed that only a stable HIF1 heterodimer (composed of both functional HIF1α and HIF1β subunits) acts as a transcriptional activator of the preconditioning protective effect. The cardioprotective effect of HIF transcription factor is mostly described based on its target genes such as heme oxygenase 1 (HMOX1; Ockaili *et al.*, 2005), adenosine receptor A2B (Eckle *et al.*, 2008), iNOS (Xi *et al.*, 2004; Natarajan *et al.*, 2006) and VEGF (Wang and Si, 2013). However, the complete mechanism of the protective pathways through HIFs is very complex and not yet fully understood.

Nuclear erythroid 2-related factor 2

During reperfusion, when blood flow is restored, significantly increasing oxidative stress can overwhelm antioxidant defence and result in cardiac dysfunction caused by cardiomyocyte damage or death. The critical component of the defence mechanism against elevated levels of ROS is the stimulation of expression of antioxidants. This function belongs to NRF2, which controls expression of more than 100 genes involved in cellular protection. Thus, NRF2 is an important candidate for resistance to ischemia-reperfusion injury (Kaspar *et al.*, 2009; Nguyen *et al.*, 2009). Under resting physiological conditions, NRF2 is associated with Kelch-like ECH-associated protein 1 (Keap1) promoting NRF2 degradation in proteasome (Itoh *et al.*, 1999). Increased levels of ROS oxidise redox-sensitive cysteine residues on Keap1 and release it from NRF2, which can thereafter translocate to the nucleus (Yamamoto *et al.*, 2008). Within the

nucleus NRF2 forms heterodimer with small Maf protein which binds to the antioxidant-responsive elements (AREs) in the promoter region of cytoprotective genes (Ma, 2013). Antioxidant enzyme systems, regulated by NRF2, include mainly enzymes involved in redox homeostasis (SOD; CAT; PRX; thioredoxin, TXN), iron metabolism (HMOX1; ferritin), glutathione synthesis and metabolism (GPX; glutathione S transferase, GST) and quinon regulation (NAD(P)H quinone reductase 1).

An importance of the NRF2 transcriptional activity in cardioprotection was mainly documented by NRF2 activators (Calvert *et al.*, 2009; Zhang *et al.*, 2010; Deng *et al.*, 2013; Katsumata *et al.*, 2014) or by the observed elevation of target antioxidant gene transcripts, mostly SOD and HMOX1 (Zhang *et al.*, 2013; Huang *et al.*, 2014). In line, *Nrf2* KO mice were sensitive to ischemia and demonstrated a twofold increase in infarct size (Xu *et al.*, 2014). Also, there is more evidence about the important involvement of NRF2 in modulation of protective action in IPC (Zhang *et al.*, 2013; Huang *et al.*, 2014; Xu *et al.*, 2014). The oxidant-sensing NRF2 transcription factor is supposed to be a promising target in approach to ameliorate injuries after I/R. This is supported by several studies using synthetic and natural NRF2 activators (reviewed in Zhou *et al.*, 2014).

Nuclear factor kappa-B

NF-κB is one of the most commonly investigated redox sensitive transcription factors. It is known by its inducibility which may rapidly respond to changes of the cellular oxidative stress, ischemia-reperfusion, and elevation of pro-inflammatory molecules within the heart tissue (reviewed in Hall *et al.*, 2006). NF-κB family consists of five members, namely p65 (RelA), RelB, c-Rel (Rel), NFKB1 (p50 and its precursor p105) and NFKB2 (p52 and its precursor p100). All members have a Rel homology domain that is essential for the dimerization, association with inhibitory proteins and DNA binding, whereas only the first 3 contain a transcriptional activation domain that serves to positively regulate gene expression. Each protein binds as homodimers or heterodimers (reviewed in Hayden and Ghosh, 2004). It is supposed that NF-κB-dependent transcriptional activation or repression in the heart is regulated primarily by p65/p50 or p50/p50 dimers, respectively (Pelzer *et al.*, 2001).

The function of NF-κB is mediated by its target genes and varies depending on the stimuli and cell types. It can be involved in a variety of cellular responses including inflammation,

immunity, cell survival, proliferation and differentiation (Hayden and Ghosh, 2011). NF-κB occurs in cytosol in inactive state associated with specific inhibitor of κB protein (IκB). Activation of NF-κB and its translocation to the nucleus is facilitated by rapid releasing of IκB and its degradation through ubiquitin proteasome pathway (Alkalay *et al.*, 1995). Two possible signalling pathways, referred to as canonical (major) and non-canonical (minor), can trigger this process (reviewed in Bonizzi and Karin, 2004). The canonical pathway, also termed the classical pathway, is specific by the inducible degradation of IκB subunit under exposure to pro-inflammatory stimuli, such as tumour necrosis factor alpha (TNFα), interleukin 1 (IL1) or bacterial LPS, and their reaction with the appropriate receptors (TNFα receptor, IL1 receptor, toll-like receptors) and other pro-inflammatory receptors (DiDonato *et al.*, 1996; Shih *et al.*, 2011).

NF-κB has been described in both cardioprotective and cardiotoxic specific cellular context. NF-κB can promote cardiomyocytes survival by repressing apoptotic cell death induced by hypoxia or I/R, through its transcriptional activity (de Moissac *et al.*, 1998; Misra *et al.*, 2003). On the other hand, chronic activation of NF-κB may cause increased expression of pro-inflammatory cytokines and produced harmful consequences, including cardiac cell death (Hamid *et al.*, 2011).

1.4.2. Superoxide dismutase

SOD is the major reductant of constantly produced $O_2^{\bullet -}$ in the cell to form H_2O_2 and O_2 and is considered the first line of enzymatic reduction of free radicals. The three known SOD isoforms differ in intracellular localization and the type of bound ferric cofactor. Copper-zinc SOD (Cu/ZnSOD, SOD1) is located primarily in the cytosol, but also in the nucleus, peroxisomes, and in the space between the outer and inner membrane of the mitochondria (Crapo *et al.*, 1992). The manganese isoform (MnSOD, SOD2) provides primarily the reduction of $O_2^{\bullet -}$ which is produced in mitochondria during oxidative phosphorylation (Weisiger and Fridovich, 1973). The third isoform is localized extracellularly (EcSOD, SOD3) and it also binds copper or zinc in the catalytic centre, but unlike the cytosolic isoform, it forms a tetramer and is found primarily in extracellular fluids such as plasma, lymph and synovial fluid (Marklund *et al.*, 1982, 1986) and also in vessels (Fukai, 2002).

Isoform of SOD1 seems to be more constitutively expressed than SOD2 isoform, which is induced in response to oxidative or other stress conditions (reviewed in Miao and St. Clair, 2009). An individual SOD gene has its own and unique regulatory mechanism, and uses common transcriptional factors. It was documented in many studies, that both isoforms are highly regulated on transcriptional level by transcriptional factor NF-κB (Xu *et al.*, 1999; Kiningham *et al.*, 2001; Rojo *et al.*, 2004), NRF2 (Park and Rho, 2002) and HIF1α (Melov *et al.*, 1998).

The SODs stand as the main antioxidants in the intracellular defence against ROS. All three isoforms have been shown to provide cardioprotective effect against I/R injury (Yoshida *et al.*, 2000; Pinto *et al.*, 2016). Wang *et al.* (1998) demonstrated importance of cytosolic isoform on transgenic mice. Over-expression of Cu/ZnSOD almost totally quenched the burst of superoxide generation and this was accompanied by decrease in infarct size and better recovery of the cardiac contractile function compared with wild-type controls (Wang *et al.*, 1998). The comparable results and protection against I/R injury were observed on the very similar model of transgenic mice with overexpressed mitochondrial isoform of MnSOD (Chen *et al.*, 1998). Asimakis *et al.* (2002) showed that mitochondrial isoforms had higher contribution in mediating tolerance to oxidative stress induced by ischemia-reperfusion than cytosolic isoforms. Similarly, Jones *et al.* (2003) demonstrated higher importance of MnSOD against I/R injury than Cu/ZnSOD. Participation of SOD in IPC modulated cardioprotective effect was also observed in other studies (Kuzuya *et al.*, 1993; Yamashita *et al.*, 1994). Besides that, activation of SOD activity by lecithin or by other mimetics could have beneficial impact on the disease associated with increased oxidative stress (Janssen and Nozik-Grayck, 2017; Tanaka *et al.*, 2017). However, the increased activity of SOD can also lead to adverse events (Kowald and Klipp, 2004; Kowald *et al.*, 2006) and loss of cardioprotective effect (Omar and McCord, 1990; Omar *et al.*, 1990). In the clinical trial, it was observed that intravenous administration of human SOD did not provide any cardioprotection for patients subjected to reperfusion following percutaneous transluminal coronary angioplasty (Flaherty *et al.*, 1994). The regulation of $O_2^{\bullet-}$ and H_2O_2 is more complex and depends on redox equilibrium within the cell. Pathophysiological changes may also significantly affect these systems (Zengin *et al.*, 2015).

1.4.3. Catalase

Catalase is ubiquitous enzyme that plays important role in defending cells against oxidative damage by degrading hydrogen peroxide to water and oxygen ($2H_2O_2 \rightarrow 2\ H_2O + O_2$) with high efficiency. Catalase is a tetrameric protein with four similar subunits each containing heme group. The enzyme use manganese or iron ion as cofactor (Chelikani *et al.*, 2004). The heme-iron is oxidised by hydrogen peroxide to form a high-valence intermediate known as compound I. Further reaction with H_2O_2 reduces compound I and forms molecular oxygen and water (Alfonso-Prieto *et al.*, 2009). The enzyme is located primarily in the peroxisomes and it is absent in the mitochondria of mammalian cells. There is only one evidence of its occurrence in mitochondria in rat heart (Radi *et al.*, 1991). The intracellular reduction of H_2O_2 and other organic peroxides in other compartments of the cell is ensured by other enzymatic antioxidants including PRX, GPX, and thioredoxin reductase (TXNRD).

The upregulation of catalase activity had infarct size limiting effect as shown on transgenic mice (Li *et al.*, 1997). Interestingly endotoxin pre-treatment increased endogenous myocardial catalase activity and decreased I/R injury (Brown *et al.*, 1989). Ability of catalase to metabolise hydrogen peroxide during hypoxia was demonstrated on catalase-overexpressing transgenic mice (Chen *et al.*, 1997). Undyala *et al.* (2011) showed that treatment with cell-penetrating derivative of catalase protected neonatal cardiomyocytes from hypoxia-reperfusion injury as well as I/R injury.

1.4.4. Peroxiredoxin system

Peroxiredoxins are thiol-specific antioxidant family of 6 enzymes, which have either one (1-Cys) or two (2-Cys) conserved cysteine residues essential for their catalytic activity (Rhee *et al.*, 2005). PRX reduce H_2O_2 and organic peroxides with utilization of thioredoxin as an electron donor (Kang *et al.*, 1998). All 6 isoforms are highly expressed in cardiac tissue (Brixius *et al.*, 2007) and differ in their location. PRX1, PRX2 and PRX6 are located in the cytosol, PRX3 in mitochondria, PRX5 in cytosol, mitochondria and peroxisomes and PRX4 is located in the extracellular space (Rhee *et al.*, 2005). There is a number of evidence for participation of each peroxiredoxin isoform in antioxidant effects protecting heart against I/R injury (Zhao *et al.*,

2009; Guo *et al.*, 2018; Wei *et al.*, 2018), oxidative stress-induced apoptosis (Zhao *et al.*, 2009) and left ventricle (LV) remodelling after myocardial infarction (Matsushima *et al.*, 2006).

PRX1 plays an important role in inhibition of oxidative stress and apoptosis during the I/R injury (Guo *et al.*, 2018). Zhao *et al.* (2009) demonstrated the specific role of PRX2 in protection against oxidative stress-induced apoptosis on cardiomyocytes and I/R injury in the heart. Overexpression of PRX3 protects the heart against LV remodelling and subsequent heart failure after myocardial infarction in mice (Matsushima *et al.*, 2006). However, Brixius *et al.* (2007) showed that expression of all 6 isoforms is regulated differently. The expression of PRX1 and PRX2 were not affected, but the expression of PRX3, PRX4, PRX5 and PRX6 isoforms was decreased in response to enhanced ROS production in human failing heart.

1.4.5. Maintenance of the redox homeostasis

The intracellular redox regulation is generally considered as reduction-oxidation based processes that respond to cyclic maintenance of the redox state in cells. Related redox signalling is essential to control physiological as well as maladaptive processes essential for a cell survival. Imbalance in the redox regulation leads to an excessive oxidative stress in the cells. This may result in diminishing of the cellular functions, lipid peroxidation and protein degradation.

Thioredoxin and glutathione antioxidant systems contributes to the main regulatory mechanism of redox balance within the cell. They maintain redox sensitive cysteine residues of the proteins in reduced form and thus protect cells from harmful oxidative stress leading to inactivation of enzymes and apoptosis.

Thioredoxin and glutathione antioxidant systems are the most versatile systems facilitating both, peroxide detoxification and disulphide reduction to sustain redox balance in the cell. The functions of both systems overlap in many cases and these systems work in parallel (He *et al.*, 2017).

1.4.5.1. Glutathione antioxidant system

Generally, the term glutathione antioxidant system includes mainly low molecular weight compound glutathione and major enzymes involved in its metabolism (glutathione reductase,

GSR; GPX; GST; GRX). Glutathione is tripeptide (L-g-glutamyl-L-cysteinyl-glycine), which is the most abundant intracellular non-enzymatic antioxidant present in all animal cells. Its synthesis from constitutively present amino acids glutamate, cysteine and glycine, is driven by consumption of ATP and occurs in cytosol of cells. Catalysts of this reaction are γ-glutamate-cysteine synthase (γ-GCS) and glutathione synthase. Increased level of glutathione inhibits the γ-GCS regulatory subunit and thereby regulates its own synthesis by positive feedback loop (Lu, 2009). It has been shown that promoter region of the γ-GCS subunits contains ARE element (Mulcahy *et al.*, 1997) and binding sites for both the redox-sensitive transcription factor NF-κB and Activator protein 1 (AP-1; Galter *et al.*, 1994). Thus, a TXN1-mediated redox stability of these transcription factors in the nucleus is required for the synthesis of glutathione.

Glutathione exists in thiol-reduced form (GSH) and disulfide-oxidised form (GSSG). In the reduced state, the thiol group on the cysteine residue is able to provide reducing equivalent to oxidised molecules such as ROS. Then, GSH itself is oxidised and produces GSSG form (Kaplowitz *et al.*, 1985). Based on this ability, GSH participates in many redox reactions and helps to maintain the redox potential of the cell. GSH may act either in direct interaction with ROS or it serves as a substrate for other redox systems such as GPX or GRX (Sáez *et al.*, 1993; Yoshida *et al.*, 1996). Under physiological conditions, the majority of glutathione within the cell is in biologically active reduced state. The ratio of reduced glutathione to oxidised form (GSH/GSSG) is a common marker of oxidative stress. The cellular concentration in the cytosol can range from 1 to 10 mM with an overall ratio GSH/GSSG in the range 30:1 to 100:1 (Hwang *et al.*, 1992). The GSSG may be reduced back to GSH by GSR in an NADPH-dependent reaction Under high oxidative stress, excessive GSSG is accumulated in the cell and exported across cell membrane to the extracellular space and it can be detected as an indicator of increased oxidative stress in plasma (Jones *et al.*, 2000).

The utilization of GSH was shown to be markedly elevated during human myocardial ischemia and led to the formation of oxidised glutathione after reperfusion period (Ferrari *et al.*, 1990). Myocardial glutathione content and high redox ratio (GSH/GSSG) plays an important role in maintaining physiological heart functions under the basal conditions, whereas the massive accumulation of the GSSG after reperfusion phase indicates detrimental oxidative stress. Such

a huge dissipation of the myocardial GSH during reoxygenation after reperfusion might cause the LV dysfunction in patients with ischemic heart disease (Vecchi *et al.*, 1998).

As mentioned above GSR is an essential enzyme, which recycles oxidised glutathione back to its reduced form by utilizing NADPH as the reductant (Argyrou and Blanchard, 2004). GSR is an flavoenzyme (Kelner and Montoya, 2000) found in cytosol and within organelles including nucleus, mitochondria, endoplasmic reticulum and lysosomes. Under oxidative stress, GSR is regulated on the transcriptional level as well as by post-translational modifications (Couto, 2016).

GPX are tetrameric enzymes catalysing the reduction of H_2O_2 or lipid peroxides to water or the corresponding alcohols, typically using GSH as reductant. GPX is highly expressed comparing to the catalase in the cardiovascular tissue and its ability to detoxify lipid peroxides beside H_2O_2 is significant. Therefore, its position in the myocardium is attributed to more important protective antioxidant role than that of catalase (Forgione *et al.*, 2002). Eight mammalian GPX isoforms have been identified. Four main isoforms contain selenocysteine in their catalytic site. GPX1 is ubiquitous, located in the cytosol and mitochondria. It reduces H_2O_2 and fatty acid peroxides, but not the esterified lipid peroxides. That is the role of membrane-bound GPX4 isoform which is characterized as the lipid peroxidation inhibiting protein. GPX2 was predominantly found in the gastrointestinal epithelial cells and play a role in carcinogenesis. GPX3 is the only extracellular isoform highly expressed in the most of the tissue. GPX5, GPX7 and GPX8 contain cysteine in the active site and GPX6 is a selenoprotein in humans, but in rats and mice contains cysteine residue in active site. The last four isoforms seem to be of minor significance (reviewed in Brigelius-Flohé and Maiorino, 2013). Yoshida *et al.* (1996) showed in transgenic mice that overexpression of GPX1 makes the heart more resistant to myocardial I/R injury. Moreover, it was found that mitochondrially-bound isoform GPX4 plays a key role in the protection of contractile function as well as in the mitochondrial functions following the I/R injury (Dabkowski *et al.*, 2008). Overexpression of GPX also protects the heart against post-infarction remodelling and heart failure in mice (Shiomi *et al.*, 2004).

1.4.5.2. Thioredoxin system

The thioredoxin antioxidant system consists mainly of the two oxidoreductase enzymes – thioredoxin and TXNRD. TXN is the ubiquitous disulphide reductase, which can provide electron to the large range of enzymes and thereby acts as a powerful antioxidant and plays an important role in maintaining critical protein thiols in the reduced state. Among the target enzymes of TXN oxidoreductive activity can be found PRX, GPX, GSH, protein disulphide isomerase, ribonucleotide reductase and several transcriptional factors (reviewed in Lee *et al.*, 2013). Moreover, reduced TXN can prevent apoptosis via an inhibitory binding to apoptosis signal-regulating kinase 1 (ASK-1), whereas this binding is lost when TXN is oxidised (Saitoh *et al.*, 1998). Therefore, TXN-dependent reduction plays a critical role in the control of protein thiol homeostasis, redox signalling and enables the reduction of the intracellular ROS in the cell.

There are two major isoforms in mammalian cells, the mainly cytosolic TXN1, which can be translocated into the nucleus after certain stimuli and control activity of redox-sensitive transcription factors in cooperation with TXNRD1 (Hirota *et al.*, 1999; Schroeder *et al.*, 2007). Besides that, TXN2 is located in mitochondrial matrix and together with TXNRD2 interplay with other mitochondrial antioxidants. They possess a strong influence on mitochondria-triggered apoptosis and cell viability. TXNRDs use $NADPH^+$ to catalyse the reduction of active disulphide site of TXN.

The critical role of TXN1 in regulating oxidative stress *in vivo* heart was investigated using transgenic mice (Yamamoto *et al.*, 2003). Thioredoxin have been implicated in a large number of cardiovascular diseases, including ischemic heart disease (Park and Suzuki, 2007), cardiac hypertrophy (Ago and Sadoshima, 2007), atherosclerosis and hypertension (Okuda *et al.*, 2001; Hägg *et al.*, 2006). Cardiac specific overexpression of *Txn1* in mice improved systolic and diastolic post-ischemic ventricular function and reduced size of myocardial infarction (Turoczi *et al.*, 2003). TXN1 refines the effects of I/R by improving the free radical-mediated damage in cardiac and mitochondrial function (Perez *et al.*, 2016). Furthermore, TXN1 reduced myocardial apoptosis and reduced infarct size after I/R of mice, which received human TXN1 before reperfusion (Tao *et al.*, 2004). Taken together, these findings support the view that also TXN1 plays a crucial role in cardioprotection against I/R injury.

As already mentioned, important role of TXN1 relates to its ability to translocate to the nucleus. Several important human transcription factors are redox regulated and contain redox-sensitive cysteines in their DNA binding domain. TXN1 is able to reduce these cysteine residues in the nucleus and thereby ensure transcriptional activity of relevant transcription factors. These include the most NF-κB (Matthews *et al.*, 1992; Hirota *et al.*, 1999), HIF1α (Welsh *et al.*, 2002), NRF2 (Hansen *et al.*, 2004), AP-1 (Wei *et al.*, 2000) and more.

1.4.6. Heme oxygenase

Heme oxygenase is enzyme considered as one of the most sensitive and reliable indicators to cellular oxidative stress and very often associated with the protection of the heart against I/R injury (Vulapalli *et al.*, 2002; Tang *et al.*, 2005; Wang *et al.*, 2010; Zhou *et al.*, 2014a). This stress responsive enzyme plays the prominent role in maintaining redox homeostasis by rapid degradation of pro-oxidant hem to bioactive signalling molecules carbon monoxide (CO), biliverdin and iron, which is stored within the iron-binding protein ferritin (Balla *et al.*, 1992; Otterbein and Choi, 2000; Abraham and Kappas, 2008). Biliverdin is reduced to bilirubin by the biliverdin reductase (BLVR), which plays important antioxidant and cytoprotective role (Stocker *et al.*, 1987).

HMOX is the member of heat shock protein family and is present in all cells and tissues. Under normal physiological conditions, HMOX1 is expressed in low levels, whereas HMOX2 is expressed constitutively. HMOX1 is rapidly increased in response to oxidative stress stimuli such as hypoxia (Mitani *et al.*, 1991) as well as to hyperoxia (Lee *et al.*, 1996), UV light (Vile and Tyrrell, 1993) and heat stress (Lu *et al.*, 2002). The expression of HMOX1 is strongly induced by its substrate heme and by numerous stress agents such as heavy metals, inflammatory cytokines, heat shock, nitric oxid and CO (reviewed in Dunn *et al.*, 2014) mostly via transcriptional factors HIF1α (Lee *et al.*, 1997), NRF2 (Alam *et al.*, 1999) and NF-κB (Lin *et al.*, 2007).

It has been shown that HMOX1 plays an important role in failing heart (Grabellus *et al.*, 2002; Wang *et al.*, 2010). Cardiac-specific *Hmox1* transgenic mice showed improved postinfarction survival and attenuated cardiac hypertrophy which was possibly based on the decrease in oxidative stress, interstitial fibrosis, and apoptosis (Vulapalli *et al.*, 2002). Additionally,

Yoshida *et al.* (2001) showed on heterozygous transgenic mice (*Hmox1*$^{+/-}$) exhibited exaggerated cardiac injury and dysfunction after I/R. The direct causative role of HMOX1 in protection against I/R-induced injury has been demonstrated on transgenic mice with over expression of *Hmox1* and KO mice (*Hmox1*$^{-/-}$). Due to the recovery of post-ischemic cardiac function, prevention of the development of reperfusion-induced ventricular fibrillation and reduction in infarct size, authors marked the targeting of HMOX1 as possible treatment of I/R injury in patients with coronary artery disease (Juhasz *et al.*, 2011).

1.4.7. Aconitase

ACO is an enzyme that catalyses the reversible isomerization of citrate to isocitrate via cis-aconitate as an intermediate and it is involved in the regulation of cellular metabolism. ACO belongs to the family of iron–sulphur-containing enzymes. Catalytic ACO activity is regulated by reversible oxidation of $[4Fe\text{-}4S]^{2+}$ cluster and cysteine residues. It undergoes reversible inactivation induced by oxidants, which cause disassembly of the Fe-S cluster. ACO activity is considered as marker of oxidative stress and has been suggested to reflect an intra-mitochondrial redox status and ROS play a central role in the regulation of ACO functions (reviewed in Lushchak *et al.*, 2014).

There are two isoforms of this enzyme, cytosolic, soluble form referred as ACO1 and mitochondrial ACO2. ACO1 possess enzymatic activity when it is in the reduced form, whereas it acts as a regulator of iron homeostasis when it is in the oxidised form. In the oxidised form is known as iron regulatory protein 1 (IRP1) which recognizes iron responsive elements of mRNAs of ferritin and transferrin receptor and in this way regulates their translation, when it is in the oxidised form (Anderson *et al.*, 2012). Iron metabolism is impaired during ischemia, and iron is released as free iron. This catalytic free iron can generate ROS through the Fenton reaction, catalysing the production of ·OH from H_2O_2 and $O_2^{\bullet-}$ (Merkofer *et al.*, 2006). The role of the second isoform ACO2 is to control cellular ATP production by regulation of reversible isomerization of citrate to isocitrate in the Krebs cycle. Active $[4Fe\text{-}4S]^{2+}$ clusters are sensitive to $O_2^{\bullet-}$ radicals (Flint *et al.*, 1993) and may inactivate its function.

1.5. Enzymes of energetic metabolism and its role in mitochondrial ROS production

1.5.1. Creatine kinase

Heart is the most oxidative tissue with a high energy demand, thus continuous production of ATP by oxidative phosphorylation in mitochondria is required. The creatine kinase (CK) system functions in cardiac muscle cells as the main energy buffer keeping a high ATP/ADP ratio and related energy homeostasis. CK catalyses phosphorylation of creatine in a reversible reaction of creatine with ATP (Kenyon and Reed, 1983).

There are three CK isoforms expressed in cardiomyocytes: two cytosolic isoforms primarily identified in the muscle and brain, CKM, CKB, respectively and one mitochondrial sarcomeric isoform, mtCKs. The isoforms are highly compartmentalized and localized in the proximity of ATPases in order to maintain ideal conditions for their function (Kitzenberg *et al.*, 2016). The major cytosolic CK isoform CKM regenerates ATP and simultaneously decreases level of free ADP. The minor cytosolic isoform CKB is predominantly expressed in the heart at the early developmental stage. In adulthood it functions as a constitutive enzyme, which can be induced under conditions of metabolic challenge (Letout *et al.*, 2005).

The mtCKs isoform plays a role during "physiological" hypoxia and operates in the mitochondria intermembrane space (Brdiczka *et al.*, 1998). It is functionally related to the adenine nucleotide translocase (ANT) located in inner mitochondrial membrane and it increases availability of ADP for complex V of the respiratory chain (Saks *et al.*, 1985) and thus regulates the membrane potential and controls formation of ROS (Meyer *et al.*, 2006). It also prevents opening of the MPTP, which is involved in apoptotic cell death signalling (Dolder *et al.*, 2003).

1.5.2. Hexokinase

Hexokinase (HK) is another important enzyme, which may affect ROS production in respiratory chain. There are two isoforms HK1 and HK2, respectively, present in heart tissue (Southworth *et al.*, 2007). They differ in their level, cellular localization, allosteric regulation and affinity for glucose and ATP. Both isoforms phosphorylate glucose to glucose-6-phosphate and when associated with mitochondria, then directly couple metabolism of glucose with ATP produced

by oxidative phosphorylation (Gottlob *et al.*, 2001). ADP produced by HK reaction activates complex V of respiratory chain and thus support mitochondrial respiration. Under normal condition, HK1 isoform is attached to outer mitochondrial membrane and HK2 is mostly located in cytosol. The attachment of HK2 to outer mitochondrial membrane can be induced under ischemic condition (Southworth *et al.*, 2007), which in the same time prevents the binding of pro-apoptotic BAX protein and activation of apoptosis (Pastorino *et al.*, 2002). Both HK1 and HK2 interact with the outer mitochondrial membrane via voltage dependent anion channel (VDAC), which is functionally coupled with ANT. Their interaction under pathophysiological conditions prevents opening of MPTP and releasing of cytochrome c (Azoulay-Zohar *et al.*, 2004; Miyamoto *et al.*, 2008). We have shown that association of HK2 can be potentiated by severe hypoxia (Waskova-Arnostova *et al.*, 2015) and short ischemic insult (Kolář *et al.*, 2007).

2. Hypothesis and Aims

Based on the analysis of available data in the literature, we hypothesized that antioxidant system and redox sensitive transcription factors play an important role in the cardioprotective phenotype elicited by adaptation to hypoxia.

To test this hypothesis, we defined the following main aims:

1. To identify the role of the main components of antioxidant systems in protective and non-protective regimens of chronic hypoxia:
    a. To analyse the expression of antioxidants after adaptation to continuous and intermittent normobaric hypoxia.
    b. To analyse the expression of antioxidants after adaptation to severe intermittent hypobaric hypoxia.
2. To investigate the effect of severe intermittent hypobaric hypoxia on TXN1 and its main redox homeostasis target transcription factor NF-κB.
3. To characterise the role of important enzymes of energetic metabolism, creatine kinase and hexokinase, in protective and non-protective regimens of chronic hypoxia.

3. Methods

The handling with animals and hypoxic chambers, as well as all *in vivo* surgical procedures and analysis of infarct size were performed on institute of Academy of Science in Prague by Dr. Ján Neckář. All molecular analyses were performed in our laboratory with contribution of cooperative group of Doc. Nováková in selected protein analyses.

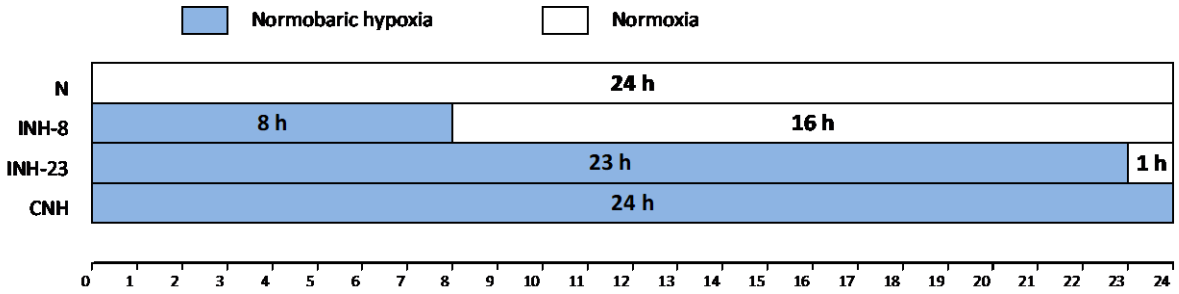
3.1. Animal models

Adult male Wistar rats purchased from Velaz, Ltd. (Prague, Czech Republic) or Charles River Laboratories (Sulzfeld, Germany) were used for all experiments. The animals were acclimatized for at least 2 weeks prior to any experiment and housed in controlled laboratory conditions (12:12 h light-dark cycle, 23°C), they had a free access to water and standard chow diet. The rats were maintained and handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication no. 85-23, revised 1996). All experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology, Czech Academy of Sciences.

3.2. Experimental models of chronic hypoxia

3.2.1. Adaptation to normobaric hypoxia

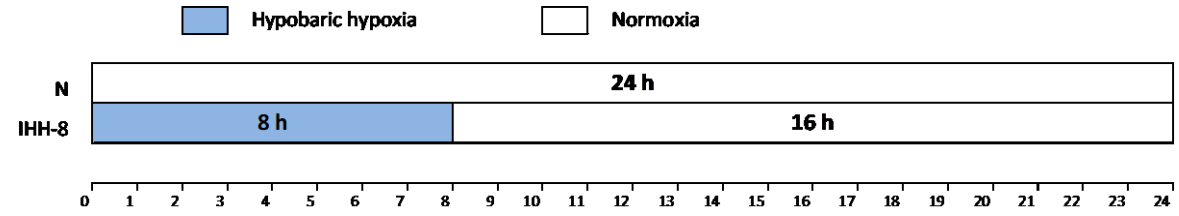
Normobaric hypoxia conditions were induced in a normobaric chamber equipped with hypoxia generators Everest Summit (Hypoxico Inc., New York, NY, USA). Rats were randomly assigned to control and experimental groups and exposed to three different experimental regimens of hypoxia (Fi_{O2} 0.1, corresponding to high altitude of 5,500 m) for three weeks. First group was exposed to normobaric hypoxia continuously for 24 h/day (CNH), second group was exposed to hypoxia intermittently for 8 h/day followed by 16 h reoxygenation at normoxic conditions (INH-8) and the third group was kept under hypoxia conditions 23 h/day followed by interruption for 1 hour at normoxic conditions (INH-23). In parallel, control group was kept in the normoxic condition (room air) for the same period of the time (Scheme 3).



Scheme 3. Time schedule of adaptation to protective and non-protective regimen of normobaric hypoxia (FiO_2 0.1). All animals were exposed to moderate normobaric hypoxia (FiO_2 0.1, corresponding to high altitude of 5,500 m) for three weeks continuously 24 h/day (CNH) or intermittently for 8 h/day (INH-8) or for 23 h/day (INH-23). The control rats (N) were kept in the normoxic (room air) conditions 24h/ day for the same period of time.

3.2.2. Adaptation to hypobaric hypoxia

In the separate experiment, the rats were exposed to hypobaric hypoxia intermittently 8h/day for 5 days a week in a hypobaric chamber (IHH-8). First, the animals were exposed to stepwise lowering of barometric pressure during the first 13 exposures to reach the level corresponding to the altitude of 7,000 m (Pb = 308 mmHg, 41 kPa; and pO_2 = 65 mmHg, 8.6 kPa). Thereafter, this level of high-altitude hypoxia conditions (7,000 m) was maintained for additional 11-17 exposures. The remaining time (16h each exposure day and 2 days after 5 days hypoxic exposure) the rats were kept in normoxic conditions. The control group (N) was housed in the normoxic conditions for the same period of total time (Scheme 4; Kolář *et al.*, 2007).



Scheme 4. Time schedule of adaptation to protective regimen of hypobaric hypoxia (7,000 m). The hypoxic animals were exposed to intermittent hypobaric hypoxia corresponding to the altitude of 7,000 m for 8 h/day, 5 days/wk. The total number of exposures was 24–30. The control rats (N) were kept in the normoxic conditions (room air) 24h/ day for the same period of time.

3.3. Myocardial ischemia-reperfusion

The rats were anesthetized (sodium pentobarbital, 60 mg/kg i.p.) and ventilated (Ugo Basile, Gemonio, Italy) with room air by tracheal cannula (68–70 strokes/min; tidal volume of 1.2 ml/100 g body weight). Another heparinized cannula was placed in the left carotid artery and the blood pressure (P23Gb, Gould, Cleveland, OH, USA) and single-lead ECG were continuously recorded and subsequently analysed by custom-designed software. The rectal temperature was measured and maintained between 36.5 and 37.5°C by a heated table throughout the experiment. Left thoracotomy was performed. After 10 min stabilization, regional myocardial ischemia was induced by tightening of a silk suture around the descending branch of the left coronary artery for 20 min. Next, the suture was released to allow reperfusion. Five minutes later, the chest was closed and the air from thorax was exhausted. Spontaneously breathing animals were maintained in deep anaesthesia for the following 3 h.

3.4. Infarct size determination

Hearts were excised and washed with 20 ml saline through cannulated aorta. The size of area at risk (AR) and the infarct size (IS) were determined by staining with 5% potassium permanganate and 1 % 2,3,5-triphenyltetrazolium chloride (pH 7.4, 37°C). The hearts were cut perpendicularly to the long axis of the ventricle into 1 mm thick slices and stored overnight in 10% neutral formaldehyde solution. The day after, the right ventricular free wall was separated and both sides of the left ventricular slices were photographed. The IS, AR, and the size of the LV were determined by computerized planimetry using software Ellipse (ViDiTo, Košice, Slovakia). The size of AR was normalized to the LV (AR/LV), and the IS was normalized to the LV (IS/LV) and to the AR (IS/AR) as described in Neckář *et al.* (2002a).

3.5. Tissue preparation

All rats were killed by decapitation. The hearts were rapidly excised, washed in ice-cold saline and dissected in to the LV, RV and septum on the ice dish. LV samples were immediately frozen in liquid nitrogen and weighed. The samples were stored in -80°C until used for analysis.

3.6. RNA isolation, Reverse Transcription and quantitative PCR

Heart LV samples were pulverized to fine powder with liquid nitrogen and immediately dissolved in 1 mL of RNAzol reagent (Molecular Research Center, Cincinnati, OH, USA). Total RNA was isolated according to manufacturer's instructions. RNA concentration and purity (expressed as 260/280 nm ratio) were assessed spectrophotometrically by Nanodrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). Integrity of RNA was verified at agarose gel. 1 μg of total RNA was used to synthesize first strand cDNA using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA) using oligo(dT) primers according to the manufacturer's protocol. Real-time quantitative RT-PCR (qPCR) was performed on a Light Cycler 480 (Roche Applied Sciences, Penzberg, Germany) using specific mono-color hydrolysis probe (Universal Probe – Roche Applied Sciences) and primers (Table 1) with the appropriate Probe Master kit (Roche Applied Sciences) according to the manufacturer's protocol with following incubations: pre-incubation (95°C/10min); amplification, 45 cycles (denaturation 95°C/10s, annealing 60°C/30s, elongation 72°C/1s); cooling (40°C/30s). Alternatively, in some experiments, MESA GREEN qPCR MasterMix Plus for SYBR Assay (Eurogentec, Seraing, Belgium) was used in combination with gene-specific primers (Table 1) designed by the Universal Probe Library Assay Design Center (Roche Applied Sciences). qPCR was performed with following amplification conditions: pre-incubation (95°C/10min); amplification, 50 cycles (denaturation 95°C/20s, annealing 60°C/20s, elongation 72°C/15s); melting (95°C/5s, 65°C/60s); cooling (40°C/30s). The specificity of products was verified by melting curve analysis. Relative levels of target genes were normalized to those of reference gene Hypoxanthine-guanine phosphoribosyltransferase 1 (*Hprt1*) and were calculated using ΔΔCT method (Pfaffl, 2001).

Table 1. Hydrolysis probe numbers and primer sequences complementary to rat target genes used in qPCR analyses.

Gene symbol	Alternative symbol	Probe number	Primer position	5'→ 3' sequence	NCBI RefSeq
Sod1	*Cu/ZnSod*	5	left	taagaaacatggcggtcca	NM_017050.1
			right	tggacacattggccacac	
Sod2	*MnSod*	67	left	tggacaaacctgagccctaa	NM_017051.2
			right	gacccaaagtcacgcttgata	
Cat		12	left	cagcgaccagatgaagca	NM_012520.2
			right	ggtcaggacatcgggtttc	
Prdx1	*Prx1*	15	left	gagacctgtagctcgactctgc	NM_057114.1
			right	aacagccgtggctttgaa	
Prdx2	*Prx2*	12	left	gactctcagttcacccacctg	NM_017169.1
			right	tattcagtgggcccaagc	
Prdx3	*Prx3*	113	left	aatgaccttccggtggtaaa	NM_022540.1
			right	gctgttggacttggcttgat	
Prdx4	*Prx4*	84	left	cgaaagctggtattgctcct	NM_053512.2
			right	agccctgcaaagcttcagt	
Prdx5	*Prx5*	53	left	gactatggccccgatcaa	NM_053610.1
			right	aaaacacctttcttgtccttgaa	
Prdx6	*Prx6*	1	left	ttgattgctctttcaatagactctg	NM_053576.2
			right	ctgcaccattgtaagcattga	
Gpx1			left	cgacatcgaacccgatataga	NM_030826.4
			right	atgccttaggggttgctagg	
Gpx4		25	left	tgggaaatgccatcaaatg	NM_001039849.3
			right	cggcaggtccttctctatca	
Gsr		64	left	ttcctcatgagaaccagatcc	NM_053906.2
			right	tgaaagaacccatcactggtta	
Txn1		126	left	atgccgaccttccagttcta	NM_053800.3
			right	ggcttcgagcttttccttgt	
Txn2		98	left	cacacagaccttgccattga	NM_053331.2
			right	acgtccccgttcttgatg	
Txnrd1		71	left	agcaccattggcatccac	NM_001351981.1
			right	ccacactggggcttaacct	
Txnrd2		97	left	gcacatggtgaagctacctaga	NM_022584.3
			right	gctccatccacatcttctcag	
Aco1		124	left	ttgctgtgtctgagattgaaaag	NM_017321.1
			right	cttgaaaacctttaaatccttgct	
Aco2		81	left	cgccttacagcctactggtc	NM_024398.2
			right	ggcagaggccacatggta	
Hmox1		4	left	gtcaagcacagggtgacaga	NM_012580.2
			right	ctgcagctcctcaaacagc	
Hmox2		128	left	tacggcaccagaaaaggaaa	NM_001277073.1
			right	gtgcttccttggtcccttc	
Blvrb			left	gtgaccgatgaccacatcc	NM_001106236.1
			right	gtgtaggccccggttagtg	
Ftl1			left	ttttgatcgggatgacgtg	NM_022500.4

			right	ttctgcaacttgaggagacg	
Nfe2l2	*Nrf2*	2	left	agcatgatggacttggaattg	NM_031789.2
			right	cctccaaaggatgtcaatcaa	
Hif1α		18	left	catgatggctccctttttca	NM_024359.1
			right	acatagtaggggcacggtca	
Hif2α		63	left	gcaactacctgttcaccaacc	NM_023090.1
			right	ttcatcaaagttctggcttcc	
Hif1an	*Fih1*	7	left	tgagaaactacaagctatccagca	NM_001113749.1
			right	cggtgtcattgagtgtttgc	
Egln1	*Phd2*	120	left	cgagcgagcaagagctaaag	XM_008772679.1
			right	ggcaactgagaggctgtagg	
Egln2	*Phd1*	66	left	cagcctatgccaccaggta	NM_001004083.1
			right	tgtcccgatgctagctgata	
Egln3	*Phd3*	29	left	acgccatgactgtctggtact	NM_019371.1
			right	gcaagagcagattcagtttttct	
Hprt1	*Hprt*	95	left	gaccggttctgtcatgtcg	NM_012583.2
			right	acctggttcatcatcactaatcac	
Ckm		16	left	ccgcagcatcaagggtta	NM_012530.2
			right	cccgtcaggctgttgaga	
Ckb		84	left	ccacttcctcttcgacaagc	NM_012529.3
			right	ggaacgtcttattgtcattgtgc	
Ckmt2	*mtCks*	83	left	gccacccctcattaagactg	NM_001127652.1
			right	caaaaaggtcagcaaacacct	
Hk1		121	left	tctgggcttcaccttctcat	NM_012734.1
			right	atcaagattccacagtccaggt	
Hk2		101	left	ccagcagaacagcctagacc	NM_012735.2
			right	agatgccttgaatccctttg	

3.7. Analysis of reduced and oxidised glutathione

The measurement of reduced and oxidised glutathione was performed according to slightly modified protocol of Reed *et al.* (1980) adapted by Yoshida (1996). We determined the concentrations of both reduced and oxidised states in heart LV samples. Homogenization of the tissue was performed in total volume of 1 ml containing cold 5% metaphosphoric acid and 10 mM EDTA. Proteins were precipitated, pelleted by centrifugation and discarded. Subsequently, 0.4 ml of supernatant was incubated with 0.1 ml of 0.4 M iodoacetic acid to block the thiol group of GSH and then with 0.1 ml of 1-fluoro-2,4-dinitrobenzene to derivatize amino groups of both GSH and GSSG. Incubation with glycine was used to remove excess reactive reagent. High-performance liquid chromatography (HPLC) system 1100 (Agilent, Santa Clara, CA, USA; Zorbax NH2 column; 4.6 mm × 150 mm; 5 μm) was utilized for the analysis of processed samples. The mobile phase for gradient elution was methanol-water mix in ratio 4:1 (v/v;

solution A) mixed with 2 mol/l sodium acetate-water-methanol mix in ratio 3:1:2 (v/v/v; solution B). Detection was performed at 365 nm of wavelength.

3.8. SDS-PAGE and Western blotting

The frozen tissue of heart LV was pulverized in liquid nitrogen and subsequently homogenized in homogenization buffer (12.5 mM Trizma base-TRIS, 2.5 mM EGTA, 1 mM EDTA, 250 mM sucrose, 5 mM DTT) supplemented with complete protease inhibitor cocktail (Roche Diagnostics). Whole LV homogenates as well as nucleus and cytoplasmic cellular fractions were prepared, aliquoted and stored at -80 °C prior to further use. The samples were loaded onto 10%, 12% or 15% bis-acrylamide gel and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed at a constant voltage of 200 mV. Proteins were subsequently transferred to nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany) using Mini-PROTEAN TetraCell (Bio-Rad, Hercules, CA, USA). After blocking with 5% dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 1 h at room temperature, the membranes were washed in TTBS and then incubated with primary antibodies against MnSOD, Cu/ZnSOD, GPX4 (Cayman Chemical Company, Ann Arbor, MI, USA), PRX3, PRX5 (AbFrontier, Soul, South Korea), ACO1, ACO2, BLVR, CAT, HMOX1, HMOX2, PRX6, TXN1, (Abcam, Cambridge, UK), TXN2 and NF-κB (sc-372; Santa Cruz Biotechnology, Dallas, TX, USA) (Table 2). Subsequently, the membranes were washed multiple times by TTBS and incubated with corresponding secondary antibodies anti-rabbit (Sigma-Aldrich, St. Louis, MO, USA; Bio-Rad) or anti-goat IgGs (Santa Cruz Biotechnology) conjugated with horseradish peroxidase for 1 h at room temperature. Incubation with enhanced chemiluminiscence substrate (SuperSignal West Dura Extended Duration Substrate, ThermoFisher Scientific) was performed prior to visualization by a LAS-4000 imaging system (Fujifilm Lifesciences, Cambridge, MA, USA). Relative protein level changes were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The samples for the groups which were subsequently compared were run on the same gel and quantified on the same membrane. GAPDH and actin were used as internal loading controls. The results were normalized to total protein amount and expressed as a percentage of the control as described in Waskova-Arnostova *et al.* (2015).

Table 2. List of used primary antibodies against target proteins and its specifications.

Protein	Manufacturer	Cat. number	Host	Clonalilty	Dilution
MnSOD	Cayman	10011390	rabbit	poly	1:2,000
Cu/ZnSOD	Cayman	10011387	rabbit	poly	1:2,000
CAT	Abcam	ab16731	rabbit	poly	1:2,000
PRX3	AbFrontier	LF-PA0030	rabbit	poly	1:2,000
PRX5	AbFrontier	LF-PA0010	rabbit	poly	1:2,000
PRX6	Abcam	ab59543	rabbit	poly	1:4,000
GPX4	Cayman	10005258	rabbit	poly	1:2,000
TXN1	Abcam	ab86255	rabbit	poly	1:1,000
TXN2	Santa Cruz Biotechnology	sc-50336	rabbit	poly	1:1,000
TXNRD1	Cell Signaling	6925	rabbit	poly	1:1,000
ACO1	Abcam	ab126595	rabbit	mono	1:3,333
ACO2	Abcam	ab129105	rabbit	mono	1:30,000
HMOX1	Abcam	ab13243	rabbit	poly	1:2,000
HMOX2	Abcam	ab90492	rabbit	poly	1:1,000
BLVR	Abcam	ab19260	rabbit	poly	1:5,000

3.9. Nuclear extract and Nrf2 transcriptional activity

Preparation of nuclear extract was performed using Dignam's protocol (Dignam *et al.*, 1983) slightly modified by Jirkovsky (Jirkovsky *et al.*, 2012) using 100 mg of heart LV tissue. The amount of transcriptionally active form of Nrf2 was analysed by commercial kit TransAM Nrf2 (Active Motif, Inc., Carlsbad, CA, USA) according to the manufacturer's instruction. 20 µg of nuclear extract were loaded into each well.

3.10. Cryosection preparation

After rapid excision, hearts were perfused under constant pressure with relaxing calcium free Tyrode´s solution (140 mM NaCl, 5.4 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2.6H_2O$, 10 mM glucose, 5 mM HEPES, pH 7.4) for 2 min followed by 4% methanol-free formaldehyde (ThermoFisher Scientific) for 2 min using Langendorff apparatus. Subsequently, hearts were immersed in 4% formaldehyde for 1 h and then incubated in 20% sucrose overnight. Ventricles were cut transversally in one-third from apex, separated, snap-frozen in liquid nitrogen, and then stored in -80°C. 5-7 µm thick cryosections were prepared from frozen ventricles using a cryostat

(Leica CM3050, Leica-microsystems, Wetzlar, Germany) and used used for indirect immunofluorescence staining.

3.11. Immunofluorescence analysis

Prepared cryosections were fixed in 4% methanol-free formaldehyde (ThermoFisher Scientific) and permeabilized in 100% ice-cold methanol. Subsequently, they were incubated in serum diluted in PBS containing 0.3% Triton X-100 and 0.3 M glycine (Sigma-Aldrich) for 1 h at room temperature to block non-specific binding sites. Thereafter, incubation with primary polyclonal antibody against TXN1 (Table 2), was performed for 1 h at room temperature followed by incubation with donkey anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (Invitrogen, Molecular Probes, Carlsbad, CA, USA). Stained sections were mounted in ProLong Gold Antifade Reagent containing a nuclei marker 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Molecular Probes). The images were acquired using a wide field fluorescence microscope (Olympus Cell-R IX2-UCB, Olympus, Hamburg, Germany) equipped with Plan-Apochromat 100x/1.4 NA objective and ORCA camera C4742-80-12AG (Hamamatsu, Shizuoka, Japan) as described in Waskova-Arnostova *et al.* (2015).

3.12. Enzyme-linked immunosorbent assay (ELISA)

Heart LV samples were pulverized in liquid nitrogen and mixed with homogenization buffer (12.5 mM Tris, 2.5 mM EGTA, 100 mM NaF, 250 mM sucrose, 0.1 mM activated orthovanadate, 6 mM mercaptoethanol, complete protease inhibitor cocktail (Roche Diagnostics), 1% Triton X-100). The samples were homogenized by sonication (amplitude:100, cycle: 0.5, 30 seconds) and centrifuged at 10,000g for 10 min in 4°C. The supernatants were used for subsequent analysis of specific protein content by ELISA. Specifically, levels of TNFα, IL10 and IL6 were measured by DuoSet ELISA capture kits (eBioscience, Vienna, Austria; TNFα: BMS622, IL6: BMS625, IL10: BMS629) according to the manufacturer's instructions. Obtained protein level values were expressed as pg/mg of total protein.

3.13. Statistical analyses

All statistical analyses were performed using the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). Differences between groups were compared by analyses of variance (ANOVA) after confirmation of the normal distribution. Myocardial IS, heart rate (HR) and mean arterial blood pressure (MAP) was determined in 7 – 10 hearts from each experimental group. Results of physiological measurements (HR and MAP) were evaluated by the ANOVA procedure for repeated measurements with subsequent Bonferroni post-hoc test. One-way ANOVA was used for evaluation of IS and weight parameters with subsequent Newmann-Keuls post-hoc test. Regression and correlation analysis (Pearson) were used to assess the relationship between IS/LV and AR/LV. Five or six hearts from each experimental group were used for WB, qPCR analyses, ELISA assays and transcriptional factor activities. One-way ANOVA followed by post-hoc test with Bonferroni correction for multiple comparisons was used for statistical analyses in case of evaluation of normobaric hypoxia groups (CNH, INH-8, INH-23) and the unpaired Student's t-test was used to determine differences in IHH-8 group and normoxic group. The WB immunoreactivity and relative mRNA abundance were expressed as a percentage of the control. Data were expressed as mean ± SEM and P values less than 0.05 were considered statistically significant.

4. Results

4.1. The analysis of antioxidant system in protective and non-protective regimens of chronic hypoxia

Our first aim was to determine the effects of different modes of hypoxia (normobaric and hypobaric) and different regimens of chronic hypoxia (continuous and intermittent) on the expression of various components of antioxidant system with respect to changes in myocardial tolerance to acute I/R injury.

4.1.1. The mRNA expression of the antioxidant enzymes in chronic continuous and intermittent normobaric hypoxia.

All regimens of adaptation to normobaric hypoxia decreased the body weight in all hypoxic group compared with normoxic controls. The most conspicuous decrease in body weight was observed in INH-23 group. Accordingly, all regimens of the hypoxia resulted in the apparent hypertrophy of the RV, when the RV/body weight (BW) ratio significantly increased by 180% in CNH, 129% in INH-8 and 163% in INH-23, respectively. Nevertheless, neither the LV weight nor LV/BW ratio was affected. Importantly, hematocrit was increased in all hypoxic groups (139% in CNH, 128% in INH-8 and 144% in INH-23, respectively) compared with the control groups. There was also seen a difference between hypoxic regimens when a less significant decline was observed in INH-8 than in both CNH and INH-23 groups (Table 3).

Table 3. Heart weight parameters

Group	n	BW (g)	RV/BW (mg/g)	LV/BW (mg/g)	Hematocrit (%)
Normoxia	10	370 ± 10	0.49 ± 0.01	1.33 ± 0.03	40.6 ± 1.0
CNH	8	337 ± 7*#	0.88 ± 0.03*†#	1.26 ± 0.03†#	56.3 ± 1.0*†
INH-8	10	334 ± 8*	0.63 ± 0.02*	1.39 ± 0.03	52.1 ± 1.3*
INH-23	7	307 ± 6*†	0.80 ± 0.03*†	1.40 ± 0.04	58.3 ± 0.9*†

*CNH, continuous normobaric hypoxia; INH-8, intermittent normobaric hypoxia for 8 h/day; INH-23, intermittent normobaric hypoxia for 23 h/day; n, number of rats; BW, body weight; RV/BW, relative right ventricular weight; LV/BW, relative left ventricular weight. Values are mean SEM; * P<0.05 vs. Normoxia; † P<0.05 vs. INH-8; # P<0.05 vs. INH-23.*

4.1.1.1. Hemodynamic parameters and analysis of infarct size

The HR and MAP values were determined at the baseline (before ischemia), immediately after 20 minutes of ischemia and at the end of 3h reperfusion. No significant changes in HR and MAP parameters were detected in baseline and after ischemia between the groups. After reperfusion period both parameters, HR and MAP, were significantly higher compared with the values at the end of ischemia in normoxic group. Rats adapted to CNH and INH-8 had a significantly higher MAP after 3h reperfusion than the normoxic animals (Table 4).

Table 4. Heart rate and mean arterial blood pressure during ischemia/reperfusion injury

	Baseline	Ischemia (20 min)	Reperfusion (3 h)
Heart rate (beats/min)			
Normoxia	413 ± 10	436 ± 10	378 ± 12†#
CNH	408 ± 10	401 ± 8	396 ± 9
INH-8	421 ± 8	429 ± 7	421 ± 11
INH-23	410 ± 16	406 ± 14	389 ± 16
Blood pressure (mmHg)			
Normoxia	115 ± 7	124 ± 5	102 ± 6#
CNH	131 ± 5	127 ± 8	121 ± 5*
INH-8	126 ± 5	134 ± 4	130 ± 2*
INH-23	124 ± 4	128 ± 4	113 ± 4

*CNH, continuous normobaric hypoxia; INH-8, intermittent normobaric hypoxia for 8 h/day; INH-23, intermittent normobaric hypoxia for 23 h/day. Values are mean SEM; *P<0.05 vs. Normoxia; † P<0.05 vs. Baseline; # P<0.05 vs. Ischemia.*

In all experimental groups the AR was between 29-32% of the respective size of LV and the AR/LV ratio did not significantly differ among the groups (Table 5). In the normoxic animals, IS reached approximately 62% of the AR. However, CNH and INH-8 regimens noticeably reduced IS/AR ratio (to about 41% and 46%, respectively). On the other hand, INH-23 did not lower IS compared with normoxic group (Fig. 1A). Fig. 1B shows a high correlation between IS/LV and AR/LV ratios. All experimental groups did not differ significantly in the slope of linear regression lines. Analysis of the relationship between IS and the size of AR revealed that the cardioprotective effect of INH-8 and CNH was manifested by a parallel downward shift of linear regression lines.

Table 5. Infarct size and area at risk normalized to the left ventricle

Group	n	AR/LV (%)	IS/LV (%)
Normoxia	10	29.5 ± 3.1	18.5 ± 2.1
CNH	8	31.1 ± 3.0	13.1 ± 2.1#
INH-8	10	32.1 ± 2.3	15.1 ± 1.9
INH-23	7	31.9 ± 4.0	21.4 ± 3.0

CNH, continuous normobaric hypoxia; INH-8, intermittent normobaric hypoxia for 8 h/day; INH-23, intermittent normobaric hypoxia for 23 h/day; n, number of rats; AR/LV, area at risk normalized to the left ventricle; IS/LV, infarct size normalized to the left ventricle. Values are mean SEM; # P<0.05 vs. INH-23

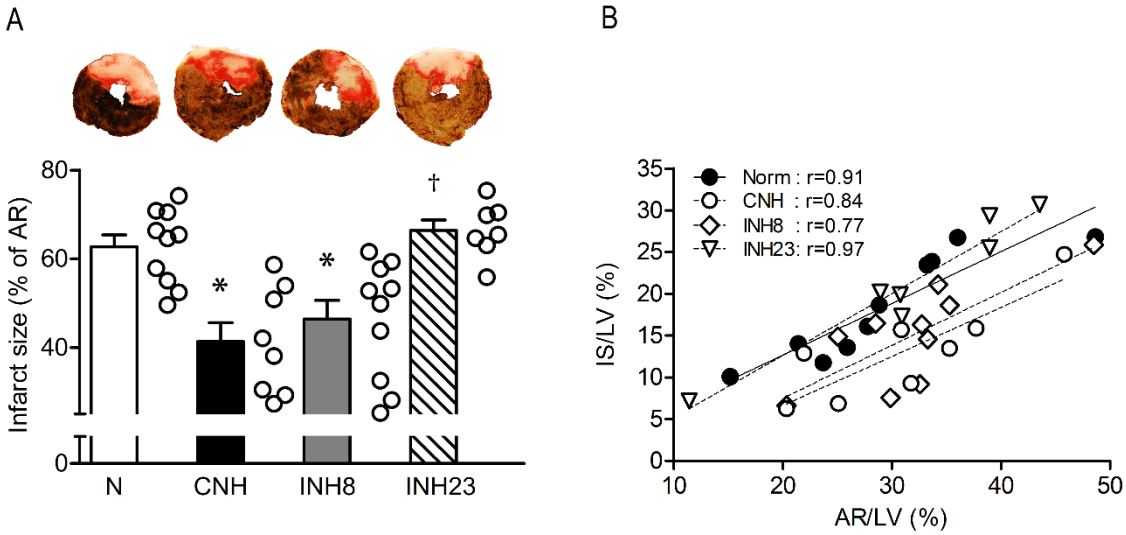


*Fig. 1. The effect of continuous and intermittent normobaric hypoxia on myocardial infarct size. Rats were exposed to hypoxia for 3 weeks continuously (CNH) or intermittently for 8 h/day (INH-8) or for 23 h/day (INH-23) and compared with animals kept at normoxia (N). A: myocardial infarct size (IS) expressed as a percentage of the area at risk (AR). B: relationship between the area at risk (AR) and infarct size (IS), both expressed as a percentage of the left ventricle (LV). Values are means ± SEM; *P<0.05 vs. N; †P<0.05 vs. other hypoxic groups (INH-8, CNH).*

4.1.1.2. mRNA expression of the antioxidant enzymes and the level of oxidative stress in LV myocardium.

Our data shows a significant increase in the expression of first line antioxidant defence against ROS, e.g. superoxide dismutase family. Both isoforms, *MnSod* and *Cu/ZnSod*, were significantly increased by about 16% in the CNH protective regimen. *MnSod* was also markedly elevated in the INH-8 group, but *Cu/ZnSod* only tended to increase (Fig. 2). No change in the expression of both Sod isoforms was observed after INH-23 compared with normoxic group.

Interestingly, *Cat* had an opposite trend and inclined to decrease in both protective regimens (CNH and INH-8) compared with the controls. The downtrend of expression in CNH and INH-8 groups were significant if compared with INH-23 group (Fig. 2).

Peroxiredoxin family (*Prx*), which members are capable of directly scavenging peroxide, was represented in our analysis by all 6 isoforms. As shown in Fig. 2, *Prx1* did not change in any of the hypoxic regimens. Both protective regimens (CNH and INH-8) elevated expression of *Prx2* by about 22% and this increase was also significant compared with INH-23 group. *Prx3* and *Prx4* remained unchanged in protective regimens, but their expression in INH-23 group was significantly lower than in INH-8 group. *Prx5* expression markedly increased in both CNH (by 65%) and INH-23 (by 89%) groups. The mRNA level of the isoform *Prx6* was significantly higher in INH-8 group by 31% compared with normoxic controls (Fig. 2).

Furthermore, we analysed components of another important antioxidant system of the cell defence against ROS, glutathione system, which was represented by glutathione peroxidases 1 and 4 (*Gpx1*, *Gpx4*), glutathione reductase (*Gsr*) and GSH/GSSG ratio. Both *Gpx*1 and *Gpx4* tended to increase in both protective regimens, but statistical significance was reached only for *Gpx4* in CNH group (Fig. 2). A significant increase of *Gsr* expression was observed in CNH and INH-8 groups (Fig. 3).

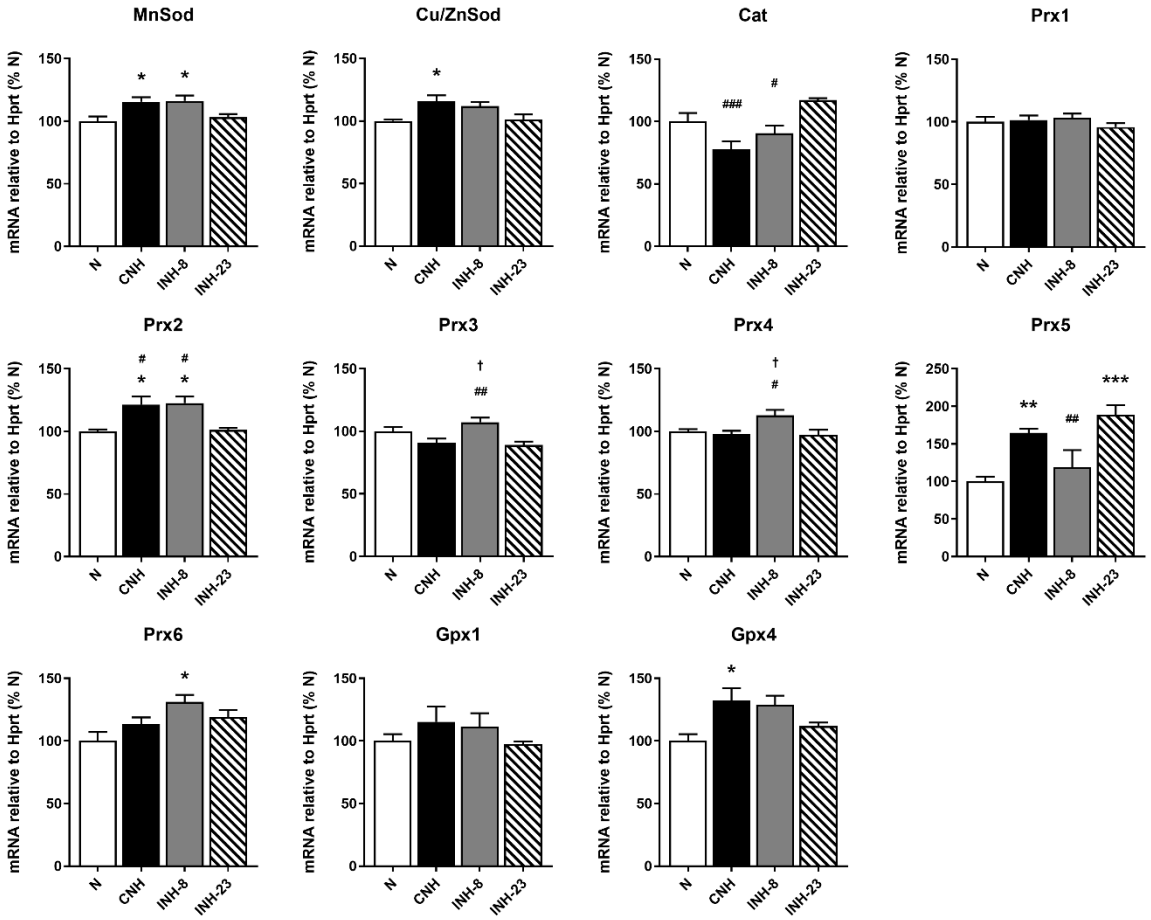


*Fig. 2. The effect of different regimens of chronic normobaric hypoxia on the mRNA expression of myocardial antioxidants. Transcripts were assessed in LV preparations from control normoxic rats (N) and those adapted to continuous (CNH) or intermittent (INH-8 and INH-23) normobaric hypoxia. The mRNA transcript levels were normalized to the reference gene Hprt1. Cu/ZnSod, cooper-zinc superoxide dismutase; MnSod manganese superoxide dismutase; Cat, catalase; Prx, peroxiredoxin; Gpx, glutathione peroxidase. The values are the means ± SEM and they are expressed as percentage of control (100%); *P<0.05 vs. N, **P<0.01 vs. N, ***P<0.001 vs. N,† P<0.05 vs. CNH, † P<0.05 vs. CNH, # P<0.05 vs. INH-23, ## P<0.01 vs. INH-23, ### P<0.001 vs. INH-23, (n=5).*

The ratio of reduced and oxidised glutathione (GSH/GSSG) reflecting the level of oxidative stress was maintained in both protective regimens, however it significantly decreased by 21% in INH-23 (Fig. 3).

Thioredoxin family is one of the main regulatory mechanism of the redox homeostasis in the cell. None of the hypoxia regimens significantly affected the mRNA levels of cytosolic isoform *Txn1* and its reductase *Txnrd1*. However, both protective regimens significantly enhanced expression of mitochondrial isoforms *Txn2* (CNH by 35% and INH-8 by 53%) and *Txnrd2* (CNH by 19% and INH-8 by 24%). No significant changes were found in the expression of *Txn* system in INH-23 group (Fig. 3).

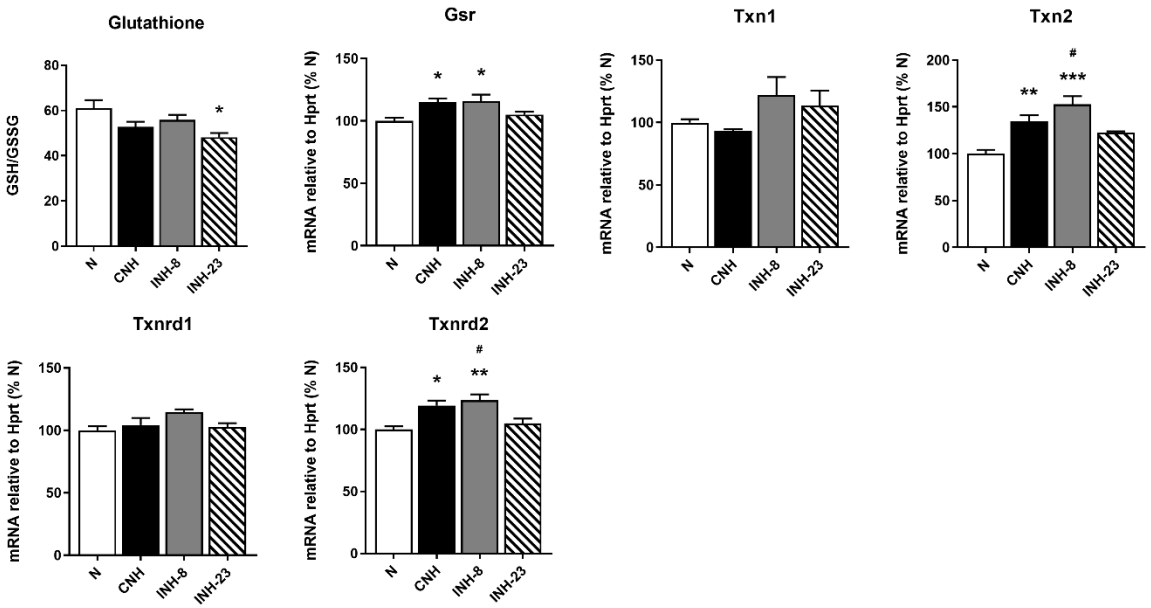


*Fig. 3. The effect of different regimens of chronic normobaric hypoxia on the GSH/GSSG ratio and on the mRNA expression of antioxidants maintaining redox state. GSH, GSSG and transcripts were assessed in LV preparations from control normoxic rats (N) and those adapted to continuous (CNH) or intermittent (INH-8 and INH-23) normobaric hypoxia. The GSH/GSSG ratio was determined by measurement of the content of reduced (GSH) and oxidised (GSSG) glutathione by HPLC. The mRNA transcript levels were normalized to the reference gene Hprt1. Gsr, glutathion reductase; Txn, thioredoxin; Txnrd, thioredoxin reductase. The values are the means ± SEM and they are expressed as percentage of control (100%), *P<0.05 vs. N, **P<0.01 vs. N, ***P<0.001 vs. N, # P<0.05 vs. INH-23, (n=5).*

Another group of ROS scavengers is composed of enzymes responsible for maintaining the levels of free iron. Whereas *Aco1* expression was elevated only after INH-8 exposure (by 33%), mRNA levels of mitochondrial isoform *Aco2* markedly increased in all hypoxic groups (by 56-74%). Constitutively expressed *Hmox1* was raised in CNH group by 70% and inducible *Hmox2* was not affected except for decreased expression in INH-23 group when compared with INH-8 group (Fig. 4).

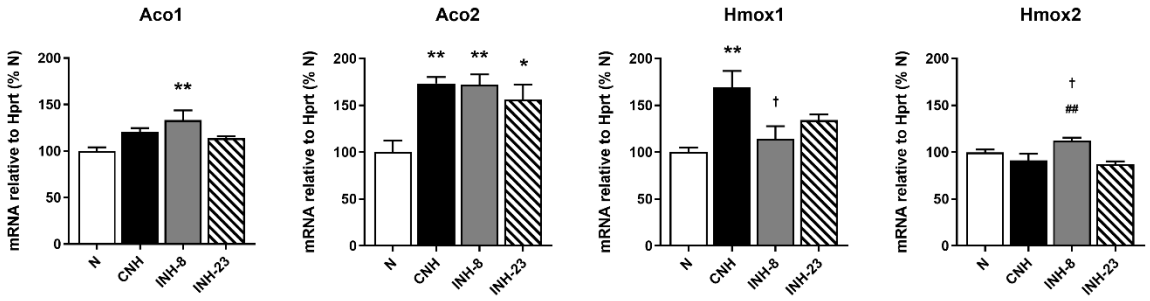


*Fig. 4. The effect of different regimens of chronic normobaric hypoxia on the mRNA expression of enzymes related to iron metabolism. Aconitase (Aco) and heme oxygenase (Hmox) transcripts were assessed in LV preparations from control normoxic rats (N) and those adapted to continuous (CNH) or intermittent (INH-8 and INH-23) normobaric hypoxia. The mRNA transcript levels were normalized to the reference gene Hprt1. The values are the means ± SEM and they are expressed as percentage of control (100%), *P<0.05 vs. N, **P<0.01 vs. N, † P<0.05 vs. CNH, ## P<0.01 vs. INH-23, (n=5).*

4.1.1.3. mRNA expression of associated transcription factors and their regulatory enzymes.

The main transcription factor regulating the expression of most antioxidants is NRF2. The mRNA levels of *Nrf2* were significantly increased only in INH-8 group by 21% (Fig. 5). HIF plays the crucial transcriptional role during the adaptation to hypoxia. *Hif1α* expression was also increased only in INH-8 group by 18%. On the other hand, *Hif2α* transcript levels were markedly elevated in both CNH and INH-8 groups (by 112% and 109%, respectively) and a similar effect was observed in expression of *Fih1* (increase by 117% and 79%, respectively). Expression of other *Phd1*, *Phd2* and *Phd3*, was not affected in any of the hypoxic group.

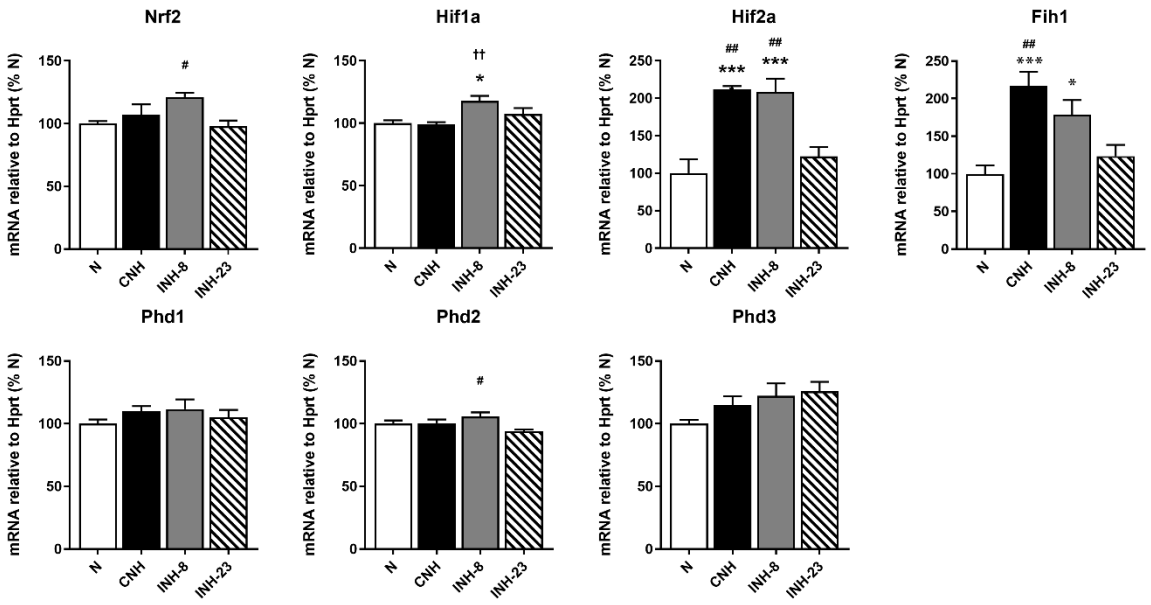


*Fig. 5. The effect of different regimens of chronic normobaric hypoxia on the mRNA expression of transcriptional factors and their regulating enzymes. Transcripts of nuclear factor, erythroid 2-like 2 (Nrf2), hypoxia inducible factor (Hif), factor inhibiting Hif1 (Fih1) and prolyl hydroxylases (Phd) were assessed in LV preparations from control normoxic rats (N) and those adapted to continuous (CNH) and intermittent (INH-8 and INH-23) normobaric hypoxia. The mRNA transcript levels were normalized to the reference gene Hprt1. The values are the means ± SEM and they are expressed as percentage of control (100%), *P<0.05 vs. N, ***P<0.001 vs. N, †† P<0.01 vs. CNH, # P<0.05 vs. INH-23, ## P<0.01 vs. INH-23, (n=5).*

Table 6 shows the comparison of changes in mRNA expression of all analysed antioxidants and enzymes in protective (CNH and INH-8) and non-protective (INH-23) regimens of chronic normobaric hypoxia. Most of the antioxidants were significantly increased at mRNA levels in protective regimens albeit with some differences between CNH and INH-8 groups. On the other hand, the non-protective regimen (INH-23) was associated with the absence of antioxidant response except for increased expression of *Prx5* and *Aco2*. Interestingly, all hypoxic groups exhibited increased expression of *Hk2* and decreased expression of *Hk1*.

Table 6. The comparison of mRNA expression of the antioxidants and enzymes in protective (CNH and INH-8) and non-protective (INH-23) regimens of chronic normobaric hypoxia.

Name of gene	Symbol		CNH	INH-8	INH-23
Cu/Zn superoxide dismutase	*Cu/ZnSod*		↑	-	-
Mn superoxide dismutase	*MnSod*		↑	↑	-
catalase	*Cat*		-	-	-
peroxiredoxin 1	*Prx1*		-	-	-
peroxiredoxin 2	*Prx2*		↑	↑	-
peroxiredoxin 3	*Prx3*		-	-	-
peroxiredoxin 4	*Prx4*		-	-	-
peroxiredoxin 5	*Prx5*		↑	-	↑
peroxiredoxin 6	*Prx6*		-	↑	-
glutathione perixodase 1	*Gpx1*		-	-	-
glutathione perixodase 4	*Gpx4*		↑	-	-
glutathione reductase	*Gsr*		↑	↑	-
thioredoxin 1	*Txn1*		-	-	-
thioredoxin 2	*Txn2*		↑	↑	-
thioredoxin reductase 1	*Txnrd1*		-	-	-
thioredoxin reductase 2	*Txnrd2*		↑	↑	-
aconitase 1	*Aco1*		-	↑	-
aconitase 2	*Aco2*		↑	↑	↑
heme oxygenase 1	*Hmox1*		↑	-	-
heme oxygenase 2	*Hmox2*		-	-	-
nuclear factor, erythroid derived 2, like 2	*Nrf2*		-	↑	-
hypoxia inducible factor 1 alfa	*Hif1a*		-	↑	-
hypoxia inducible factor 2 alfa	*Hif2a*		↑	↑	-
factor inhibiting HIF1	*Fih1*		↑	↑	-
HIF-prolyl hydroxylase 1	*Phd1*		-	-	-
HIF-prolyl hydroxylase 2	*Phd2*		-	-	-
HIF-prolyl hydroxylase 3	*Phd3*		-	-	-
creatine kinase, muscle	*Ckm*		-	-	↓
creatine kinase, brain	*Ckb*		-	-	-
mitochondrial creatine kinase, sarcomeric	*mtCks*		↑	↑	-
hexokinase 1	*Hk1*		↓	↓	↓
hexokinase 2	*Hk2*		↑	↑	↑

CNH, continuous normobaric hypoxia; INH-8, intermittent normobaric hypoxia for 8 h/day; INH-23, intermittent normobaric hypoxia for 23 h/day. Arrows indicate significantly increased or decreased expression compared to normoxic group.

4.1.2. The mRNA and protein expression of antioxidant enzymes in severe chronic intermittent hypobaric hypoxia.

Adaptation to chronic severe intermittent hypobaric hypoxia (IHH-8) significantly decreased mRNA levels of main antioxidants in rat myocardium when compared with normoxic group (Fig. 6), namely: *MnSod* (by 18 %), *Cu/ZnSod* (by 22 %), *Cat* (by 20 %), *Prx3* (by 18%), *Prx5* (by 14%), *Prx6* (by 14%) and *Gpx4* (by 32%). These changes were partially reflected at the protein level when trends to decrease were observed (Fig. 7). The significant decline in the protein levels was found only in the case of PRX3 (by 27%) and PRX5 (by 27%).

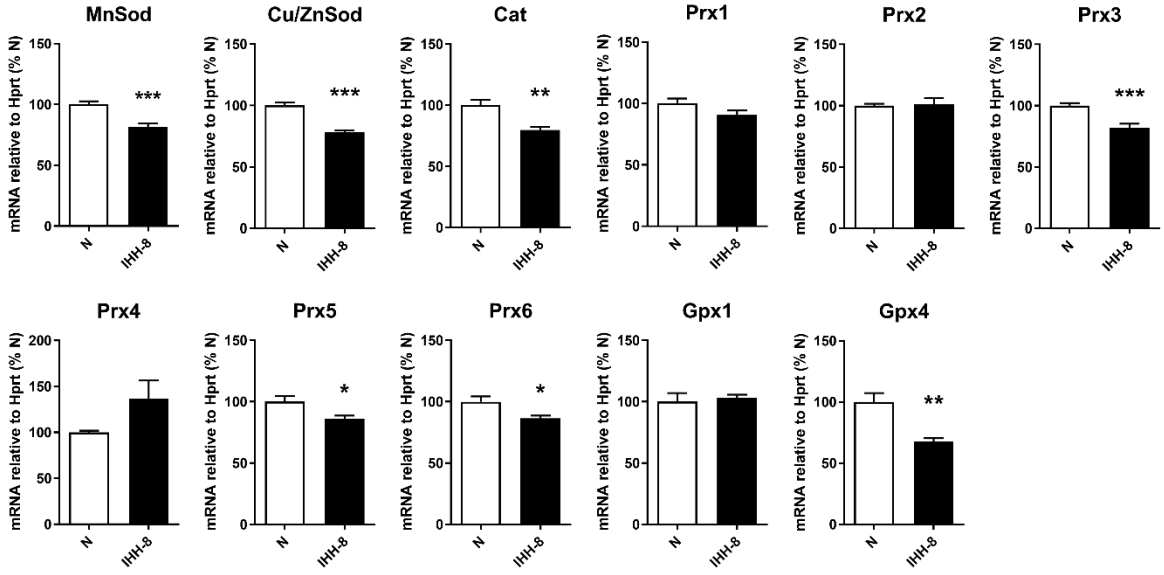


*Fig. 6. The effect of chronic intermittent hypobaric hypoxia (IHH-8) on the mRNA expression of manganese superoxide dismutase (MnSod), cooper-zinc superoxide dismutase (Cu/ZnSod), catalase (Cat), peroxiredoxin (Prx) and glutathione peroxidase (Gpx). Transcripts were determined in the left ventricles (LV) from control (N) and hypoxic (IHH-8) rats. The mRNA levels were normalized to the reference gene Hprt1. The values are the mean ± SEM, expressed as a percentage of control; *P < 0.05 vs. N, **P<0.01 vs. N, ***P<0.001 vs. N, (n=6).*

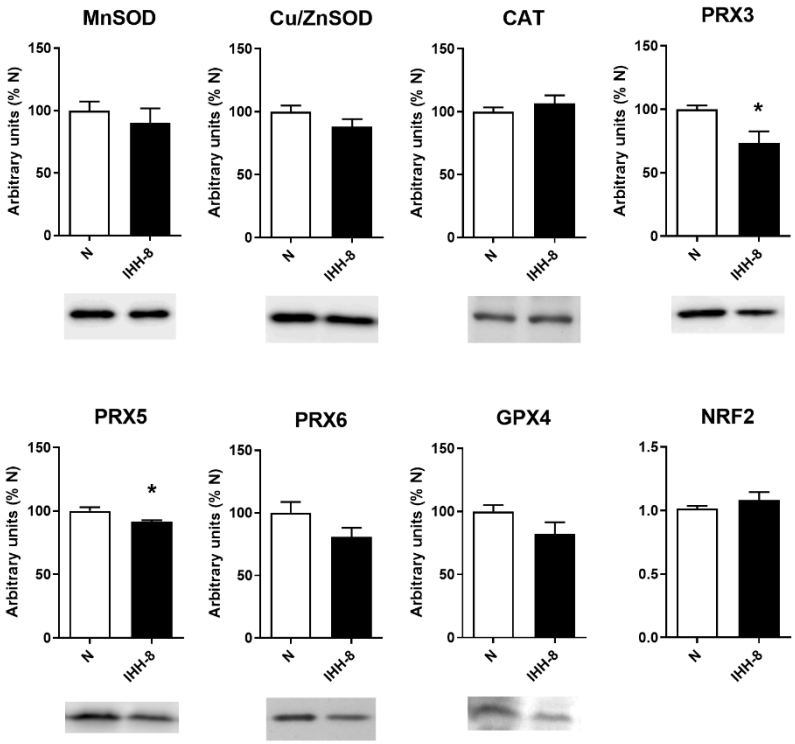


*Fig. 7. The effect of chronic intermittent hypobaric hypoxia (IHH-8) on the protein levels of manganese superoxide dismutase (MnSOD), cooper-zinc superoxide dismutase (Cu/ZnSOD), catalase (CAT), peroxiredoxin (PRX) and glutathione peroxidase (GPX) and nuclear factor, erythroid 2-like 2 (NRF2). Protein levels were determined in the left ventricle (LV) homogenates from control (N) and hypoxic (IHH-8) rats by Western blot, except NRF2, which transcriptionally active form was determined in LV nuclear fraction by TransAM kit. The values are the mean ± SEM, expressed as a percentage of control; *P < 0.05 vs. N, (n=6).*

On the other hand, the only significant increases at mRNA levels (Fig. 8) were seen in cytosolic *Aco1* (by 71%), inducible *Hmox1* (by 23%) and *Ftl1* (by 72%). However, this increase was confirmed on protein level (Fig. 9) only in case of HMOX1 (by 149%). The mRNA and protein levels of the mitochondrial isoform *Aco2*, constitutive *Hmox2* and biliverdin reductase B (*Blvrb*) remained unchanged after IHH-8 (Fig. 8 and 9).

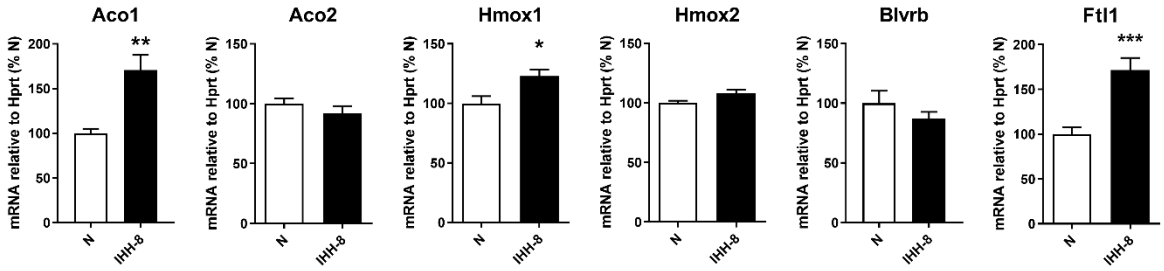


*Fig. 8. The effect of chronic intermittent hypobaric hypoxia (IHH-8) on the mRNA expression of aconitase (Aco), heme oxygenase (Hmox), biliverdin reductase (Blvrb) and ferritin (Ftl1). Transcripts were determined in the left ventricles (LV) from control (N) and hypoxic (IHH-8) rats. The mRNA levels were normalized to the reference gene Hprt1. The values are the mean ± SEM, expressed as a percentage of control; *P < 0.05 vs. N, **P<0.01 vs. N, ***P<0.001 (n=6).*

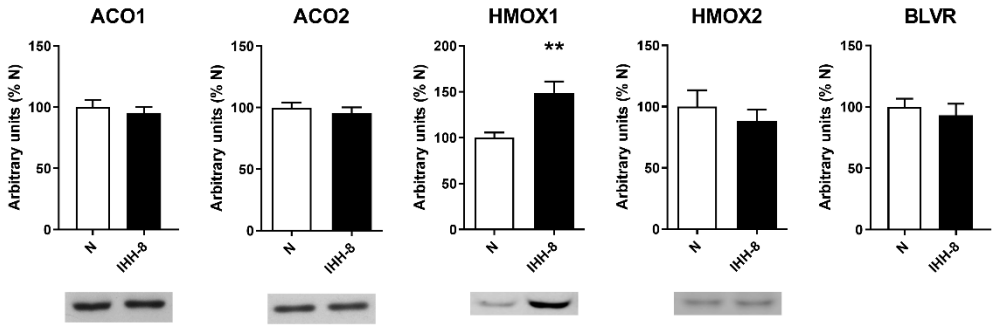


*Fig. 9. The effect of chronic intermittent hypobaric hypoxia (IHH-8) on the protein levels of aconitase (ACO), heme oxygenase (HMOX) and biliverdin reductase (BLVR). Protein levels were determined by Western blot in the left ventricle (LV) homogenates from control (N) and hypoxic (IHH-8) rats. The values are the mean ± SEM, expressed as a percentage of control; **P < 0.01 vs. N, (n=6).*

The Fig. 11 shows that the expression of cytosolic isoform TXN1 significantly increased at the protein level after adaptation to IHH-8 by 122 %. However, mitochondrial TXN2 and also cytosolic thioredoxin reductase, TXNR1, changed neither at mRNA nor at protein level (Fig. 10 and 11). Only *Txnrd2* was decreased by 17% on mRNA levels in IHH-8 group (Fig. 10).

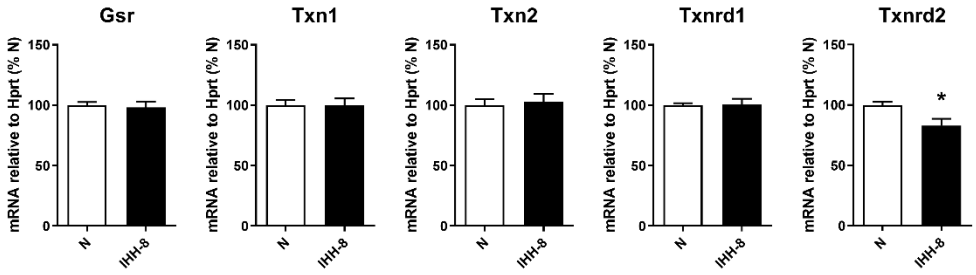


*Fig. 10. The effect of chronic intermittent hypobaric hypoxia (IHH-8) on the mRNA expression of glutathione reductase (Gsr), thioredoxin (Txn) and thioredoxin reductase (Txnrd). Transcripts were determined in the left ventricles (LV) from control (N) and hypoxic (IHH-8) rats. The mRNA levels were normalized to the reference gene Hprt1. The values are the mean ± SEM, expressed as a percentage of control; *P < 0.05 vs. N, (n=6)*

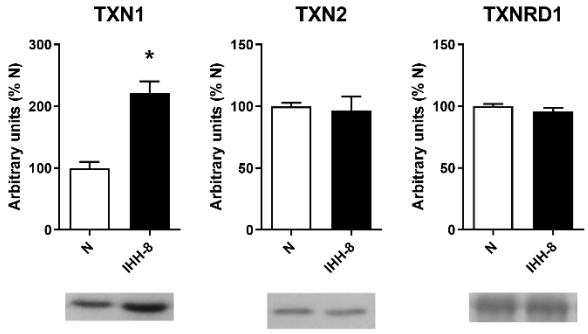


*Fig. 11. The effect of chronic intermittent hypobaric hypoxia (IHH-8) on the protein levels of thioredoxin (TXN) and thioredoxin reductase (TXNRD). Protein levels were determined by Western blot in the left ventricle (LV) homogenates from control (N) and hypoxic (IHH-8) rats. The values are the mean ± SEM, expressed as a percentage of control; *P < 0.05 vs. N, (n=6).*

The main transcription factor for most of the antioxidants, *Nrf2*, increased at mRNA level by 17 % (Fig. 12), but its transcriptionally active form of the protein did not change after adaptation to IHH-8 (Fig. 7). The mRNA levels of both *Hif1α* and *Hif2α* remained unchanged, too. Likewise, there were no differences in expression of *Fih1* or *Phd* isoforms at mRNA levels when compared with the normoxic group (Fig. 12).

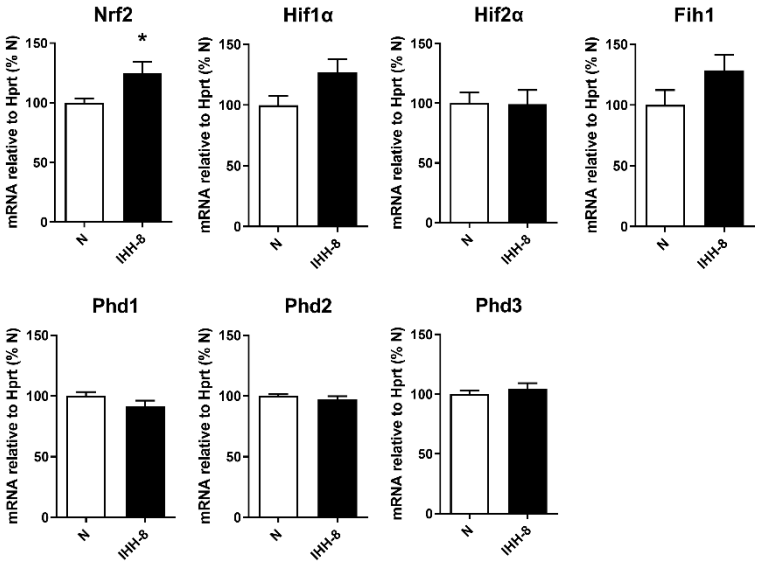


*Fig. 12. The effect of chronic intermittent hypobaric hypoxia (IHH-8) on the mRNA expression of nuclear factor, erythroid 2-like 2 (Nrf2), hypoxia inducible factor (Hif), factor inhibiting Hif1 (Fih1) and prolyl hydroxylases (Phd). Transcripts were determined in the left ventricles (LV) from control (N) and hypoxic (IHH-8) rats. The mRNA levels were normalized to the reference gene Hprt1. The values are the mean ± SEM, expressed as a percentage of control; *P < 0.05 vs. N, (n=6).*

4.2. TXN1 and its main redox homeostasis target transcription factor NF-κB in severe chronic intermittent hypobaric hypoxia.

TXN1, well known reductant, can translocate to the nucleus under conditions of oxidative stress where it reduces redox-sensitive cysteines of transcription factors and thus enable their transcriptional activity on target genes. NF-κB is one of these transcriptional factors being very important in the regulation of inflammation, redox homeostasis and cell survival acting via transcriptional activation of its target genes.

The immunofluorescence analysis revealed translocation of TXN1 from the cytosol into the nucleus after IHH-8 (Fig. 13A). This observation was confirmed by western blotting of both LV homogenates and nuclear fractions. While the total amount of cytosolic TXN1 decreased by 28%, the level of its active reduced form increased by 53% in the nuclear fraction after IHH-8 (Fig. 13B).

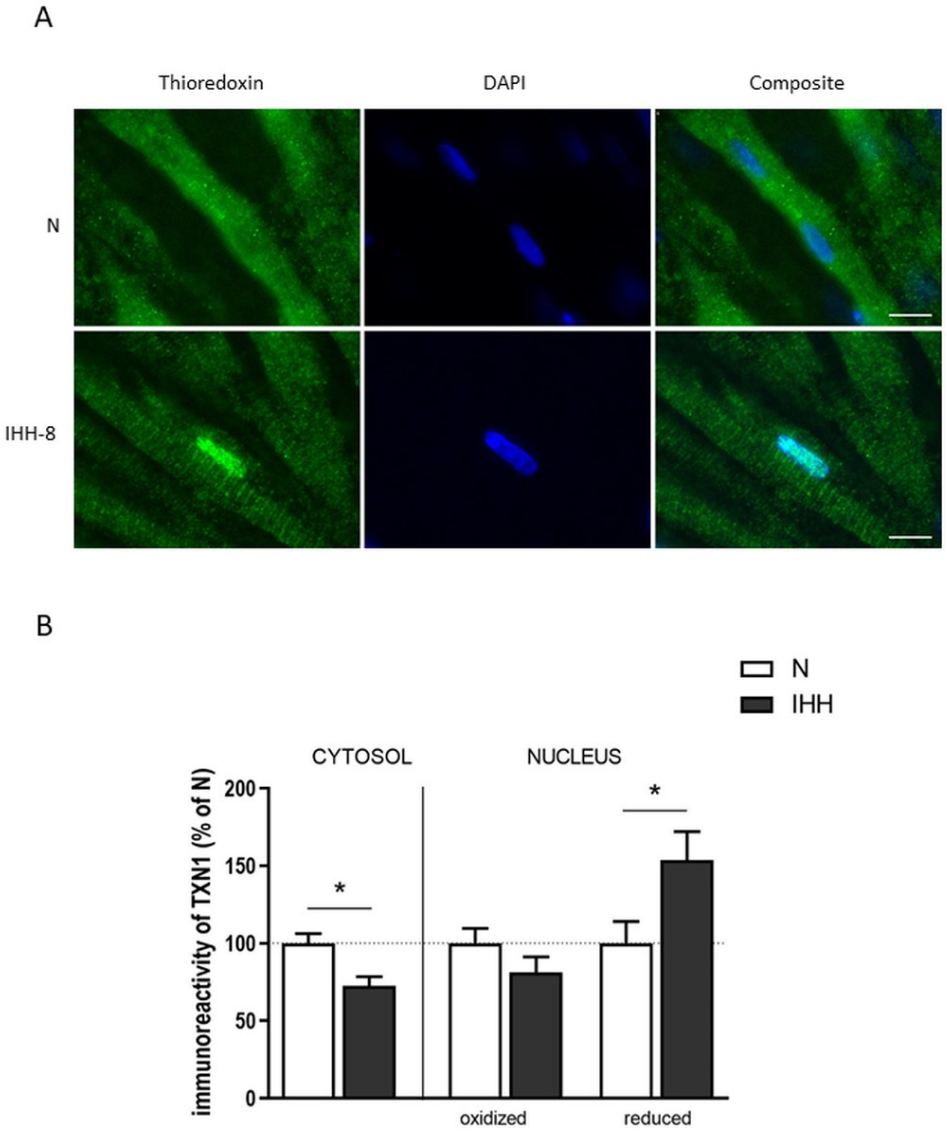


*Fig. 13. Cellular localization of thioredoxin 1 (TXN1). (A) The effect of chronic intermittent hypobaric hypoxia (IHH-8) on the translocation of TXN1 into the nucleus assessed by immunofluorescence. The green color represents the specific TXN1 staining (left) and blue color indicates the nucleus staining by DAPI (middle). The left ventricle (LV) from normoxic control stained only by TXN1 (left upper) and by both TXN1 and DAPI (right upper) is compared with IHH-8 LV stained by TXN1 (left lower) and both TXN1 and DAPI (right lower). The light blue color represents increased co-localization of TXN1 with the nucleus (right) in IHH-8. (B) The redistribution of oxidised and reduced form of TXN1 detected by Western blot. The relative protein levels were determined in LV from control (N) and hypoxic (IHH-8) groups. The values are the mean ± SEM, expressed as a percentage of control, *P < 0.05 vs. N, (n=6).*

IHH-8 significantly increased protein level of NF-κB p65/p50 subunit by 35% in homogenate and markedly elevated its level in the nuclear fraction by 197% (Fig. 14). In line with that, the protein levels of TNFα increased by 86% after IHH-8 (Fig. 15). Also, cytokines IL6 and IL10 increased by 76% and 57%, respectively. The ratio of IL10 to TNFα did not change after adaptation.

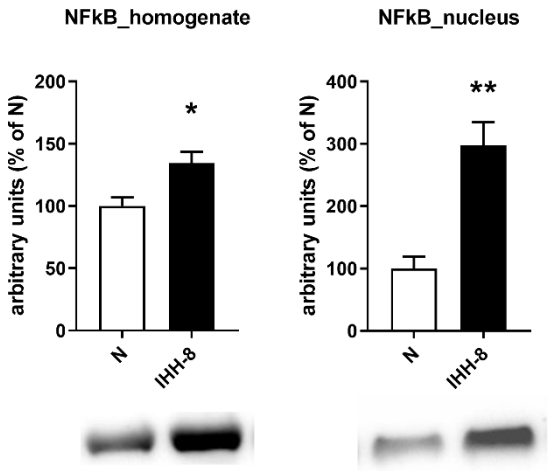


*Fig. 14. The effect of chronic intermittent hypobaric hypoxia (IHH-8) on nuclear factor kappa B (NF-κB) p65/p50 subunit protein levels in homogenate and in the nuclear fraction. The protein levels were determined in the left ventricle (LV) from rats in control (N) and hypoxic (IHH-8) groups. The values are the mean ± SEM, expressed as a percentage of the control (100%), *P < 0.05 vs. N, **P < 0.01 vs. N, (n=6)*

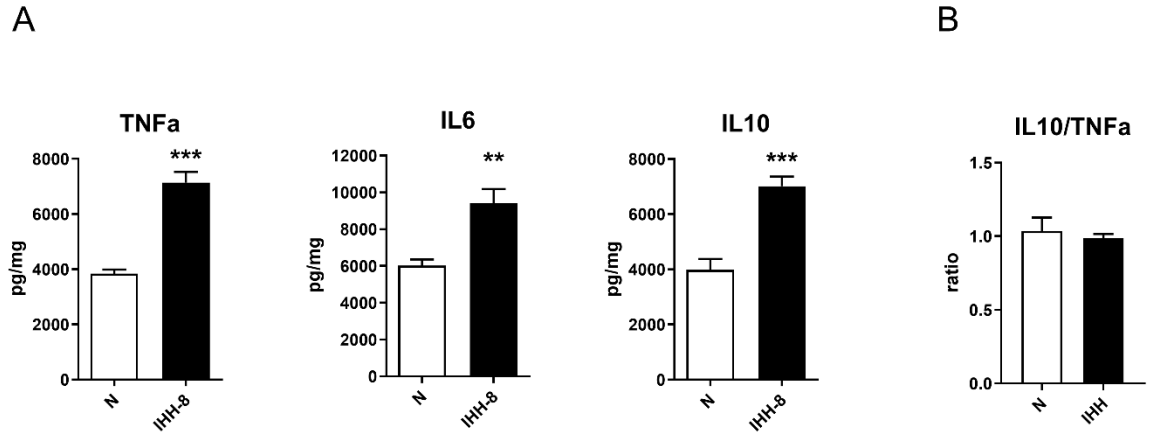


*Fig. 15. Cytokine levels in the left ventricle (LV) from rats in control (N) and hypoxic (IHH-8) groups. (A) Tumor necrosis factor α (TNFα), interleukin 6 (IL6), interleukin 10 (IL10) determined by specific ELISA. (B) IL10/TNFα ratio. The values are expressed as the mean ± SEM, **P < 0.01 vs. N, ***P < 0.001 vs. N, (n=6).*

4.3. The mRNA expression of creatine kinase and hexokinase in the protective and non-protective regimens of chronic hypoxia.

Creatine kinase and hexokinase enzyme systems substantially contribute to the maintenance of energy homeostasis within the heart. The mitochondrial isoforms control the mitochondrial membrane potential and ROS production. Compared with controls, none of the hypoxic regimens affected the mRNA levels of *Ckm* and *Ckb* isoforms, except for a slight decrease of *Ckm* in INH-23 group (Fig. 16). The expression of *mtCks* was significantly increased in CNH and INH-8 (by 14% and 17%, respectively) compared with normoxic group and there was also significant difference when compared to INH-23. All hypoxic regimens significantly decreased expression of *Hk1* isoform (by 33%, 38% and 24% in CNH, INH-8 and INH-23, respectively). Interestingly, an opposite effect was observed for *Hk2* isoform expression, when the mRNA levels were markedly elevated in all hypoxic groups (by 99% in CNH, by 112% in INH-8 and by 74% in INH-23) compared with normoxic group (Fig. 16).

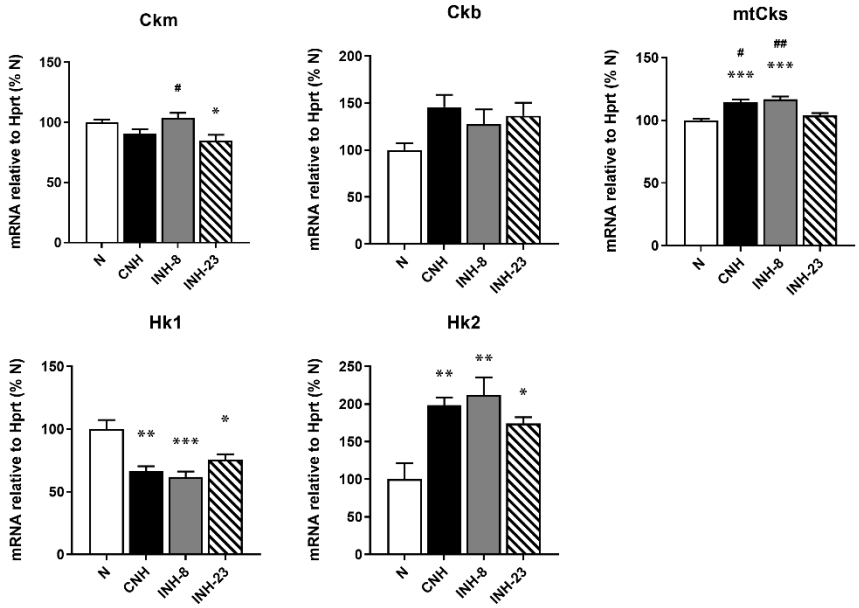


*Fig. 16. The effect of different regimens of chronic hypoxia on the mRNA expression of the enzymes of energetic metabolism. Transcripts of creatine kinase, muscle (Ckm), creatine kinase, brain (Ckb), mitochondrial creatine kinase, sarcomeric (mtCks) and hexokinase (Hk) were assessed in LV preparations from control normoxic rats (N) and those adapted to continuous (CNH) and intermittent (INH-8 and INH-23) normobaric hypoxia. The mRNA transcript levels were normalized to the reference gene Hprt1. The values are the means ± SEM and they are expressed as percentage of control (100%), *P<0.05 vs. N, **P<0.01 vs. N, ***P<0.001 vs. N, # P<0.05 vs. INH-23, ## P<0.01 vs. INH-23 (n=5).*

In contrast, IHH-8 adaptation significantly decreased mRNA expression of *Ckm* isoform by 37%, but levels of *Ckb* and *mtCks* did not change (Fig. 17). mRNA levels of cytosolic *Hk1* were reduced after IHH-8 (by 25%), whereas mitochondrial *Hk2* mRNA level was raised in IHH-8 by about 68%.

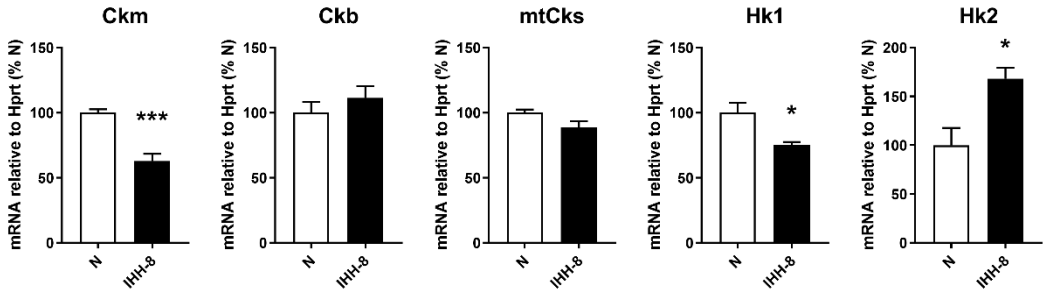


*Fig. 17. The effect of chronic intermittent hypobaric hypoxia on the mRNA expression of creatine kinase, muscle (Ckm), creatine kinase, brain (Ckb), mitochondrial creatine kinase, sarcomeric (mtCks) and hexokinase (Hk). Transcripts were determined in the left ventricles (LV) from control (N) and hypoxic (IHH-8) rats. The mRNA levels were normalized to the reference gene Hprt1. The values are the mean ± SEM, expressed as a percentage of control; *P < 0.05 vs. N, ***P < 0.001 vs. N, (n=6).*

5. Discussion

The present thesis has contributed to the understanding of cardiac effects of different regimens of adaptation to hypoxia in terms of hypoxia severity as well as with respect to the time course of adaptation. It has been shown previously, that a models of intermittent and continuous chronic hypoxia have beneficial effects such as reduced infarct size, lowered incidence of ischemic arrhythmias and an improvement of contractile dysfunction (reviewed in Mallet *et al.*, 2006; Ošťádal and Kolář, 2007). Milano *et al.* (2002) hypothesized that in the intermittent adaptation model, periodic reoxygenation produces sudden hyperoxic conditions similar to preconditioning, which results in myocardial adaptation to the I/R insult and triggers protective signalling pathways. However, in our study, 1-hour reoxygenation abolished the cardioprotective effect elicited by chronic hypoxia (Kasparova *et al.*, 2015). It is already known, that most of intermittent hypoxic adaptations improved the resilience of the heart to the I/R insult, but the degree of protection varies among the used protocols (reviewed in Mallet *et al.*, 2018). Our results confirmed the previous finding of Neckář *et al.* (2013), when adaptation to CNH increased myocardial resistance to I/R damage, whereas 1-hour daily interruption of hypoxic adaptation (INH-23) abolished protection. Chronic intermittent exposure to normobaric hypoxia (INH-8) exhibited protective effect on infarct size and this result is in an agreement with protection observed in both chronic continuous (Neckář *et al.*, 2013) and intermittent hypobaric hypoxia (Neckář *et al.*, 2004). Yeung *et al.* (2007) also showed that even shorter period (6h/day, 14 days) of the same intensity of normobaric hypoxia (Fi_{O2} 0.1) displayed infarct reduction after I/R. Besides that, it was repeatedly shown, that adaptation to hypoxia lowers the body weight and leads to hypertrophy of the RV (Neckář *et al.*, 2002b, 2005; Kolář *et al.*, 2007). Our results were in line with these observations in all hypoxic regimens and the most obvious decrease in body weight was observed in INH-23 group probably due to higher stress associated with the regimen of short term reoxygenation (Kasparova *et al.*, 2015). The RV hypertrophy has been shown to be related to the rapid development of pulmonary vasoconstriction and subsequent hypertension after exposure to hypoxic environment (Herget *et al.*, 1978).

The main finding of our study (Kasparova *et al.*, 2015) using normobaric model of adaptation to chronic hypoxia is that the expression of important components of antioxidant enzymatic system and their regulatory factors are increased after adaptation to the both protective regimens (CNH and INH-8), whereas non-protective regimen with 1h-daily reoxygenation (INH-23)

elicited no response of the antioxidants at expression level. Based on these results we suggest that antioxidant enzyme system plays an important role in the development of ischemic-tolerant phenotype of chronically hypoxic heart. Interestingly, the activated antioxidant genes belong mostly to the mitochondrial antioxidants.

The intracellular antioxidant system is highly complex machinery consisting of low molecular weight antioxidants, which are functionally dependent on the high molecular weight antioxidant proteins. From a functional point of view, the antioxidant defence can be divided into two parts: direct control of ROS scavenging and maintaining of redox homeostasis by recycling of oxidised molecules (Limón-Pacheco and Gonsebatt, 2009). Adequate levels of ROS are maintained by SOD, CAT, PRX, and GPX; meanwhile, redox homeostasis is principally sustained by the TXN and glutathione system. Also, very important part of the intracellular antioxidant defence is the HMOX and ACO which are considered as markers of oxidative stress and are involved in iron metabolism.

Both regimens used in our study that exhibited cardioprotection (CNH and INH-8) increased *MnSod* mRNA expression. Mitochondrial MnSOD is located in the mitochondrial matrix and reduces local concentration of superoxide formed in the heart mainly by complexes I and III of the respiratory chain (Buettner *et al.*, 2006). In contrast, *Cu/ZnSod* mRNA was elevated only in the CNH group. Cytosolic Cu/ZnSOD dismutates superoxide produced by NOX, lipoxygenase or cyclooxygenase. Most of the studies investigating the expression of SODs under hypoxic conditions refer to the mitochondrial MnSOD isoform. Guo *et al.* (2009) documented an elevation of MnSOD and no change in Cu/ZnSOD protein level after chronic intermittent exposure to hypoxia for 6 hours per day (5,000 m, 28 days). Nakanishi *et al.* (1995) examined mRNA expression of *MnSod* in myocardium of rats adapted to hypobaric hypoxia corresponding to 5,500 m altitude (12 hours per day for 1, 3, 5, 7, 4 and 21 days). They detected increased mRNA amount of *MnSod* at early stages but there were no further changes in its level after the fifth day of adaptation. Interestingly, both cardioprotective adaptations (CNH and INH-8) investigated in our study increased *MnSod*, but *Cu/ZnSod* mRNA was elevated only in CNH regimen. Likewise, a short (5 h) exposure to normobaric hypoxia (6% O_2) increased the amount of *MnSOD* mRNA three times (Schülke *et al.*, 2012). Importantly, overexpression of MnSOD was shown to improve myocardial function after I/R injury (Chen *et al.*, 1998; Woo *et al.*, 1998). In the previous study of Neckář *et al.* (2013), increased protein expression of MnSOD

was detected in CNH but not in INH-23, which is in line with our current observations of mRNA levels. Importantly, infarct size negatively correlated with MnSOD expression and activity in myocardial mitochondria from chronically hypoxic rats (Balková *et al.*, 2011).

We also observed the upregulation of PRXs. Hydrogen peroxide produced by SODs may be eliminated also by PRXs (Kang *et al.*, 1998). Interestingly, it was demonstrated that overexpression of PRXs induced cytoprotective effects in cells as well as in cardiac tissue and their depletion increased infarct size and apoptosis (Zhao *et al.*, 2009). In the present study, we did not observe any changes at the mRNA level of cytosolic *Prx1* isoform under different hypoxic conditions. However, *Prx2* was significantly increased in both protective regimens suggesting its involvement in the cardioprotective effect. It is the only antioxidant located specifically in the cytosol which was increased in both protective regimens. Based on the fact, that *Prx2* mRNA level is much higher in heart than in other tissues (Kim *et al.*, 2000), it can be assumed that this isoform could play the main role among cytosolic antioxidants. Moreover, Zhao *et al.* (2009) suggested that the antioxidant properties of PRX2 in the heart might be independent of other antioxidant enzymes, indicating a unique effect of PRX2 in the process of cardiac I/R injury or H_2O_2-induced cell death. PRX3 acts predominantly in the mitochondrial matrix and PRX5 is present in different subcellular compartments including mitochondria and peroxisomes (Yamashita *et al.*, 1999; Van der Eecken *et al.*, 2011). *Prx3* mRNA level did not change compared with the control but there was significant difference between INH-8 and non-protective regimen INH-23. Since *Cat* mRNA tended to decrease in both protective regimens, the increased amount of *Prx5* could substitute the diminished function of mitochondrial CAT, which is involved in mitochondrial SOD product elimination (Radi *et al.*, 1991). PRX4, which is located in the endoplasmic reticulum, and also secreted into the extracellular space, may function as a regulatory factor for NF-κB (Jin *et al.*, 1997) and its importance in intracellular redox signalling was previously demonstrated (Wong *et al.*, 2000). *Prx4* mRNA was significantly higher in INH-8 than in INH-23. INH-8 also markedly enhanced the expression of *Prx6*, compared with the corresponding control. PRX6 is a bifunctional enzyme with GPX and phospholipase A2 activities (Fisher, 2011). As seen in Table 6, *Prxs* mRNA levels were suppressed in non-protective regimen INH-23, except for *Prx5*. Interestingly, *Prx*2 seems to be the most important isoform in our model since it reacted to both protective regimens by elevated expression (Table 6).

Oxidised PRXs are reduced by the thioredoxin superfamily proteins, comprised of TXNs and their reductases. TXN operates as a donor of electrons to oxidised forms of molecules and by reducing them it is itself oxidised. TXNRD ensures regeneration of oxidised form of TXN by utilizing NADPH and H^+ as substrates as well as other specific agents such as lipid hydroperoxides. Two isoforms are known in the heart tissue, TXN1, coupled with TXNRD1, is mainly located in cytosol and nucleus, while TXN2 and its reductase TXNRD2 operate in mitochondria (reviewed in Lu and Holmgren, 2013). In our study, *Txn1* and its reductase *Txnrd1* mRNA levels did not change in any of the analysed regimens. On the other hand, mitochondrial isoform of *Txn2* and its reductase *Txnrd2* increased in both protective regimens. TXN2 is implicated in cellular resistance to apoptosis induced by mitochondrial ROS (Huang *et al.*, 2015). Also, TXNRD2 exerts a crucial function during postischemic reperfusion via thiol regeneration (Horstkotte *et al.*, 2011). Our results suggest that maintaining of redox homeostasis in mitochondria could contribute to cellular adaptation to mild hypoxia.

We also focused on the glutathione system. Reduced glutathione can donate reducing equivalent ($H^+ + e^-$) to the other molecules or can serve as a cofactor in the reduction reactions for GPX, GRX and GST. GSR, which is in cytosol and also in mitochondria, reduces GSSG to GSH and independently scavenges highly reactive hydroxyl radicals (Sáez *et al.*, 1993; Winterbourn, 1993). Decreased GSH/GSSG ratio is generally considered as an indicator of oxidative stress (Jones *et al.*, 2000). Our results (Kasparova *et al.*, 2015) indicate that both protective regimens did not substantially change the GSH/GSSG ratio. We assume that the ratio can be kept during I/R injury by the elevated levels of *Gsr*, which were observed in both protective regimens of adaptation. These results are in contrast with finding of Kolář *et al.* (2007), when adaptation to chronic intermittent hypobaric hypoxia (7,000 m for 8 h/day, 5 days/week) lowered the GSH/GSSG ratio, probably due to severe hypoxic conditions. The non-protective regimen INH-23 decreased the GSH/GSSG ratio possibly reflecting increased oxidative stress in the absence of *Gpxs* and *Gsr*.

It has been reported that INH-23 nearly doubled the myocardial concentration of malondialdehyde as compared with CNH protective regimen (Neckář *et al.*, 2013), indicating increased oxidative stress and lipid peroxidation. We observed an increase of *Txnrd2* after adaptation to both protective regimens, *Gpx4* in CNH group and *Prx6* in INH-8 group, while in contrast none of these genes changed in INH-23 group. All these enzymes have been

demonstrated to play an important role during detoxification of lipid peroxides in mitochondria (Ursini and Bindoli, 1987; Sakai *et al.*, 2015) and cytosol (Manevich and Fisher, 2005). Therefore, it could be suggested that increased capability to scavenge lipid peroxides might be the part of the cardioprotective phenotype induced by CNH and INH-8 adaptation.

HMOX is very often associated with a cellular protective function during oxidative stress and I/R injury (Vulapalli *et al.*, 2002; Zhou *et al.*, 2014a). The isoform HMOX1 is stress-inducible enzyme, whereas HMOX2 is a ubiquitous enzyme expressed constitutively under physiological conditions. Both isoforms catalyse the oxidative degradation of heme to iron, CO and biliverdin, which is reduced to bilirubin by bilirubin reductase (Sedlak *et al.*, 2009). In our hypoxic models, *Hmox1* was substantially elevated only in CNH group, while *Hmox2* mRNA levels did not change when compared with normoxic group. Expression of *Hmox* genes was shown to increase in the defence against cardiotoxic stimuli (Jirkovsky *et al.*, 2012) or severe hypoxic or anoxic conditions (Kacimi *et al.*, 2000; Issan *et al.*, 2014).

ACO is another antioxidant from iron-dependent group of enzymes, which has been considered as the marker of oxidative stress (reviewed in Lushchak *et al.*, 2014). ACO1 is cytosolic isoform also known as IRP1 and has been demonstrated to serve as a key upstream regulator of HIF2α expression (Wilkinson and Pantopoulos, 2013). Mitochondrial ACO2, beside its metabolic role, can function as an indicator of ROS formation (Chouchani *et al.*, 2014) and specific pro-oxidant in mitochondria. When increased oxidative stress leads to oxidation of Fe-S clusters in ACO2, its enzyme activity is inhibited and danger of formation of hydroxyl radicals and Fenton reaction rises (Vasquez-Vivar *et al.*, 2000). The substantial increase of *Aco2* mRNA levels under all hypoxic regimens observed in our results suggests that insufficient activity of this enzyme resulted in its increased transcription. Inactivated ACO2 could impact mitochondrial metabolism by lowering the entry of reducing equivalents to the electron transport chain, decreasing membrane potential and slowing the rates of $O_2^{\bullet -}$ and H_2O_2 production (Scandroglio *et al.*, 2014).

The observed increased cytosolic and mitochondrial ROS scavengers and no change in the GSH/GSSH ratio confirm optimal activation of the antioxidant system in both cardioprotective regimens of adaptation (Table 6). The activity and capacity of the antioxidant system are orchestrated at the protein level by activation of specific transcription factors, such as NRF2.

NRF2 controls expression of cytoprotective genes including antioxidants in a redox state-dependent manner and via the ARE (Itoh *et al.*, 1997; Jeyapaul and Jaiswal, 2000). Nevertheless, only INH-8 regimen significantly increased mRNA levels of *Nrf2* in our study (Kasparova *et al.*, 2015). HIF, as a key transcriptional factor under low oxygen conditions; together with its regulatory hydroxylases also control response of cellular antioxidant defence in hypoxia (Jaakkola *et al.*, 2001). We found a slight but significant elevation of *Hif1α* mRNA after adaptation to INH-8 regimen and very perspicuous upregulation of *Hif2α* mRNA in both protective regimens (CNH and INH-8). Interestingly, none of all three isoforms of prolyl hydroxylases (*Phd1-3*) changed the expression after adaptation to any regimen of hypoxia, while HIF1 inhibiting factor (*Fih1*) was markedly increased after CNH and INH-8. Additionally, the correlation coefficient between the expression of *Fih1* and *Hif2α* ($r = 0.82$, $p < 0.0001$) was markedly higher than between *Fih1* and *Hif1α* ($r = 0.3$, $p < 0.196$), which may suggest stronger connection rather between FIH1 and HIF2α than between FIH1 and HIF1α. The differences between *Hif1α*, *Hif2α* and *Nrf2* mRNA expression shown in our study are quite interesting. It seems that excessive oxidative stress under INH-23 regimen induced by 1-hour daily reoxygenation does not induce mRNA expression of these transcription factors. In contrast, regimen with longer daily episode of normoxia (16 h/day; INH-8) upregulated the expression of important transcriptional factors *Nrf2* and *Hif2α* as well as HIF inhibitor *Fih1*, possibly due to moderate ROS formation. All the mentioned transcriptional factors are strongly regulated by the TXN1/TXNRD1 system and they are sensitive to the cellular redox state (reviewed in Nagarajan *et al.*, 2017). The TXN2/TXNRD2 system maintains redox homeostasis in mitochondria and mitochondrial ROS formation is required for HIF activation during hypoxia (Hamanaka and Chandel, 2009) as well as for NRF2 transcriptional activity (Hansen *et al.*, 2004; Malec *et al.*, 2010).

We can conclude that cardioprotective effect of CNH and INH-8 regimens was accompanied by increased expression of antioxidant enzymes which maintained unchanged GSH/GSSG ratio. In contrast, only 1-hour daily reoxygenation during the adaptation protocol (INH-23) abolished protective effect of hypoxia. In the same time, antioxidant system did not respond at the transcriptional level, except the *Prx5*, which protein product operates both in cytosol and in mitochondria. It is apparent that its activation was not sufficient to induce cardioprotection. It can be concluded that the increase of cytosolic isoforms *Prx2* and *Gsr* together with

mitochondrial *MnSod*, *Txn2/Txnrd2*, and *Gsr* play an important role in cardioprotection conferred by continuous and intermittent regimen of adaptation to chronic well tolerable normobaric hypoxia.

Based on these results, we further focused on the antioxidant system under another cardioprotective regimen of severe intermittent hypobaric hypoxia (IHH-8; 7,000 m, 8h/ day, 5 weeks) which also attenuates infarct size and decreases the number of ischemia-reperfusion arrhythmias (Neckář *et al.*, 2002a, 2002b, 2005; Kolář *et al.*, 2007). Surprisingly, we observed that under these severe conditions the most of antioxidant enzymes decreased at mRNA level and simultaneously did not change or slightly decreased at protein level. The only elevated antioxidants were *Aco1* and *Ftl1* at mRNA levels, stress-inducible HMOX1 at both mRNA and protein levels, and TXN1 was markedly increased at protein level. We did not find any changes in transcriptional activity of antioxidant transcriptional factor NRF2, which conforms well to the attenuated antioxidant response. These data are in line with previous finding of increased oxidative stress, which was demonstrated by the reduced GSH/GSSG ratio in the model of severe IHH-8 (Kolář *et al.*, 2007). Although, there is abundant evidence that myocardial tissue exposed to oxidative stress strengthen antioxidant defence, which contributes to the reduction of infarct size after I/R (Nakanishi *et al.*, 1995; Guo *et al.*, 2009; Balková *et al.*, 2011; Neckář *et al.*, 2013), our findings well demonstrate the importance of adaptive conditions to hypoxia (type of hypoxia, duration, oxygen levels, etc.) in resulted phenotype. Besides that, model of severe IHH-8 induces also hypertrophy of the left ventricle (Neckář *et al.*, 2005; Kolář *et al.*, 2007) and activates expression of pro-apoptotic genes such as *Bad* and *Bax* (Holzerová *et al.*, 2013). Importantly, ROS have been found to play a key role in the development of a cardioprotective phenotype (Kolář *et al.*, 2007).

As mention above, HMOX1 is characterized as stress-responsive protein induced by various oxidative agents and operates as strong cytoprotective and anti-inflammatory component of cell survival pathways. The transcriptional activity of the *Hmox1* gene is controlled, besides NRF2 (Alam *et al.*, 1999), also by stress induced transcription factor NF-κB (Lin *et al.*, 2007) and by HIF (Lee *et al.*, 1997; Wright *et al.*, 2003; Jürgensen *et al.*, 2004), which influence might be here more pronounced under these conditions of severe hypoxia. High levels of *Hmox1* could play substantial part in modulating of cytoprotection. *Hmox1* is well known as hypoxia-induced

gene with cardiovascular regulatory functions which may improve antioxidant defence during hypoxia in cardiomyocytes (Borger and Essig, 1998). Also, Yet *et al.* (2001) showed that HMOX1 has an important role in regulation of infarct size and post-ischemic recovery of cardiac function. In addition, overexpression of HMOX1 improved recovery after I/R and limited cardiomyocyte apoptosis *in vivo* (Vulapalli *et al.*, 2002). Furthermore, inflammation was shown to stimulate HMOX1 activity (Willis *et al.*, 1996; Lee and Chau, 2002), which metabolizes heme and produces biliverdin, free iron and CO. Enhancement of HMOX activity might lead to increased formation of bilirubin, a powerful antioxidant, via BLVR (Baranano *et al.*, 2002; Maghzal *et al.*, 2009; Song *et al.*, 2013), which protects unsaturated fatty acids against oxidation (Neuzil and Stocker, 1994). Surprisingly, our results did not confirm activation of this pathway, as neither mRNA nor protein levels of BLVR changed after IHH-8 adaptation. On the other hand, elevated free iron production and oxidative stress leads to oxidation of ACO1, which then functions as IRP1 and affects transcription of ferritin (*Ftl*). Ferritin is the major intracellular iron storage protein, which prevents an initiation of the Fenton reaction and dangerous hydroxyl radical formation (Haile *et al.*, 1992; Pantopoulos and Hentze, 1995). Our results show increased expression of *Aco1* and *Ftl1*, thus it could be suggested that this pathway contributes to protection against formation of ROS. Another product of HMOX activity is CO, which plays important role in improved post-ischemic recovery, reduction of infarct size and ventricular fibrillations (Bak *et al.*, 2005). There is an evidence that anti-inflammatory effect of HMOX1 consists of its production of CO which leads to induction of anti-inflammatory protein IL10 (Lee and Chau, 2002; Otterbein, 2002; Piantadosi *et al.*, 2011). Our results indicate the same consequence that upregulated levels of anti-inflammatory protein IL10 are probably due to released levels of CO mediated by HMOX1. Therefore, the increased expression of HMOX1 likely represents important part of modulating cardioprotective phenotype during severe hypoxia, but not during well tolerable moderate normobaric hypoxia (Chytilová *et al.*, 2015; Kasparova *et al.*, 2015).

Also, we detected markedly increased protein expression of TXN1 and substantially elevated level of its reduced form in the nucleus. It is in accordance with the fact that elevated oxidative stress releases TXN1 from dimerization with TXN-interacting protein and keeps it free to scavenge ROS and possibly translocate to nucleus (Watson and Jones, 2003). Increased oxidative stress also activates redox-sensitive transcriptional factors such as HIF, NF-κB and

NRF2, and their signalling pathways which are supposedly involved in the cardioprotective response (Baetz *et al.*, 2005; Eckle *et al.*, 2008; Deng *et al.*, 2013). Then, translocated TXN1 plays an essential role for these redox-sensitive transcriptional factors by directly increasing their transcriptional activity (Matthews *et al.*, 1992; Ueno *et al.*, 1999; Hansen *et al.*, 2004). It is well known that TXN1 reduces oxidised cysteine residues of NF-κB in the nucleus and potentiates NF-κB-dependent transcription (Hirota *et al.*, 1999).

Finally, we observed increased expression of NF-κB and its target genes related to the inflammatory signalling, TNFα, IL6 and IL10. NF-κB is a key transcriptional factor activated during stress conditions and regulating transcription of many genes involved in cell survival and immunomodulation. Hirota *et al.* (1999) showed that treatment by inflammatory cytokine TNFα caused quick translocation of TXN1 in nucleus where it can directly associate with NF-κB. TNFα is one of the pro-inflammatory cytokine and has been well described as an inducer of NF-κB translocation to nucleus by releasing its inhibiting IκB protein (Yang *et al.*, 2001). As TNFα can contribute to translocation of TXN1, we decided to determine the levels of inflammatory markers during adaptation to hypoxia. Chytilová *et al.* (2015) described pivotal role of TNFα against I/R injury during protective adaptation to CNH. Elevated levels of pro-inflammatory cytokines, such as TNFα and IL6, are mostly related to the heart failure. But there are evidences that activation of immune system (by TNFα and IL6) in the heart can promote survival signalling pathways involving stress transcription factors (reviewed in Lecour and James, 2011). Our results showed increased levels of TNFα and IL6 after IHH-8 adaptation compared with normoxic rats; likewise, significant elevation of NF-κB in homogenate and markedly increased presence in nucleus. These outcomes supposedly led to increased transcriptional activity of NF-κB and probably positive feedback loop with TNFα as one of the target genes of NF-κB. We suggest that oxidative stress, present during protective adaptation to IHH-8, induces increased amount of inflammatory markers such as TNFα and IL6, which initiate translocation of TXN1 and NF-κB into the nucleus. Our data provide compelling evidence that nuclear TXN1 is the important signalling molecule, which modulates transcription factor activities. TNFα/NF-κB pathway may mediate cytoprotective effect (Chytilová *et al.*, 2015). Increased levels of NF-κB target genes, HMOX1 and IL10, can compensate levels of pro-inflammatory cytokines and contribute to developing of protective phenotype during adaptation to severe hypobaric hypoxia.

Increased production of ROS in mitochondria directly leads to increase mitochondrial membrane potential and decreased availability of ADP (Korshunov *et al.*, 1997). Therefore, the enzymes of energetic metabolism such as mitochondrial mtCKs and all three HK isoforms are important stimulants of oxidative phosphorylation by producing ADP and lowering the membrane potential in the heart (Gottlob *et al.*, 2001; da-Silva *et al.*, 2004; Santiago *et al.*, 2008). Adaptation to protective regimens of normobaric hypoxia (CNH and INH-8) led to increased mRNA expression of *mtCKs*, while both cytosolic isoforms remained unchanged. These data are in accordance with elevated protein levels of this mitochondrial isoform in the same regimens; then, increased total activity of CK under hypoxic conditions can be attributed mainly to mtCKs (Waskova-Arnostova *et al.*, 2014). In all regimens of normobaric hypoxia, we observed elevated mRNA levels of *Hk2* isoform, while *Hk1* mRNA was decreased. Adaptation to hypoxia stimulates expression of *Hk2* through HIF1 transcriptional activity (Riddle *et al.*, 2000). Moreover, it has been shown that HK2 isoform can activate its own expression and repress the transcription of *Hk1* (Rodríguez *et al.*, 2001). In this mild model of hypoxia, where the oxidative stress is well compensated, we did not observe increased HK interaction with mitochondria (Waskova-Arnostova *et al.*, 2014).

On the other hand, adaptation to IHH-8 regimen represents model of severe hypoxia which leads to increased association of hexokinase 2 with mitochondria (Waskova-Arnostova *et al.*, 2015). Also, mRNA levels of *Hk2* were significantly increased, which was again in concordance with elevated protein levels and total HK activity (Waskova-Arnostova *et al.*, 2015). This phenomenon probably appears when antioxidant defence is not sufficient, oxidative stress rises and then HK2 directly protects mitochondria from activation of apoptosis in heart tissue (Pastorino *et al.*, 2002; Majewski *et al.*, 2004). It has been repeatedly shown that increased mitochondrial association of HK2, but also HK1, is cytoprotective under conditions of hypoxia or I/R (Schindler and Foley, 2010; John *et al.*, 2011; Pasdois *et al.*, 2012). Both isoforms bind to VDAC on the outer mitochondrial membrane which is coupled with ANT and this interaction blocks the binding of pro-apoptotic proteins under pathophysiological conditions and thus prevents opening of MPTP and cytochrome c release (Azoulay-Zohar *et al.*, 2004; Miyamoto *et al.*, 2008). Importantly, we also observed increased association of HK2 with mitochondria after short ischemia (Kolar *et al.*, 2017).

These data suggest that increased oxidative stress that occurs under ischemic conditions as well as in the present model of severe hypoxia is an important factor for increased interaction of HK2 with mitochondria and thus may promote survival of cardiomyocytes and play a role in cardioprotective mechanisms.

6. Conclusion

Aim 1: To identify the role of the main components of antioxidant systems in protective and non-protective regimens of chronic hypoxia.

We demonstrated that the antioxidant enzyme system, especially mitochondrial isoforms, plays a key role in regulation of oxidative stress in adaptation to protective regimens of normobaric hypoxia. Our results also show, that only 1-hour daily reoxygenation during the adaptation protocol prevented response of the antioxidant system and abolished protective effect of hypoxia. Nevertheless, we can not conclude, if the protective response is based on coordinated expression of individual components of the antioxidant system or whether some of its components play a dominant role. On the other hand, cardioprotective regimen of severe hypobaric intermittent hypoxia did not increase most of the antioxidant enzymes. Markedly elevated expression of stress-inducible HMOX1 support the view that this enzyme represents important part of modulating cardioprotective phenotype during severe hypoxia by activation of anti-inflammatory response to increased oxidative stress in the cell.

Aim 2: To investigate the effect of severe intermittent hypobaric hypoxia on TXN1 and its main redox homeostasis target transcription factor NF-κB.

We showed, that IHH-8 increased translocation of TXN1 into the nucleus and the ratio of reduced and oxidised form also increased. The observed increased expression of NF-κB in homogenate and in the nucleus led to elevated expression of its target genes, pro-inflammatory cytokines tumour necrosis factor α (TNFα) and interleukin 6 (IL6) and anti-inflammatory cytokine interleukin 10 (IL10). We may conclude that increased levels of NF-κB target genes, HMOX1 and IL10, can compensate levels of pro-inflammatory cytokines and contribute to developing of protective phenotype during adaptation to severe hypobaric intermittent hypoxia.

Aim 3: To characterise the role of important enzymes of energetic metabolism, creatine kinase and hexokinase, in protective and non-protective regimens of chronic hypoxia.

Our results indicate, that mtCKs together with both isoforms of HK play an important role in stimulation of oxidative respiration in mitochondria during adaptation to all regimens of hypoxia and contribute to prevent oxidative stress. Moreover, IHH-8 regimen increased interaction of HK2 with mitochondria and thus may promote survival and decrease probability of apoptosis activation.

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List of abbreviations

·OH	Hydroxyl radical
ACO	Aconitase
ADP	Adenosine diphosphate
Akt	V-akt oncogene homolog/protein kinase B
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocase
AP-1	Activator protein 1
AR	Area at risk
ARE	Antioxidant-responsive element
ASK-1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
BAX	Bcl-2-associated X protein
BLVR	Biliverdin reductase
BLVRB	Biliverdin reductase B
BW	Body weight
CAT	Catalase
cDNA	complementary DNA
cGMP	Cyclic guanosine monophosphate
CK	Creatine kinase
CKB	Creatine kinase, brain isoform
CKM	Creatine kinase, muscle isoform
CNH	Continuous normobaric hypoxia
CoQ	Coenzyme Q
Cu/ZnSOD	Copper-zinc superoxid dismutase
CVD	Cardiovascular disease
Cys	Cystein
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECG	Electrocardiogram
EcSOD	Extracellular superoxide dismutase
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
eNOS	constitutive NO synthase
FMN	Flavin mononucleotide
FIH1	Factor inhibiting HIF1
FiO2	Fractional concentration of inspired oxygen
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPX	Glutathione peroxidase
GRX	Glutaredoxines
GSH	Thiol-reduced form of glutathione
GSR	Glutathione reductase
GSSG	Disulfide-oxidised form

GST	Glutathione S transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia-induced transcription factor
HIF1α	Hypoxia inducible factor 1α
HK	Hexokinase
HMOX	Heme oxygenase
HPLC	High-performance liquid chromatography
Hprt1	Hypoxanthine-guanine phosphoribosyltransferase 1
HR	Heart rate
HRE	Hypoxia responsive element
I/R	Ischemia-reperfusion
IgG	Immunoglobulin G
IHH	Intermittent hypobaric hypoxia
IHH-8	intermittent hypobaric hypoxia for 8 hours per day
IL1	Interleukin 1
IL6	Interleukin 6
IL10	Interleukin 10
INH-23	Intermittent normobaric hypoxia for 23 hours per day
INH-8	Intermittent normobaric hypoxia for 8 hours per day
iNOS	inducible NOS
IPC	Ischemic preconditioning
iPost	Ischemic postconditioning
IRP1	Iron regulatory protein 1
IS	Infarct size
IκB	Inhibitor of κB protein
JNK	c-Jun N-terminal kinase
K_{ATP}	ATP-sensitive K^+ channel
Keap1	Kelch-like ECH-associated protein 1
KO	Knock out
LV	Left ventricle
Maf	V-maf musculoaponeurotic fibrosarcoma oncogene homolog
MAP	Mean arterial blood pressure
MAPK	Mitogen-activated protein kinase
MnSOD	Manganese superoxid dismutase
MPTP	Mitochondrial permeability transitions pore
mtCKs	Creatine kinase, mitochondrial sarcomeric isoform
N	Normoxia
NAD^+/NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
NRF2	Nuclear factor erythroid 2-related factor 2
O2•−	Nuperoxide anion
PCR	Polymerase chain reaction
PHD	Prolyl hydroxylase

PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKC-α	Protein kinase C alpha
PKC-δ	Protein kinase C delta
PKC-ε	Protein kinase C epsilon
PLC-γ	Phospholipase C-γ
pO_2	partial pressure of oxygen
PRX	Peroxiredoxins
qPCR	Real-time quantitative RT-PCR
Rac1	Rac family small GTPase 1
Rel	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog
RelA	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A
RelB	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog B
RIC	Remote ischemic conditioning
RIPerC	Remote ischemic perconditioning
RIPostC	Remote ischemic postconditioning
RIPreC	Remote ischemic preconditioning
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transription
RV	Right ventricle
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard error of mean
SOD	Superoxide dismutase
TNFα	Tumor Necrosis Factor Alpha
TRIS	Tris(hydroxymethyl)aminomethane
TTBS	Tris-buffered saline with Tween 20
TXN	Thioredeoxin
TXNRD	Thioredoxin reductase
VDAC	Voltage dependent anion channel
VEGF	Vascular endothelial growth factor
WB	Western blot
WHO	World Health Organization
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase
γ-GCS	γ-glutamate-cysteine synthase

List of publications

Publications related to the thesis results:

Kasparova D, Neckar J, Dabrowska L, Novotny J, Mraz J, Kolar F, Zurmanova J. Cardioprotective and non-protective regimens of chronic hypoxia diversely affect the myocardial antioxidant systems. Physiol Genomics 2015, 47(12): 612-20. (IF, 2018 = 2.58)

Author´s contribution: *performed experiments (with J Neckar, LD and JZ), performed data analysis (with J Neckar, LD, JM and JZ), interpreted results of experiments (with J Neckar, JM and JZ), wrote the article (J Neckar, J Novotny, FK and JZ).*

Waskova-Arnostova P, Elsnicova B, **Kasparova D**, Hornikova D, Kolar F, Novotny J, Zurmanova J. Cardioprotective adaptation of rats to intermittent hypobaric hypoxia is accompanied by the increased association of hexokinase with mitochondria. J Appl Physiol 2015, 119(12): 1487-93. (IF, 2018 = 3.14)

Author´s contribution: *performed experiments (with PW-A, BE, DH, FK and JZ), performed data analysis (with PW-A, BE and JZ), contributed in writing of the article (with PW-A, BE, FK, JN and JZ).*

Waskova-Arnostova P, **Kasparova D**, Elsnicova B, Novotny J, Neckar J, Kolar F, Zurmanova J. Chronic hypoxia enhances expression and activity of mitochondrial creatine kinase and hexokinase in the rat ventricular myocardium. Cell Physiol Biochem 2014, 33(2): 310-20. (IF, 2017 = 5.50)

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Publications not related to the thesis results:

Benak D, **Sotakova-Kasparova D**, Neckar J, Kolar F, Hlavackova M. Selection of optimal reference genes for gene expression studies in chronically hypoxic rat heart. Mol Cell Biol. 2019. (on line published 12 July 2019; DOI: 10.1007/s11010-019-03584-x) (IF, 2018 = 3.74)

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