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Ischemická tolerance srdcí potkanů adaptovaných na chronickou hypoxii a fyzickou zátěž: úloha TNF-α

Cardiac ischemic tolerance in rats subjected to adaptation to chronic hypoxia and physical exercise: the role of TNF-α

PhD. thesis

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Declaration

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

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Declaration of co-author

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

Prague ....

RNDr. Jan Neckář, Ph.D.

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Abstract

Cardiovascular diseases represent the most important health risk factors because they are responsible for more than 50% of total mortality. Among them, the ischemic heart disease is leading cause of mortality. From the whole spectrum of different cardioprotective phenomena we have selected: 1) adaptation to chronic normobaric hypoxia (CNH) as the traditional experimental model in our laboratory area and 2) protective effect of exercise which in recent years represents promising and clinically relevant protective mechanism.

The whole thesis is based on two studies. Aim of the first study was to characterize the expression of the main pro-inflammatory cytokine, TNF-α, in hearts of rats adapted to CNH. Chronic TNF-α inhibition by infliximab was used for discovering of certain role of TNF-α in CNH. We showed that increased myocardial level of TNF-α during adaptation to CNH was contributed via its receptor TNFR2 and nuclear factor κB-dependent activation of protective redox signalling with increased antioxidant defence. This adaptive pathway participates on the infarct size-limiting effect of CNH. Aim of the second study was find out whether exercise training and CNH could play synergy in cardiac protection in rats model. We reported that CNH and exercise reduced infarct size but their combination provided the same degree of protection as CNH alone. High ischemic tolerance of the CNH hearts persists after exercise, possibly by maintaining the increased antioxidant capacity despite attenuating TNF-α-dependent protective signalling.

In conclusion, TNF-α is involved in the cardioprotective mechanism afforded by CNH, and regular exercise training of rats during their adaptation to CNH conferred the same infarct size-limiting effect as CNH alone. All these findings significantly contribute to the actual information about the cardioprotective mechanisms of adaptation to CNH and physical training.

key words: ischemia, reperfusion, cardiac protection, chronic hypoxia, exercise, tumor necrosis factor alpha

Abstrakt

Kardiovaskulární choroby jsou zodpovědné za více než 50% celkové úmrtnosti a proto patří mezi nejvýznamnější rizikové faktory zdraví. Ischemická choroba srdeční patřící mezi ně je hlavní příčinou úmrtnosti vůbec. Z celého spektra různých kardioprotektivních fenoménů jsme si vybrali: 1) adaptaci na chronickou normobarickou hypoxii (CNH) jako tradiční experimentální model používaný v naší laboratoři a 2) protektivní efekt fyzické zátěže, který v posledních letech představuje slibný a klinicky významný protektivní mechanismus.

Disertační práce je založena na dvou publikacích. Cílem první studie bylo charakterizovat expresi hlavního prozánětlivého cytokinu, TNF-α, v srdcích potkanů adaptovaných na chronickou normobarickou hypoxii (CNH). Chronické podávání inhibitoru TNF-α, infliximabu, bylo použito pro upřesnění role TNF-α v CNH. Ukázali jsme, že zvýšená hladina TNF-α v srdci během adaptace na CNH se podílela skrz svůj receptor TNFR2 a jaderný faktor κB na aktivaci protektivní redoxní signalizace se zvýšenou antioxidační ochranou. Tato adaptativní cesta se účastní protektivního efektu CNH na snížení velikosti infarktu myokardu. Cílem druhé studie bylo zjistit, zda fyzická zátěž a CNH mohou mít aditivní účinek v ochraně potkaního srdce. Zjistili jsme, že jak CNH tak i fyzická zátěž snižují velikost infarktu, ale jejich kombinace poskytuje stejnou úroveň protekce jako CNH samotná. Vysoká ischemická tolerance CNH srdcí přetrvává i po skončení fyzické zátěže, pravděpodobně udržením zvýšené antioxidační kapacity navzdory snižující se TNF-α dependentní protektivní signalizace.

Závěrem lze říci, že TNF-α je zapojen do kardioprotektivního mechanismu CNH a, že pravidelná fyzická zátěž potkanů prováděná v hypoxických podmínkách poskytla stejný efekt na zmenšení velikosti infarktu myokardu jako CNH samotná. Všechny tyto poznatky významně přispívají k aktuálním poznatkům o kardioprotektivních mechanismech adaptace na CNH a fyzickou zátěž.

klíčová slova: ischemie, reperfuze, ochrana srdce, chronická hypoxie, cvičení, tumor nekrotizující faktor alfa

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LIST OF ABBREVIATIONS

AA	arachidonic acid
AC	adenylate cyclase
ALDH	aldehyde dehydrogenase
AMPK	adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
AR	area at risk
CAT	catalase
CD	cardiovascular diseases
cGMP	cyclic guanosine monophosphate
CH	chronic hypoxia
CIH	chronic intermittent hypoxia
CNH	chronic normobaric hypoxia
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
DNA	deoxyribonucleic acid
DAG	diacylglycerol
eNOS	endothelial NO synthase
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FADD	Fas associated death domain
FAS	apoptosis antigen 1; CD95
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Grp	glucose-regulated proteins
HIF	hypoxia-inducible factor
HO	heme oxygenase
HR	heart rate
HSPs	heat shock proteins
IF	impact factor
IKK	inihibitor of NF-κB kinase
IL	interleukin
IMF	intermyofibrillar mitochondria

iNOS	inducible NOS
IP	ischemic preconditioning
IP_3	inositol triphosphate
I/R	ischemia/reperfusion
JNK	c-Jun kinase
K_{ATP}	ATP dependent potassium channel
LPS	lipopolysacharide
LV	left ventricular
MAP	mean arterial pressure
MAPK	mitogen activated protein kinase
MCP	monocyte chemoattractant protein
MDA	malondialdehyde
MI	myocardial infarction
mK_{ATP}	mitochondrial K_{ATP}
MPTP	mitochondrial permeability transition pore
NADH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor κB
NOS	nitric oxide synthase
NT	nitrotyrosine
PB	barometric presure
PH	pulmonary hypertension
PGE2	prostaglandin E2
PK	protein kinase
PL	phospholipase
PostC	postconditioning
PV	pulmonary vasoconstriction
EPO	erythropoietin
RIC	remote ischemic conditioning
RIP	receptor interacting protein
ROS	reactive oxygen species
RV	right ventricular
SaO_2	O_2 saturation
sK_{ATP}	sarcolemmal K_{ATP}

SOD	superoxide dismutase
SS	subsarcolemmal mitochondria
TACE	TNF-α-converting enzyme
TRAF	TNFR-associated factor
TRAP	TNF-α receptor-associated protein
TNF-α	tumor necrosis factor alpha
TNFR	TNF-α receptor
VE	minute ventilation
VO_2max	maximal oxygen uptake

1. INTRODUCTION

Cardiovascular diseases (CD) represent the most important health risk factors because they are responsible for more than 50% of total mortality. Among them, ischemic heart disease is the leading cause of morbidity and mortality, and according to the World Health Organization, will be the major global cause of death by the year 2020 (Murray and Lopez, 1997). Although the cardiovascular health status of our population has improved substantially and cardiovascular mortality has declined in recent years, we are still far behind the ideal situation.

The history of ischemic heart disease is relatively brief, showing the rapid development of cardiology as a scientific discipline (Braunwald, 2003; Ostadal, 2004; Stanek, 2002). Myocardial infarction (MI) was first described clinically in 1910, but the precise diagnosis was only possible after the introduction of the electrocardiogram into clinical practice in the 1920s. Before 1961, patients with acute myocardial infarction who were fortunate enough to reach the hospital were treated largely by benign neglect. They were sedated and placed on bed rest for five to six weeks. In 1961, Julian articulated the concept of a coronary care unit, which includes the treatment of arrhythmias, cardiopulmonary resuscitation with external ventricular defibrillation and well-trained nurses. The introduction of the coronary care units caused an immediate 50% reduction in in-hospital mortality. Since 1963, in-hospital mortality has decreased stepwise by almost 70% with the introduction of thrombolysis, acetylsalicylic acid, invasive cardiology and cardiac surgery. Modern therapy, together with effective secondary prevention, has increased the two-year survival of patients after MI by 75% over the past 30 years (Stanek, 2002). The progress in the prognosis, diagnosis and therapy of ischemic heart disease would have been impossible without several notable achievements of the 20th century, which were critical for further progress in the field of cardiology (Mehta and Khan, 2002), e.g., the electrocardiogram, the Framingham Heart Study, the lipid hypothesis of atherosclerosis, coronary care units, echocardiography, thrombolytic therapy, heart catheterization and percutaneous coronary intervention, open-heart surgery and implantable defibrillators.

It should be noted that the described achievements are the results of very close collaborations between theoretical and clinical cardiologists (Braunwald, 2003). This suggests that cardiology belongs to medical disciplines in which the cooperation of basic and clinical cardiologists has a long-lasting tradition of acting as an engine, driving scientific progress forward. Although the management of ischemic heart disease centers on the development of effective primary prevention, the impact of these strategies may be limited. There is, therefore, an urgent need for effective forms of secondary prevention and, in particular, treatment that will limit the extent of evolving myocardial infarction during the acute phase of coronary occlusion. Based on these presumptions, cardiovascular research should concentrate on three consecutive periods during the development of myocardial injury: mechanisms involved in cardiac protection against ischemia, factors responsible for myocardial cell death and positive and negative consequences of myocardial reperfusion. Preserving the viability of ischemic myocardium should be the major therapeutic target (reviewed in Ostadal, 2009).

1.1. Ischemic heart

Ischemic heart is characterized by reduction of blood flow to the myocardium, which causes metabolic, functional and morphological changes (Fig. 1). At the level of the myocyte, reversible and irreversible injuries are induced by impaired excitation-contraction coupling, electrical instability, altered ionic homeostasis and a shift from aerobic to anaerobic glycolysis, which could contribute not only to disease progression but, on the other hand, to higher toleration too. Cardiomyocytes can undergo cell death by two mechanisms: necrosis and apoptosis (Majno and Joris, 1995). Necrosis is characterized by cell and organelle swelling with subsequent rupture of surface membranes and the spilling of their intracellular contents. Necrosis provokes inflammatory-cell infiltration and cytokine production (Eltzschig and Eckle, 2014). Apoptosis can be initiated extrinsically by activation of sarcolemmal receptors, notably apoptosis antigen 1 (FAS) and tumor necrosis factor α receptors (TNFRs), or intrinsically by mitochondrial release of cytochrome c, which initiates a cascade of caspase activation leading to intracellular proteolysis (Ibanez et al., 2015). Cardiologists have tried to understand the mechanism of apoptosis and provide new strategies to prevent myocyte loss. A major determinant for the success of this novel approach is the degree to which apoptosis contributes to total myocyte loss and to reduce functional deterioration and mortality. However, only a few studies provide evidence of the potential of anti-apoptotic therapy to improve the outcome in CD.

During the past several years, another form of cell death, authophagic cell death, has drawn considerable attention (Di Lisa and Bernardi, 2006). Authophagy is the natural, destructive mechanism that disassembles, through a regulated process, unnecessary or dysfunctional cellular components (Kobayashi et al., 2015). Although autophagy was described in the myocardium in the beginning of history of ischemic heart, the interest about autophagy as an intracellular phenomenom began much later (Decker and Wildenthal, 1980). Autophagy has been attributed to a number of cardiac disorders, such as ischemia and cardiac hypertrophy; it enables the cardiac cell to remove the "biological wastes", such as defective mitochondria and lipofuscin, accumulated in lysosomes and thus maintain cellular homeostasis (Chen et al., 2006); stimulation of autophagy may thus have a cardioprotective effect. However, similarly to apoptosis, the mechanism of autophagic cell death remains unclear.

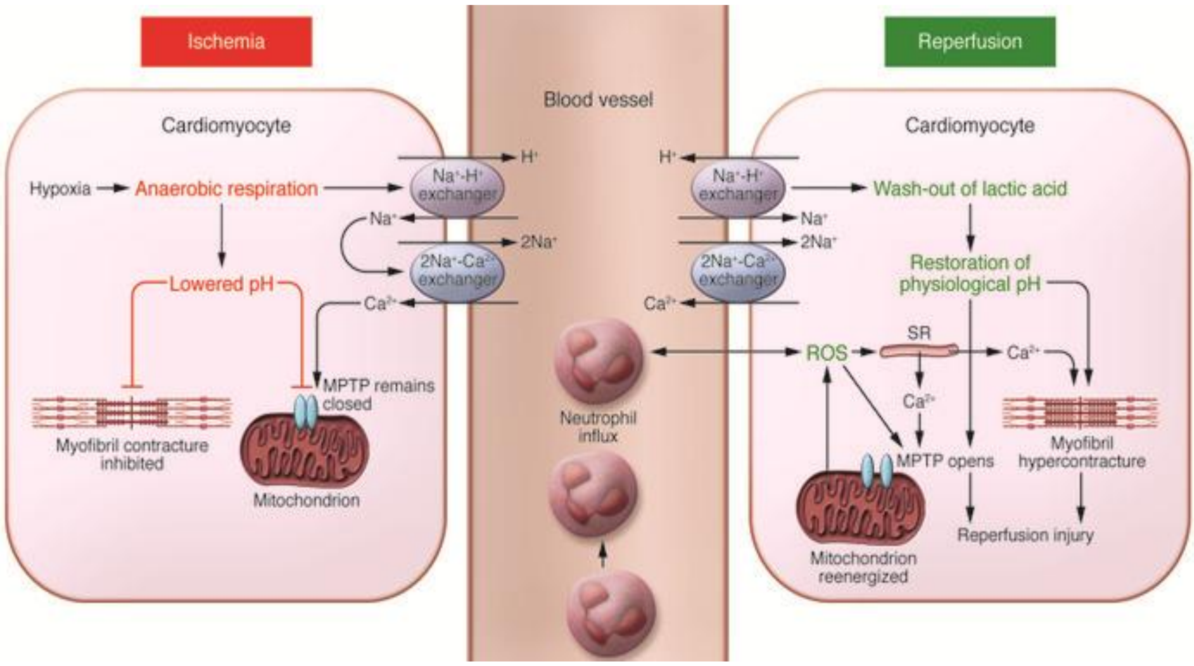


Figure 1: *Myocardial ischemia-reperfusion injury. (borrow from Hausenloy and Yellon, 2013)*

1.2. Reperfusion injury

Early coronary reperfusion represents the only effective way to limit the infarct size after ischemic period. However, there is also evidence from many studies that reperfusion may contribute to further tissue damage.

Reperfusion-induced death of cardiomyocytes that were viable at the end of the index ischemic event is defined as lethal myocardial reperfusion injury (Piper et al., 1998). The major contributory factors include oxidative stress, calcium overload, mitochondrial permeability transition pore (MPTP) opening, and hypercontracture (Yellon nad Hausenloy, 2007). During acute myocardial ischemia, the intracellular pH decreases to less than 7.0, whereas at reperfusion, physiological pH is rapidly restored by the washout of lactate and the activation of the Na^+/H^+ exchanger. This pH shift starts lethal myocardial reperfusion injury by permitting MPTP opening (Fujita et al., 2007; Milerova et al., 2010). Opening of MPTP results in mitochondrial membrane depolarization and uncoupling of oxidative phosphorylation, leading to mitochondrial membrane potential collapse, adenosine triphosphate (ATP) depletion and cell death (Hausenloy and Yellon, 2003). Therefore, pharmacological inhibition of MPTP opening at the time of reperfusion represents an important therapeutic target for preventing lethal myocardial reperfusion injury (Gomez et al., 2008).

The existence of lethal myocardial reperfusion injury has been inferred in both experimental MI models and in patients with acute ST-segment elevation MI by the observation that therapeutic interventions applied solely at the onset of myocardial reperfusion reduced MI size by 40%–50% (Hausenloy and Yellon, 2013). This observation suggests that lethal myocardial reperfusion injury may account for up to 50% of the final MI size. Lethal myocardial reperfusion injury attenuates the full benefits of myocardial reperfusion in terms of MI size reduction and thus represents an important target for cardioprotection in primary percutaneous coronary intervention patients. However, no effective therapy currently exists for reducing lethal myocardial reperfusion injury in patients who have undergone primary percutaneous coronary intervention.

Nevertheless, the reperfusion therapy still represents more or less beneficial outcome, depending on the circumstances, in particular on how early it is applied (Widimsky et al., 2003). For this reason, clinical cardiologists consider reperfusion injury to be either non-existent (reperfusion associated phenomena as accelerated expression of pre-existent injury) or clinically irrelevant (in relation to the importance of ischemic injury; Garcia-Dorado, 2004). It should be noted that many cardiovascular surgeons are associated with the existence of the potentially adverse effects of restoration of normal myocardial perfusion (Ramzy et al., 2006). The agreement of experimental and clinical cardiologists is based on that main target in reperfusion is the restoration of microcirculation; the most striking example of postischemic microvascular incompetence is the so-called no-reflow phenomenon (Ošťádal, 2005).

1.3. Cardiac protection

The degree of ischemic injury depends not only on the intensity and duration of the ischemic stimulus, but also on level of cardiac tolerance to O_2 deprivation and other components of ischemia. Therefore, it is not surprise that researches have been focused on cardioprotective mechanisms, which might increase ischemic tolerance.

In the late 1950s, the first observations appeared showing that the incidence of MI was lower in people living at high altitude (Hurtado, 1960). These epidemiological observations were later repeatedly confirmed in experimental studies using simulated hypoxia (reviewed in Ostadal and Kolar, 2007; Ostadal et al., 1998). In the early 1970s, interest concentrated on the possibility of limiting infarct size pharmacologically (Maroko et al., 1971); however, this effort was not successful because it became increasingly obvious that clinical observations did not correspond to the optimism of experimental results. After the period of skepticism, the discovery of a short-term adaptation of the myocardium, so-called “ischemic preconditioning”, by Murry et al. (1986) opened the door to the new era of cardiac protection. Several years after the description of acute cardiac protection by ischemic preconditioning (IP), a second delayed window of protection was observed (Marber et al., 1993). In 2003, Zhao et al. reported that intermittent reperfusion, after ischemia, can also reduce infarct size (Zhao et al., 2003). This phenomenom is called postconditioning (PostC). Last but not least, exercise belongs to the cardioprotective phenomena, which has big potential. The history of cardiac protection is summarized in Fig. 2.

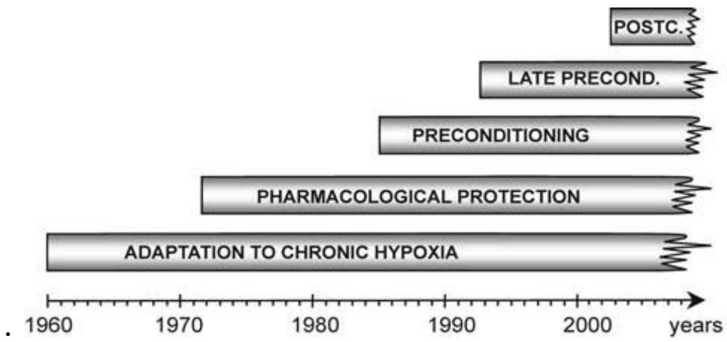


Figure 2: *History of cardioprotection. (borrow from Ostadal, 2009)*

There are several interesting aspects of IP that provide potential insight into the mechanisms. The protection afforded by IP is lost if the time between the initial IP protocol and the sustained period of ischemia is extended beyond ~1 hour. A “second window” of IP, which involves upregulation of genes, occurs ~24 hours after IP (Rizvi et al., 1999; Yellon and Baxter, 1995). The initial “early” IP does not appear to depend on new protein synthesis because of the rapid onset and since inhibition of protein synthesis does not block early IP (Thornton et al., 1990). Signalling for IP involves triggers (e.g., adenosine, several G-protein coupled cell-surface receptors and second messengers) and mediators (e.g., different protein kinases, free radicals, and NO), resulting in the activation of ATP dependent potassium channels (K_{ATP}) at the sarcolemma and in the mitochondria (sK_{ATP} and mK_{ATP}; Bolli, 2007; Hausenloy and Yellon, 2006; Murphy and Steenbergen, 2007).

Interestingly, it has been shown that the protection afforded by PostC occurs via activation of many similar signalling kinases that are involved in IP mediated protection (Hausenloy et al., 2005). Furthermore, the protection afforded by IP and PostC are not additive (Tsang et al., 2004). PostC has been suggested to involve activation of phosphoinositode 3 kinase, protein kinase B, endothelial NO synthase, protein kinase G, protein kinase Cε, extracellular signalling-regulated kinase and mK_{ATP} (Murphy and Steembergen, 2008).

Remote ischemic conditioning (RIC) describes an endogenous phenomenon in which the application of one or more brief cycles of non-lethal ischemia and reperfusion to an organ or tissue protects a remote organ or tissue from a sustained episode of lethal I/R injury. Although RIC protection was first demonstrated to protect the heart against acute MI, its beneficial effects are also seen in other organs (lung, liver, kidney, intestine, brain) and tissues (skeletal muscle) subjected to acute I/R injury (Aimo et al., 2015). The recent discovery that RIC can be induced non-invasively by simply inflating and deflating a standard blood pressure cuff placed on the upper arm or leg, has facilitated its translation into the clinical setting, where it has been reported to be beneficial in a variety of cardiac scenarios (Heusch et al., 2015). The mechanisms underlying the cardioprotective effect of RIC involve multiple intricate endogenous signalling pathways: activation of adenosine (Pell et al., 1998), bradykinin-2 (Schoemaker and van Heijningen, 2000), opioid (Patel et al., 2002), angiotensin-1 (Singh and Chopra, 2004), and CB2 endocannabinoid receptors (Hajrasouliha et al., 2008), opening of K_{ATP} channels (Pell et al., 1998), calcitonin gene-related peptide (Tang et al., 1999), ROS (Weinbrenner et al., 2004), noradrenaline (Oxman et al., 1997), NO (Wang et al., 2001), and heat shock proteins (HSPs; Tanaka et al., 1998).

From the whole spectrum of different protective phenomena we have selected: 1) adaptation to chronic hypoxia (CH) as the traditional experimental model in our laboratory area and 2) protective effect of exercise which in recent years represents promising and clinically relevant protective mechanism.

2. CHRONIC HYPOXIA

Chronic myocardial hypoxia as the result of disproportion between oxygen supply and demand at the tissue level may be induced by several mechanisms. The most common causes are undoubtedly (i) ischemic hypoxia (often described as “cardiac ischemia”), induced by the reduction or interruption of the coronary blood flow, and (ii) systemic (hypoxic) hypoxia, characterized by a drop in pO_2 in the arterial blood but adequate perfusion. For the sake of completeness we could add (iii) anemic hypoxia, in which the arterial pO_2 is normal but the oxygen transport capacity of the blood is decreased. In terms of relevant chronic clinical syndromes, ischemic hypoxia is manifested primarily in chronic ischemic heart disease whereas systemic hypoxia is associated with chronic cor pulmonale of varying origin, sleep apnea, cyanosis due to a hypoxemic congenital heart disease, and changes in the cardiopulmonary system induced by a decrease in barometric pressure at high altitude (Fig. 3).

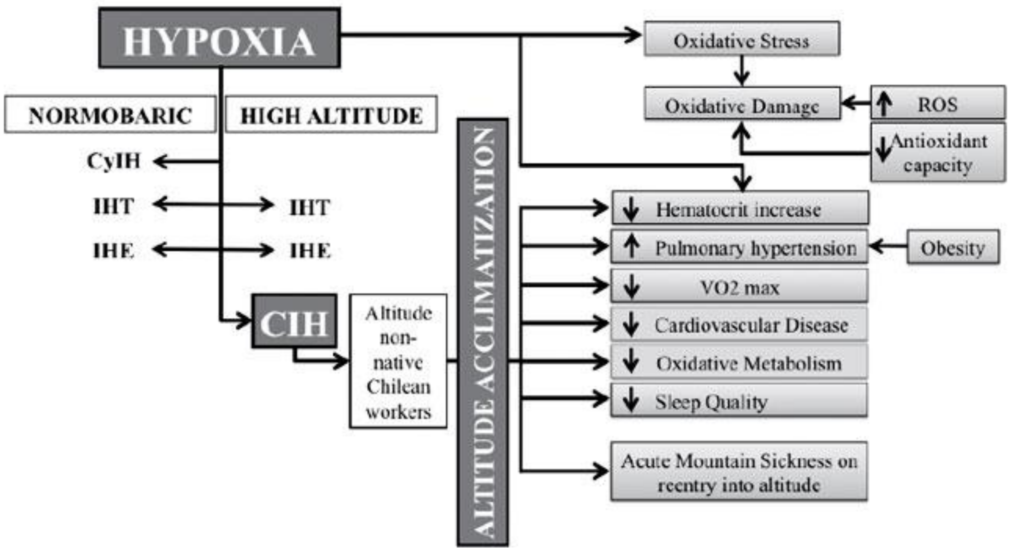


Figure 3: *Physiological responses to hypoxia and effects of high altitude acclimatization. (borrow from Farias et al., 2013)*

In two cases, however, systemic hypoxia can be considered as physiological: (i) the fetal myocardium adapted to hypoxia corresponding to an altitude of 8000m and (ii) the myocardium of subjects living permanently at high altitudes. In both situations the myocardium is significantly more resistant to acute oxygen deficiency but in populations in lowlands this property is lost soon after birth (Moret, 1980; Heath and Williams, 1995).

2.1. Experimental models of adaptation to chronic hypoxia

It should be pointed out that the term “adaptation” has been described in different ways, which occasionally leads to semantic problems in biology. According to the glossary edited by the International Union of Physiological Sciences (Bligh and Johnson, 1973), adaptation is “change which reduces the physiological strain produced by a stressful component of the total environment”. In contrast, the definition by Adolph (1956) discards the notion of benefit: “adaptations are modifications of organisms that occur in the presence of particular environments and circumstances . . . not limited, as is often done, to modifications that seem favourable to the individual”. In fact, adaptation to CH is an adjustment that does not imply, in an obligatory sense, that it is beneficial. Functional adaptive changes require time to materialize; they occur through (i) genotypic adaptations, which result from genetically fixed attributes to those species that have lived for generations in their environment, and (ii) phenotypic adaptations (including accommodation, acclimation, and acclimatization) which are labile processes occurring within the lifetime of an organism, and decay when these circumstances no longer exist (Bouverot, 1985).

The term “adaptation” as used in this thesis, refers to changes in cardiac structure and function that result from chronic exposure to natural or simulated CH. The most frequently used experimental model in research on CH is either the natural mountain environment or hypoxia simulated under laboratory conditions in a normobaric or hypobaric chamber (Table 1). This model permits the study of the time-course of development of beneficial and adverse adaptive changes, the possibility of their spontaneous reversibility when the animals are removed from the hypoxic atmosphere, and/or the pharmacological protection against unwanted manifestations. As compared to simulated high altitude, other factors such as cold or physical activity have to be taken into account in a natural mountain environment, though hypoxia remains the main stimulus. CH is, however, not always permanent; it is often of intermittent nature, e.g. in repeated ascents in mountains, in exacerbations of chronic obstructive lung disease during an acute respiratory infection, or in sleep apnea. Likewise, hypoxia is not continual in myocardial ischemia, when it depends on the actual regional coronary blood flow (Ostadal and Widimsky, 1985; Ostadal et al., 1994). Experimental data comparing the effects of permanent and intermittent CH on the myocardium are, however, very sporadic. In addition, current experimental protocols of intermittent hypoxia vary greatly in cycle length, severity and number of hypoxic episodes per day and number of exposure days. It is evident that these factors are critical in determining whether intermittent hypoxia is beneficial or harmful (Beguin et al., 2005). Another interesting methodological problem is the difference between effects of normobaric and hypobaric hypoxic exposures. Similarly as in the previous case, the available literature is not conclusive. Whereas Sheedy et al. (1996) have found that both hypobaric and normobaric hypoxia induced the same degree of right ventricular (RV) hypertrophy, remodelling of pulmonary arterioles, and increases in hematocrit, Savourey et al. (2003) have demonstrated that, compared to normobaric hypoxia, hypobaric hypoxia led to a greater hypoxemia, hypocapnia and lower arterial oxygen saturation (myocardial parameters were not investigated). Sensitivity to hypoxia is characterized by marked interspecies differences; this raises the question of suitable experimental animals. Cattle and pigs are among the most sensitive animals, sheep and dogs seem less liable to develop hypoxic pulmonary hypertension and RV hypertrophy, while rats and rabbits fall between these two groups (Tucker et al., 1975; Herget and Palecek, 1978; Reeves et al., 1979; Wauthy et al., 2004). The significance of experimental results for clinical practice depends on the extent to which observed changes are comparable to findings in humans. Pulmonary hypertension, RV hypertrophy, muscularization of the pulmonary arterioles and the enlargement of the carotid body occur in both rats and humans; the development of their ventilatory adaptation to chronic hypoxia is comparable (Heath and Williams, 1995; Ostadal et al., 1998). It is obvious, that the attempt to summarize the existing data on the effects of CH on the myocardium is complicated by different experimental models, duration and degree of hypoxic stimulus as well as by the selected experimental animals.

Adaptation to CH is characterized by a variety of functional changes to maintain homeostasis with minimum expenditure of energy (Durand, 1982). Such adjustments may protect the heart under conditions that require enhanced work and consequently increased metabolism. Adaptation thus increases cardiac tolerance to all major deleterious consequences of acute oxygen deprivation. Furthermore, chronic permanent hypoxia may have a significant antihypertensive effect, due to decreased peripheral resistance in the systemic circulation (Henley et al., 1992). In addition to protective effects, adaptation to CH also induces other adaptive responses including hypoxic pulmonary hypertension and RV hypertrophy, which may under excessive hypoxia result in congestive heart failure. We shall, therefore, deal with the development of both beneficial and adverse effects of myocardial adaptation to chronic CH.

Table 1: *Types of chronic hypoxia. (borrow from Ošťádal and Kolář, 2007)*

Type	**Human relevance**
Hypobaric hypoxia	
Permanent	Life at high altitude
Intermitent	Repeated ascents in mountains (mountaineering, tourism, pilgrim), high-altitude training
Normobaric hypoxia	
Permanent	Hypoxemic congenital heart disease, severe chronic obstructive lung disease, severe chronic ischemic heart disease
Intermitent	Exacerbations of chronic obstructive lung disease, ischemic heart disease (acute coronary syndrome, exercise), sleep apnea

2.2. Cardioprotective effects of chronic hypoxia

In CH, the myocardium must preserve adequate contractile function in spite of lowered oxygen tension in the coronary circulation. Such an environment requires genotypical adaptation or acclimatization (in lowlanders after prolonged residence at high altitude), which may have cardioprotective effects. It was reported already in the late 1950s (Hurtado, 1960) that the incidence of MI is lower in people who live at high altitude (Peru, 4000 m). An epidemiological survey from New Mexico (Mortimer et al., 1977) gave some evidence that even living at moderate elevations (2100 m) could result in protection against death from ischemic heart disease. In addition to CH, however, other factors such as relatively increased physical activity and reduced obesity have to be taken into consideration while explaining the protective effects of living at high altitude.

Epidemiological observations on the protective effect of high altitude were confirmed in experimental studies using a model of CH simulated in a hypobaric chamber. In this connection, it should be pointed out that the first experiments were carried out in Prague in 1958 by Kopecky and Daum. They found that cardiac muscle isolated from rats exposed every other day for 6 weeks to an altitude of 7000m recovered its contractile function during reoxygenation following a period of acute anoxia to a higher level than that of control animals. These results were later confirmed by Poupa et al. (1966, acute anoxia *in vitro*, isoproterenol-induced cardiac necrosis) and McGrath and Bullard (1968, acute anoxia *in vitro*). Furthermore, it has been reported (Widimsky et al., 1973; McGrath et al., 1973) that a similar protective effect can be induced by a relatively short intermittent exposure of rats to simulated high altitude (4 h/day, a total of 24 exposures up to 7000 m). Moreover, a significant sex difference was demonstrated in the resistance of isolated cardiac muscle to oxygen deficiency; the myocardium of female control rats proved to be more tolerant to hypoxia. CH resulted in enhanced resistance in both sexes, yet the sex difference was maintained (Ostadal et al., 1984). These findings were later repeatedly confirmed in studies using various experimental models, adaptation protocols, and different end points of injury. It has been found that the heart of animals adapted to CH develop smaller myocardial infarction (Meerson et al., 1973; Turek et al., 1980; Neckar et al., 2002), exhibit better functional recovery following ischemia (Tajima et al., 1994), and had a lower number of ventricular arrhythmias (Meerson et al., 1987, 1989; Asemu et al., 2000).

Examples of these cardioprotective effects are shown in Fig. 4. The antiarrhythmic protection was critically dependent on the experimental model and the degree and duration of hypoxic exposure (Asemu et al., 2000). Moreover, Henley et al. (1992) observed that adaptation to CH attenuated the development of systemic hypertension and left ventricular (LV) hypertrophy in spontaneously hypertensive rats. Zong et al. (2005) demonstrated robust cardioprotection in a novel canine model of chronic intermittent normobaric hypoxia (FIO_2 10%): 20-day program of 5–8 daily cycles of short hypoxia (5–10 min), with intervening 4-min periods of normoxia prevented development of ventricular tachycardia and fibrillation upon reperfusion. In contrast to protective effects of adaptation to CH, Joyeux-Faure et al. (2005) have recently observed that an extreme model of chronic intermittent hypoxia (FIO_2

5%, 40-s cycles of hypoxia followed by 20 s of normoxia, 8 h/day, a total of 35 days) makes the heart more sensitive to ischemic injury, probably through the excess of ROS production. In addition, persistent systemic hypertension is a common maladaptation to severe intermittent hypoxia (e.g. Kolar et al., 1989) and in models of obstructive sleep apnea, obviously as a result of increased sympathetic activity and oxidative stress (Fletcher, 2001, Zoccal et al., 2007).

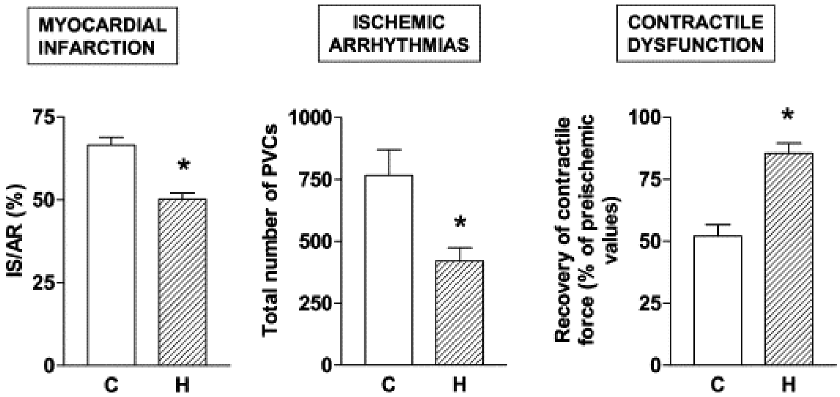


Figure 4: *Typical examples of cardioprotective effects of CH. (borrow from Ostadal and Kolar, 2007)*

2.3. Molecular mechanisms of adaptation to chronic hypoxia

Although the cardioprotective effect of CH against various manifestations of acute I/R injury has been known for half a century, its molecular mechanism (Fig. 5) did not receive major attention until recently and thus it remains far from being understood. Among numerous potentially protective factors associated with CH, only a few have been addressed experimentally so far. The situation is further complicated by the fact that various experimental models of hypoxia, animal species and methodological approaches employed as well as various end points of injury examined do not allow to compare data from different research laboratories and extrapolate them to a common picture. It cannot be excluded that the detailed involvement of individual factors is species-dependent and may differ in the protective mechanisms induced by, e.g. sustained or intermittent, mild or severe, hypoxia, etc.

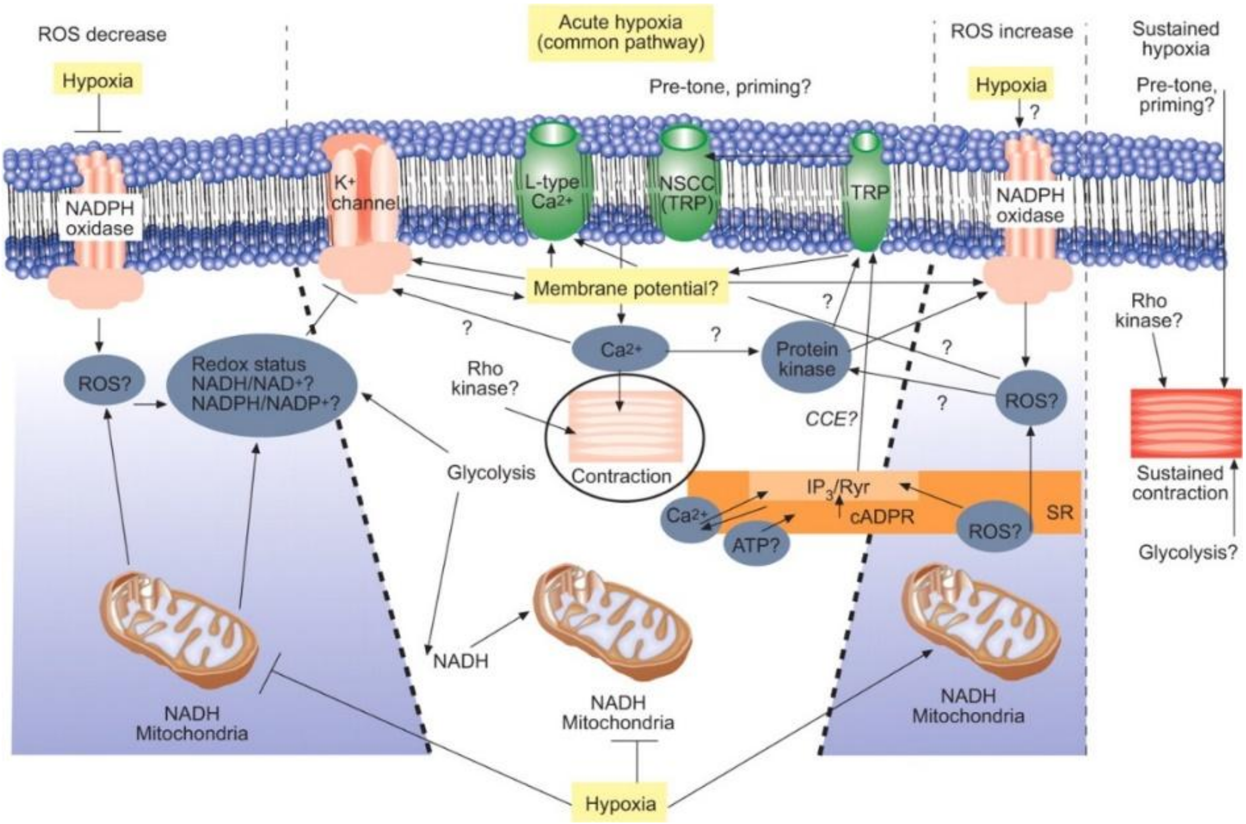


Figure 5: *Molecular pathways induced by hypoxia. (borrow from Sajkov et al, 2013)*

Nevertheless, it seems that various protective phenomena, including both short-lived IP and long-lasting effects of CH, utilize essentially the same endogenous pool of protective pathways, although with different efficiency (Neckar et al., 2002). In contrast to classic IP, CH not only activates these signalling pathways but it also affects the expression of their components and other proteins associated with maintaining oxygen homeostasis *via* transcription factors such as, e.g. hypoxia-inducible factor 1α (HIF-1α, for rev. see Semenza, 2004). It is well known that exposure to chronic intermittent hypoxia (CIH) is initially associated with oxidative stress (Yoshikawa et al., 1982; Herget et al., 2000; Chen et al., 2005; Kolar et al., 2007) and increased adrenergic stimulation (Ostadal et al., 1984a). Both events were traditionally considered as injurious but now it appears that they are also involved in the development of cardiac ischemia-resistant phenotype. Recent observations suggest that increased sympathetic activity results from the elevated carotid chemoreceptor response to hypoxia that is mediated by ROS dependent signalling and HIF-1α (for rev. see Prabhakar et al., 2007). The experiments of Mallet et al. (2006) demonstrated that robust cardioprotection in terms of infarct size-limitation and elimination of life-threatening ischemic ventricular arrhythmias in a dog model of CIH was completely prevented by administration of the β1-adrenoceptor antagonist metoprolol before each hypoxic session. In the experiments on rats, antioxidant interventions (administration of *N*-acetylcysteine or exposure to hypercapnia) during adaptation of rats to intermittent (Kolar et al., 2007) or sustained (Neckar et al., 2003) hypoxia significantly attenuated the protective effect on infarct size reduction. Milano et al. (2002) suggested that repeated reoxygenation is crucial for the induction of protective response: the recovery of contractile function was better in hearts isolated from rats that had been reoxygenated for 1 h/day throughout the hypoxic adaptation protocol compared to those maintained under sustained hypoxia.

These data suggest that both ROS and catecholamines contribute to the induction of cardioprotection by CH but the mechanism is unknown. It should be mentioned that adaptation to hypoxia decreases cardiac adrenergic responsiveness by inhibition of myocardial β-adrenoceptor-adenylyl cyclase signalling system (e.g. Voelkel et al., 1981; Hrbasova et al., 2003) that may protect the heart against excessive stimulation by catecholamines in the setting of I/R. It seems likely that chronic β1-adrenoceptor antagonism could prevent this beneficial adaptation in the study of Mallet et al. (2006). Based on the effects of NOS inhibitors or NO donors, it has been proposed that increased generation of NO plays a positive role in the protective mechanism induced by CH in neonatal rabbit (Baker et al., 1999) and rat hearts (Ostadalova et al., 2002). It remains unclear whether NO produced by the hypoxic myocardium originates from constitutive NO synthase (eNOS; Baker et al., 1999) or inducible NOS (iNOS; Rouet-Benzineb et al., 1999; Ferreiro et al., 2001; Grilli et al., 2003; Ding et al., 2005). However, it should be perceived that there is an optimal concentration of NO for protection: too little or too much may be detrimental. The role of NO in myocardial I/R injury and in adaptive protective responses of CH heart is extremely complex (for rev. see Manukhina et al., 2006; Zaobornyj et al., 2007).

Both adrenergic stimulation and increased production of ROS and NO can change the activity and/or expression of numerous signalling and effector molecules. Among them, various protein kinases (PK) were studied regarding their role in protection of CH hearts. Several reports demonstrated up-regulation and permanent activation of PKC in the myocardium following adaptation to CH (Rouet- Benzineb et al., 1999; Morel et al., 2003; Ding et al., 2004). It has been revealed that, unlike PKC isoform-ε, PKC-δ was strongly upregulated in CH rat myocardium and redistributed mainly to mitochondria and nuclear/perinuclear area (Neckar et al., 2005). These effects were ROS-dependent as they were prevented by antioxidant treatment during the hypoxic adaptation (Kolar et al., 2007). Cardioprotective effects of CH were inhibited by the general PKC inhibitor chelerythrine or PKC-δ-selective inhibitor rottlerin (Ding et al., 2004; Neckar et al., 2005) suggesting the involvement of this enzyme in the protective mechanisms. Another study demonstrated that PKC-δ and members of the family of mitogen activated protein kinases (MAPK), p38 MAPK and c-Jun N-terminal kinase (JNK), were activated and translocated from the cytosolic to the particulate fractions in CH infant human and rabbit myocardium, and inhibitors of these kinases abolished cardioprotection in hypoxic rabbits (Rafiee et al., 2002). Limited evidence suggests that many other PK such as phosphatidylinositol 3-kinase (Crawford et al., 2003; Ravingerova et al., 2007), PKA, Ca^{2+}-calmodulin-dependent protein kinase (Xie et al., 2005), cyclic guanosine monophosphate (cGMP)-dependent protein kinase (Baker et al., 1999) or extracellular signal-regulated kinase (ERK; Crawford et al., 2003) may contribute to the protective mechanism of various types of CH. Significance of these pathways, their regulation and mutual interactions remain to be elucidated. Activated PK may exert their protective effects by phosphorylation of numerous target proteins. Concerning CH, the identity of these proteins is a matter of debate, and the evidence available so far is mostly indirect and not sufficiently conclusive.

One of the potential candidates is K_{ATP}, which was studied by several groups. It was demonstrated that CH led to the activation of K_{ATP} in various tissues (Cameron and Baghdady, 1994) and already 24 h of mild hypoxia in culture increased transcription of the channel subunit SUR2A in rat heart-derived H9c2 cells (Crawford et al., 2003). Several recent reports point to the role of K_{ATP} in the cardioprotective mechanism of CH though certain controversy exists regarding the importance of the channel type that is localized either on the sarcolemma (sK_{ATP}) or the mitochondrial inner membrane (mK_{ATP}). Because the molecular identity of mK_{ATP} is unknown, the majority of these studies rely on pharmacological tools in order to distinguish which of the two types are involved in protection. Thus, experiments performed mostly on rats using selective mK_{ATP} blockers, 5-hydroxydecanoate or MCC-134, and openers, diazoxide or BMS-191095, suggest that mitochondrially located K_{ATP} plays a crucial role in the protection of CH hearts against all major end points of I/R injury (Asemu et al., 1999; Neckar et al., 2002b; Ostadalova et al., 2002; Zhu et al., 2003; Kolar et al., 2005). Activation of both mK_{ATP} and sK_{ATP} seems to contribute to improved postischemic recovery of the contractile function of CH immature rabbit hearts (Baker et al., 1997; Kong et al., 2001). As pharmacology of K_{ATP} does not seem to be sufficiently discriminative, novel methodological approaches are needed to resolve this issue.

Recent reports demonstrated that CH protects cardiac myocytes against I/R-induced cytosolic Ca^{2+} overload by preserving functions of transport and regulatory proteins that are involved in maintaining intracellular Ca^{2+} homeostasis, such as Na^{+}/Ca^{2+} exchanger, sarcoplasmic reticulum Ca^{2+} pump, ryanodine receptors (Chen et al., 2006) and phospholamban (Xie et al., 2005). Another study from the same group showed that CH protected mitochondria against Ca^{2+} overload, and delayed mitochondrial permeability transition and cytochrome *c* release upon reperfusion (Zhu et al., 2006). The latter effect, together with increased expression of antiapoptotic factor Bcl-2 and decreased expression of proapoptotic Bax, can be responsible for the reduced rate of cardiac myocyte apoptosis induced by I/R insult in CH hearts (Dong et al., 2003). However, it should not be neglected that CH *per se* can stimulate apoptosis (Bianciardi et al., 2006). Studies of Cai et al. (2003) showed that production of erythropoietin (EPO), dependent on activation of HIF-1α pathway, plays an important role not only in the stimulation of hematopoiesis but also in the increased ischemic tolerance of CH mouse heart. Angiotensin II type 1 receptor-mediated effects seem to underlie the improved postischemic recovery of the contractile function afforded by CH in neonatal rat heart (Rakusan et al., 2007). Last but not least, opioid peptides seem to contribute to the antiarrhythmic protection in CH rats (Lishmanov et al., 1998). Obviously, the list of factors and molecular pathways mentioned above is far from complete and many others (stress proteins, antioxidant enzymes, thyroid hormones, prostanoids, etc.) can be expected to play a role in the complex cardioprotective mechanism of CH. Better understanding to this phenomenon is the subject of further focused research.

2.4. Adverse effects of adaptation to chronic hypoxia

Despite to prevailing beneficial effect (increased ischemic tolerance), CH has also adverse impact. First evidence was recorded in epidemiological study of Rotta et al. (1956), who found out in healthy population living at high altitude pulmonary hypertension (PH) and RV hypertrophy. Next studies, such as by Penaloza et al. (1962) and Sime et al. (1963), confirmed these observations for the same geographical region of Peruvian Andes. Vogel et al. (1962) independently reached the same results for residents living at high altitude in the United States and Singh et al. (1965) for temporary residents in Himalayas. 3000 m was discovered as a border for developing pulmonary hypertension and RV hypertrophy in men (Hurtado, 1960).

It was observed that RV hypertrophy could be developed without full progress of chronic PH (Widimský et al., 1973). LV hypertrophy doesn't appear, only gentle increased of LV weight after prolonged exposure to high altitude (Cazarola et al., 2006). The effect of hypoxia on the pulmonary circulation results in pulmonary hypertension caused by an increase in pulmonary vascular resistance. The effect of hypoxia on the pulmonary circulation is even more pronounced during exercise, as demonstrated in studies carried out on subjects of Operation Everest II (Groves et al., 1987). Pulmonary vasoconstriction (PV) during CH can be regulated by many factors like vasoconstrictors, such as endothelin-1, angiotensin II, and vasodilatators, such as NO and prostacyclin (Aoshima et al., 2009). Hypoxic PH belongs to an important physiological mechanism that optimises ventilation-perfusion matching and pulmonary gas exchange by diverting blood flow from poorly ventilated areas of the lung (Herget et al., 2000). Hypoxic PH leads to detrimental increase in pulmonary artery pressure (Ward and McMurtry, 2009). Oxidative stress contributes to both events: hypoxic vasoconstriction and hypoxic PH (Waypa et al., 2001; Hoshikawa et al., 2001).Treatment with antioxidants (tempol or NAC) or NO donor molsidomine attenuate the effect of CH on right ventricular systolic pressure and RV hypertrophy (Elmedal et al., 2004; Andersen et al., 2005).

3. EXERCISE

The beneficial effects of exercise on the cardiovascular system have been well characterized over the last several decades and it is now accepted that exercise can be used as primary prevention for CD (Alleman et al., 2015; Fig. 6). Manifestations of CD are blunted with exercise in experimental animal models, and epidemiological data in humans further support these findings (Wang et al., 1993; Hamalainen et al., 1995).

Exercise-induced protection against acute coronary syndromes encompasses a reduction in MI (Brown et al., 2005b; Lee et al., 2012; Frasier et al., 2013), arrhythmia (Frasier et al., 2011b; Frasier et al., 2013), and stunning (Bowles et al., 1992; Taylor et al., 1999; Lennon et al., 2004; Taylor et al., 2007).

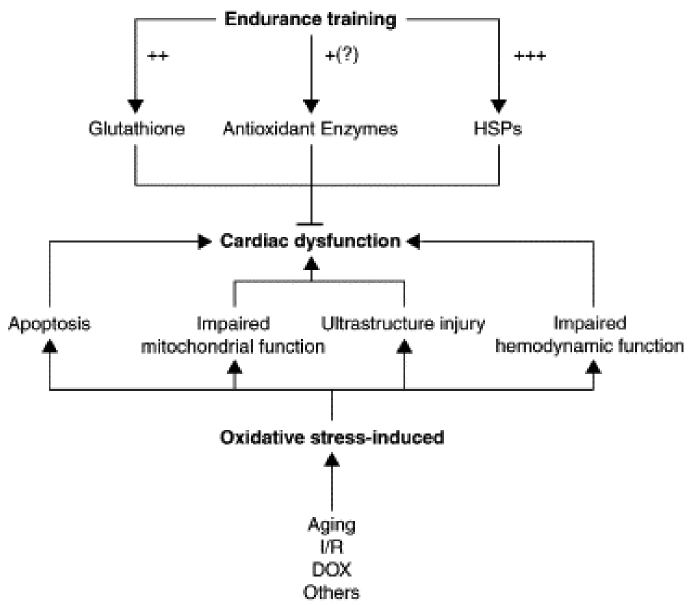


Figure 6: *Enduramce training represents effective prevention against cardiovascular diseases. (borrow from Ascensao et al., 2005)*

While there is an abundance of literature on proposed mechanisms that seek to explain the protective effects of exercise (Starnes and Taylor, 2007; Frasier et al., 2011a; Lee et al., 2012), a large portion of this research focuses on end points of protection as well as the downstream signalling events that protect the myocardium. During exercise, an increase in cardiac output is warranted so that the heart can meet the demands of exercising muscles. Aside from matching cardiac output with peripheral demand, exercise also induces preconditioning whereby the heart is more resistant to injury even long after the exercise has ceased. The proverbial “triggers” that induce cardioprotective signalling are clearly multi-factorial, and include neural, endocrine, and paracrine factors, as well as autocrine signalling and adaptations that arise from within the heart itself. Exercise can be thought of as stress; positive stress that a cell responds to in a way that allows it to better cope with that stressor. The adaptive mechanisms associated with exercise ultimately induce a cardioprotective phenotype, resulting in increased tolerance to metabolic stressors (i.e. ischemia). Proposed triggers of exercise cardioprotection include: adenosine, opioids, adenosine monophosphate-activated protein kinase (AMPK), cytokines, mitochondrial and cytosolic derived ROS, NO, and adrenergic signalling (reviewed in Alleman et al., 2015). Moreover, exercise is associated with increased hematocrit and EPO level (Ekblom, 2000).

3.1. Experimental models of exercise

"Physical activity," "exercise," and "physical fitness" are terms that describe different concepts. However, they are often confused with one another, and the terms are sometimes used interchangeably. Physical activity is defined as any bodily movement produced by skeletal muscles that results in energy expenditure. The energy expenditure can be measured in kilocalories. Physical activity in daily life can be categorized into occupational, sports, conditioning, household, or other activities. Exercise is a subset of physical activity that is planned, structured, and repetitive and has as a final or an intermediate objective the improvement or maintenance of physical fitness. Physical fitness is a set of attributes that are either health- or skill-related. The degree to which people have these attributes can be measured with specific tests. These definitions are offered as an interpretational framework for comparing studies that relate physical activity, exercise, and physical fitness to health (Caspersen et al., 1985).

Different treadmill running protocols have been developed, lasting from weeks to months, with individual running session durations ranging from minutes to hours and running speeds ranging 10-97 m/min, and with the treadmill inclinations ranging 0-25° (0-47 %) (Fenning et al., 2003; Kemi et al., 2002; Wisloff et al., 2001; Zhang et al., 2002). Most studies in rats and mice have applied continuous treadmill running, characterized by fixed or progressively increasing speed, inclination, and duration during the session. In rats, these protocols increase heart:body weight ratios up to 30 % (Diffee and Nagle, 2003; Fenning et al., 2003; Moore et al., 1993), but have also failed to induce cardiac hypertrophy, despite long exercise periods (Moran et al., 2003). In mice, continuous running protocols have induced only a limited degree of cardiac hypertrophy, either observed as modest increases in ventricular mass or cardiomyocyte dimensions (Bellafiore et al. 2007; Rosa et al. 2005), or no hypertrophy at all (Fewell et al. 1997).

The reason for the varied results is unknown. However, the relative exercise load during an exercise training period decreases if the absolute load is kept constant as the exercise capacity (maximal oxygen uptake; VO_2max) increases. This may potentially obscure the response to exercise training. Therefore, the exercise training intensity should be set relative to the individual fitness level. Interval training models have been used progressively more for studying exercise-induced cardiac hypertrophy and adaptation. This mode of exercise allows for high-intensity running bouts, in which exercise time in the high intensity zone is accumulated over time; the argument being that high aerobic intensity appears more effective than lower intensities for inducing structural and functional adaptations to the heart (Haram et al., 2009; Kemi et al., 2005). Interval training by successive 4- to 8- min high-intensity treadmill running bouts at 90 % of VO_2max; achieved by running speeds of >30 m/min on a 25° inclined treadmill, interspersed by 2-min low intensity intervals (~50 % of VO_2max), induced observable hypertrophy within 4 weeks, and resulted in 25-35 % increased LV and RV weights, and ~15 % increased cardiomyocyte dimensions after 7-13 weeks of exercise training (Kemi et al., 2002, 2005, 2008; Wisloff et al., 2001, 2002). This is superior to continuous (Iemitsu et al., 2006; Moore et al., 1993) and intensity controlled moderate intensity treadmill running programs at 65-70 % of VO_2max (Haram et al., 2009; Kemi et al., 2005). The exercise intensity in these studies was controlled by weekly measures of VO_2max, whereby the running speed was adjusted to maintain constant relative exercise intensity. Only guidance by VO_2max can achieve this (Hoydal et al., 2007). A different approach to interval running, by reducing the duration and increasing the speed of each running bout well into anaerobic intensities (97 m/min at 15°), showed only a modest degree of hypertrophy (Zhang et al. 2002). Thus, it is conceivable that the accumulated time at a high aerobic intensity accentuates cardiac hypertrophy and/or that anaerobic intensities may also cause counterproductive responses. Although most of the studies suggest that the growth response of the LV is greater or equal to the RV, it has also been reported that RV hypertrophy may be greater (Anversa et al. 1983). This may be explained by the RV performing greater relative work during exercise because of smaller mass, thinner wall, and fewer cardiomyocytes, compared to the LV.

Voluntary running programs carried out on running wheels with either no or various degrees of resistance offer less control of the exercise, since running periods and effort levels are determined by the animal itself, and may only be recorded and limited, but not reliably instigated, by the researchers. Nonetheless, voluntary wheel running has been reported to induce robust physiological hypertrophy (Allen et al., 2001; Konhilas et al., 2004; Moraska et al., 2000; Natali et al., 2001), demonstrating sufficient inherent motivation to induce adaptation. However, voluntary daily running distance peaks after ~2-4 weeks at ~10-15 km/day, and thereafter declines to <4 km/day. Accordingly, complete hypertrophy has been observed after only 3-4 weeks of voluntary wheel running, whereas longer exercise training programs have not produced further hypertrophy (Natali et al., 2001; Yancey and Overton, 1993).

Swim training is initiated by placing animals in water tanks for a given period of time. The exercise load may be regulated by attaching weights or floating devices to the animals. Although the duration of the swim exercise has varied considerably; 1-6 hours/day and 1-24 months, it generally induces cardiac hypertrophy by as much as 15 % (Kaplan et al., 1994; Medeiros et al., 2004), observable already after 1 hour/day of 1 week swim training (Edwards, 2002). It has also been demonstrated that duration (60 vs. 90 minutes) per session or of the program (4 vs. 6 weeks) or frequency (1 vs. 2/week) may not affect the magnitude of the response, whereas external weights (+2 vs. +4 % of body weight) in contrast may affect the response, with the heavier load being more effective due to greater cell hypertrophy (Evangelista et al., 2003). Hence, swim training appears equally effective as treadmill or voluntary wheel running programs for inducing physiological hypertrophy. Water temperature, by regulating core temperature, has been observed to affect the physiological hypertrophy non-linearly. Swim training at 25 ºC induced greater cardiac hypertrophy than at 35 ºC in young rats, but the opposite was true in old rats (Prathima and Devi, 1999). However, varying the water temperature 30-36 ºC yielded similar hypertrophy responses, whereas swim training at 38 ºC failed to induce hypertrophy (Harri and Kuusela, 1986). Water tank depth, density of animals, and water movement may also affect the outcome (Abel, 1994; Iemitsu et al., 2003).

3.2. Cardioprotective effects of exercise

Although there are benefits of exercise across intensities, both epidemiological and animal studies suggest that moderate to high-intensity exercise is best for the heart. The dose-response aspect relating the quantity of exercise that results in a reduction in cardiovascular risk has been extensively investigated across a number of human epidemiological studies. In a longitudinal study, Lee et al. (2000) tracked physical activity in 482 males (average 66 years of age) over a five year period and showed that energy expenditure was the key variable in reducing coronary heart disease risk. They found shorter intervals of exercise at a higher intensity provides the same protective benefit as longer intervals of exercise at a lower intensity, as long as the overall energy expenditures were equal. The study also supports the idea that exercise intensity is an important determinant of cardioprotection following an acute exercise regimen (e.g. days to weeks), and that multiple small bouts of intense exercise may have the same net result as one extended bout of exercise. Mora et al. (2007) investigated differing levels of physical activity in a group of 27,055 healthy women, determined by kcal/wk expended. They showed a dose-dependent relationship with 200-599, 600-1499 and >1500 kcal/wk groups having a 27%, 32% and 41% reduction in cardiovascular disease risk, respectively, compared to the baseline group which expended less than 200 kcal/wk.

Although the scientists acknowledged more research was necessary to determine the exact biological mechanisms that resulted in this protection, they found that the reduction in risk seen with increasing levels of physical activity can be explained in large part by a reduction in inflammatory/hemostatic biomarkers. In animal studies, cardioprotection from I/R injury has been shown to occur after only a single bout of exercise and is sustainable if the exercise continues for many months (reviewed in Frasier et al. 2011; Quindry and Hamilton 2013). The majority of our focus herein is on factors released during exercise itself. Long-term chronic exercise is likely a combination of acute factors (reaping the benefits of each individual exercise session) and additional adaptations that include shifted autonomic nervous system tone, heightened levels of cardioprotective proteins (described below), and beneficial hypertrophy. In terms of acute exercise, cardioprotection (reductions in MI) is observed after moderately high-intensity exercise (>70% VO_2 max; Yamashita et al. 1999; Hoshida et al., 2002; Brown et al., 2003; French et al., 2008; Quindry et al,. 2010b), consistent with the notion that higher intensity appears to be the most beneficial for the heart. In the following sections, we will describe the different factors released during exercise that initiate the protective phenotypic shift.

3.3. Molecular mechanisms of exercise

The mechanisms responsible for exercise-induced myocardial protection against I/R injury remain a debated issue as numerous putative mediators have been proposed. In theory, exercise-induced cardioprotection could be achieved by any physiological adaptation (Fig. 7) that attenuates one or more of the damaging events that occur during ischemia and/or reperfusion. For example, exercise-induced cardioprotection could be acquired by changes in the coronary arteries (i.e., increased collateral circulation) and/or intrinsic changes in the cardiac myocyte. Potential intrinsic changes in the cardiac myocyte that could provide cellular protection against I/R injury include increased glycolytic flux, altered NO signalling, increased levels of heat HSPs, amplified myocardial cyclooxygenase-2 (COX-2) activity, elevated endoplasmic reticulum (ER) stress proteins, enhanced function of sK_{ATP} and mK_{ATP}, increased cytosolic antioxidant capacity, and/or altered mitochondrial antioxidant capacity (Powers et al., 2014). More details are discussed below.

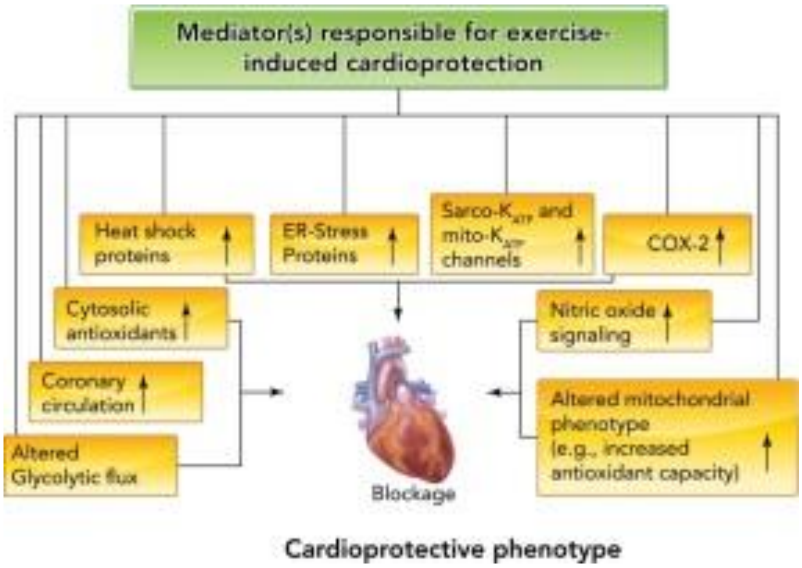


Figure 7: *Molecular mechanism of endurance training. (borrow from Powers et al., 2014)*

Myocardial survival during an I/R insult is dependent, at least in part, on cellular energy status. Hence, limiting glycolysis during long-duration myocardial ischemia could be a protective strategy to minimize I/R injury. In this regard, it has been reported that PostC results in a decrease in glycolytic flux in the ischemic heart (Vogt et al., 2002). Similarly, evidence indicates that endurance exercise training decreases the rate of glycolysis in the rat heart during ischemia (Burelle et al., 2004). Although the mechanism by which exercise training alters the metabolic phenotype of the heart to produce this response is unknown, it is feasible that a reduction in glycolysis during ischemia could be cardioprotective. Nonetheless, to date, no direct evidence exists to mechanistically connect exercise-induced changes in myocardial glycolytic flux to cardioprotection.

NO is produced in tissues from L-arginine, oxygen, and nicotinamide adenine dinucleotide phosphate (NADPH) by NOS enzymes (Powers and Jackson, 2008). Numerous studies reveal that endurance exercise training results in increased phosphorylation and activity of endothelial NOS (eNOS) in both humans and animals (Davis et al., 2004; Green et al., 2004; Hambrecht et al., 2003). This exercise-induced rise in eNOS activity is associated with increased production of NO, as evidenced by augmented levels of both nitrite and nitrosothiols in tissue and blood. In this regard, nitrite is produced by the oxidation of NO in aerobic conditions (Webb et al., 2004), whereas nitrosothiols are formed when cysteine thiols in proteins are modified by NO via a process known as *S*-nitrosylation (Foster et al., 2009). It follows that circulating levels of nitrite and nitrosthiols are commonly used as biomarkers of NO availability (Calvert and Lefert, 2013). Importantly, nitrite is a potentially important storage form of NO in both blood and tissues because nitrite can be converted to NO by either acid reduction or nitrite reductases during ischemia (Lefer, 2006).

It is clear that transgenic overexpression of HSP72 protects the heart against I/R-induced injury (Hutter et al., 1996; Jayakumar et al., 2001; Suzuki et al., 2002). Furthermore, repeated bouts of endurance exercise result in a three- to fivefold increase in cardiac HSP72 levels (Demirel et al., 2003; Hamilton et al., 2003; Powers et al., 1998). In theory, elevated cellular levels of HSP72 can protect the myocardium against I/R injury by augmenting myocardial antioxidant capacity, protecting mitochondria against I/R injury, and preventing apoptosis (Jayakumar et al, 2011; Steel et al., 2004; Suzuki et al., 2002).

Over the past decade, COX-2 has emerged as an obligatory mediator of the late phase of ischemic preconditioning-induced cardioprotection, and it follows that COX-2 could also be a candidate molecule to explain exercise-induced cardioprotection (Bolli, 2006; Bolli et al., 2002; Shinmura et al., 2002).

ER stress proteins represent a group of cardioprotective proteins that could contribute to exercise-induced cardioprotection, since recent evidence indicates that ER stress contributes to I/R-induced myocardial injury (Okada et al., 2004). Indeed, I/R-induced ER dysfunction can promote both mitochondrial-dependent and -independent cell death resulting from a disturbance in calcium homeostasis and/or impaired protein folding (Vitadello et al., 2003). Two proteins that could protect against ER stress are the glucose-regulated proteins (Grp), Grp78 and Grp94. Both Grp78 and Grp94 function in ER protein folding and also exhibit calcium-binding properties, and overexpression of Grp94 and Grp78 can protect cardiomyocytes against both calcium overload and oxidative damage (Vitadello et al., 2003; Zhang et al., 2000). Moreover, increased Grp78 and Grp94 expression is linked to a reduction in IR-induced necrosis and apoptosis in the heart (Martindale et al., 2006). Nevertheless, exercise does not elevate Grp78 and Grp94 (Murlasits et al., 2007). Therefore, the existing evidence indicates that increased ER stress proteins are not a requirement for exercise-induced cardioprotection against I/R injury.

The role that sK_{ATP} channels play in exercise-induced protection against I/R injury has received limited attention, but two studies suggest that endurance exercise training increases the expression of sK_{ATP} channels in the cardiac myocyte (Brown et al., 2005; Zingman et al., 2011). Furthermore, other reports reveal that pharmacological blockage of the sK_{ATP} channels impairs the exercise-induced protective benefits against I/R-induced myocardial necrosis (Brown et al., 2005; Quindry et al., 2012). Nonetheless, because of concerns associated with the pharmacological inhibitors used in these studies, it is difficult to form a firm conclusion regarding the mechanistic role that sK_{ATP} channels play in exercise-induced cardioprotection. Finally, using pharmacological inhibitors of the mK_{ATP} channel, it appears that mK_{ATP} channel activation protects the heart against I/R-induced ventricular arrhythmias (Quindry et al., 2010) but does not protect against I/R-induced infarction (Brown et al., 2005). Nonetheless, the inability to detect the molecular identity of the mK_{ATP} channel and concerns associated with the specificity of the channel blocker used in these experiments does not permit firm conclusions regarding the role that the mK_{ATP} channel plays in exercise-induced cardioprotection.

Superoxid dismutase (SOD) is the first line of defense against superoxide in cells, and SOD-mediated dismutation of superoxide results in formation of the nonradical ROS hydrogen peroxide (H_2O_2). Cardiac myocytes are equipped to eliminate H_2O_2 via several routes, including the enzymatic removal by catalase, thioredoxins, and glutathione peroxidase (Powers and Jackson et al., 2008). However, most studies report that the activities of catalase (CAT), thioredoxins, and glutathione peroxidase are not increased in the heart following exercise (Frasier et al., 2011; French et al., 2008; Judge et al., 2005). Nonetheless, growing evidence suggests that exercise training increases the activity of glutathione reductase in the heart via posttranslational modifications (Frasier et al., 2013). An increase in glutathione reductase activity would amplify the heart's ability to replenish cardiac levels of glutathione that is required for glutathione peroxidase to remove H_2O_2. In this regard, a recent report concludes that increases in glutathione reductase activity play an essential role in exercise-induced cardioprotection (Frasier et al., 2013). However, it is currently unclear whether this exercise-induced increase in glutathione reductase activity in cardiac myocytes is confined to the cytosolic compartment alone or whether glutathione reductase activity also increases in other cellular compartments such as the mitochondrion. Regardless of the cellular location of this enzyme, it appears likely that increases in myocardial glutathione reductase activity contribute to exercise-induced cardioprotection (Powers et al., 2014).

Emerging evidence reveals that exercise induces a mitochondrial phenotype that resists apoptotic stimuli and I/R-induced mitochondrial damage (Ascensao et al., 2006; Kavazis et al., 2009, Lee et al., 2012). Indeed, both subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria undergo biochemical adaptations in response to endurance exercise that lead to decreased apoptotic susceptibility (Kavazis et al., 2009). For example, in vitro experiments using isolated cardiac mitochondria reveal that exercise training results in a mitochondrial phenotype that resists cytochrome *c* release from both SS and IMF mitochondria exposed to ROS and/or calcium challenges (Kavazis et al., 2008). The concept that exercise training results in a mitochondrial phenotype that resists I/R-mediated damage is also supported by experiments using isolated cardiac mitochondria exposed to anoxia followed by reoxygenation. These studies reveal that, following anoxia-reoxygenation, state 3 respiration is better preserved in mitochondria isolated from the hearts of exercised rats (Ascensao et al., 2006). This finding was associated with attenuated oxidative damage to mitochondrial proteins and is in contrast to the severe metabolic dysfunction and oxidative damage observed in cardiac mitochondria isolated from sedentary animals (Powers et al., 2014).

3.4. Adverse effects of exercise

A routine of regular exercise is highly effective for prevention and treatment of many common chronic diseases and improves cardiovascular health and longevity. However, long-term excessive endurance exercise may induce pathologic structural remodelling of the heart and large arteries. Emerging data suggest that chronic training for and competing in extreme endurance events such as marathons, ultramarathons, ironman distance triathlons, and very long distance bicycle races, can cause transient acute volume overload of the atria and RV, with transient reductions in RV ejection fraction and elevations of cardiac biomarkers, all of which return to normal within 1 week. Over months to years of repetitive injury, this process, in some individuals, may lead to patchy myocardial fibrosis, particularly in the atria, interventricular septum, and RV, creating a substrate for atrial and ventricular arrhythmias. Additionally, long-term excessive sustained exercise may be associated with coronary artery calcification, diastolic dysfunction, and large-artery wall stiffening. However, this concept is still hypothetical and there is some inconsistency in the reported findings. Furthermore, lifelong vigorous exercisers generally have low mortality rates and excellent functional capacity. Notwithstanding, the hypothesis that long-term excessive endurance exercise may induce adverse cardiovascular remodelling warrants further investigation to identify at-risk individuals and formulate physical fitness regimens for conferring optimal cardiovascular health and longevity (reviewed in Okeefe et al., 2012).

In an elegant animal model of excessive endurance exercise, rats were trained (in part by prodding with electrical shocks to maintain high-intensity effort) to run strenuously and continuously for 60 minutes daily for 16 weeks, and then they were compared with control sedentary rats (Michaelides et al., 2011; Benito et al., 2011) The running rats developed hypertrophy of LV and RV, diastolic dysfunction, and dilation of the left atria and the right atria; they also showed increased collagen deposition and fibrosis in both the atria and ventricles. Ventricular tachycardia was inducible in 42% of the running rats vs. only 6% of the sedentary rats. Importantly, the fibrotic changes caused by 16 weeks of intensive exercise had largely regressed to normal by 8 weeks after the daily running regimen ceased. This animal study found that daily excessive, strenuous, uninterrupted running replicated the adverse cardiac structural remodelling and proarrhythmia substrate noted in observational studies of extreme endurance athletes. These findings support the hypothesis that in some individuals, long-term strenuous daily endurance exercise, such as marathon running or professional long-distance cycling, in some individuals may cause cardiac fibrosis (especially in the atria and the RV and interventricular septum), diastolic dysfunction, and increased susceptibility to atrial and ventricular arrhythmias. Many previous animal studies have also found acute, adverse cardiac effects of prolonged (up to 6 hours) endurance exercise, sometimes employing a rat model of cold water swimming in which the animals were forced to swim to avoid drowning (Prathasoma et al., 2014). These studies are of uncertain clinical relevance because of the excessively stressful nature of the imposed exercise.

3.5. Exercise training in hypoxia

Altitude training is frequently used by competitive athletes in a wide range of sports in the belief that it will improve sea level performance (Brosnan et al., 2000). However, the published scientific data on performance increases at sea level after extended training at altitude are contradictory. While a number of studies have reported an improvement in sea level work performance and maximal oxygen uptake following exposure to high altitude, others have observed no change (Meeuwsen et al., 2001). When training is performed under hypoxic conditions, it induces muscular and systemic adaptations which are either absent or found to a lesser degree after training under normoxic conditions (Vogt et al., 2001). Terrados et al. (1990) demonstrated that when hypoxia is combined with exercise, significantly greater increases occur in oxidative enzyme activity and myoglobin than when the same training is performed in normoxia. Thus, it seems that training in hypoxic conditions may increase the stimulus adaptation and thereby magnify the normal sea level responses to training. Conversely, altitude induced hypoxia may reduce the intensity at which athletes can train resulting in a relative deconditioning (Brosnan et al., 2000). Acute mountain sickness, problems with acclimatization and detraining due to decreased intensity are believed to influence the effectiveness of altitude training (Vogt et al., 2001). One of the major factors that can reduce the potential beneficial effect of altitude training is the reduction in training workload. Due to this reduction in aerobic power, athletes, and especially elite ones, may not reach and sustain their normal training workloads during their stay at altitude (Levine et al., 1992). It has been proposed that interval training undertaken at even moderate altitude (2500m) would result in lower absolute work rates and/or speeds, with lower heart rates (HR) and blood lactate concentrations compared with those at sea level. Indeed, investigations that compared submaximal exercise of the same relative intensity reported higher HR, a reduced training pace and higher blood lactate concentrations for exercise under hypoxic vs. normoxic conditions (Brosnan et al., 2000). A reduction in environmental oxygen at high altitude induces hypoxemia in skeletal muscle, which, in turn, causes the limitation in exercise performance, although the cause-and-effect relationship for muscle-hypoxia limiting performance is debated (Bender et al., 1989). Ascent to high altitude is accompanied by an increase in minute ventilation (VE) and a decrease in arterial O_2 saturation (SaO_2) at rest (Bender et al., 1989). The increase in VE is caused by increases in tidal volume and respiratory frequency (Ward and Nguyen, 1991). Katayama et al. (2001) have also reported that a sojourn at high altitude leads to increases in resting hypoxic ventilatory responses accompanied by increases in pulmonary ventilation and SaO_2 at rest. Moreover, Engelen et al. (1996) showed that hypoxia, which as it was said before reduces the percentage of O_2 in the arterial blood, reduces both peaks O_2 and the lactic acidosis threshold. Indeed, several studies showed that all these metabolic responses are potentiated during exercise in a hypoxic environment. Nakajono and Miyamoto (1987) showed that there was a 10.7% increase in VE during exercise in hypoxia compared to normoxia. Hogan et al. (1983) have previously reported that during an incremental maximal test performed under hypoxic conditions (17%

O_2) blood lactate concentration was elevated at moderate-to high power output (200 w) compared with normoxia.

4. TUMOR NECROSIS FACTOR ALPHA

Accumulating evidence indicates that cytokines are important mediators of CD (Biasucci et al., 1996; Giroir et al., 1992; Latini et al., 1994; Levine et al., 1990). A working understanding of inflammatory cytokines and their relationship to myocardial disease is of growing importance to basic and clinical cardiovascular scientists, immunologists, and clinicians. In this regard, tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine that has been implicated in the pathogenesis of CD, including acute MI, chronic heart failure, atherosclerosis, viral myocarditis, cardiac allograft rejection, and sepsis associated cardiac dysfunction (Neumann et al., 1995; Oral et al., 1997; Torre-Amione et al., 1996). Although initially described solely as a lipopolysaccharide (LPS)-induced macrophage product, evidence now indicates that cardiac myocytes themselves produce substantial amounts of TNF-α in response to ischemia as well as LPS (Giroir et al., 1992; Kapadia et al., 1995). Indeed, ischemia-provoked myocardial TNF-α production may prove more clinically significant than sepsis-induced myocardial TNF-α production by an order of magnitude. Ischemia and LPS are two of several clinically relevant stimulants that induce TNF-α production in the heart. The intracellular signal pathways that provoke TNF-α production are being elucidated with increasing clarity (Sweet and Hume, 1996). The discovery of the MAPKs and TNF-α transcription factors offer feasible targets for anti-TNF-α strategies. Furthermore, activation of endogenous anti-inflammatory strategies such as ligands for the gp130 subunit, induction of HSPs, and infusion of TNF-α-binding proteins now hold therapeutic promise. Control of TNF-α’s destructive role in cardiovascular disease represents a realistic goal for clinical medicine (Meldrun, 1998).

4.1. TNF-α generation

TNF-α is generated as a precursor form called transmembrane TNF-α that is expressed as a cell surface type II polypeptide consisting of 233 amino acid residues (26 kDa) on activated macrophages and lymphocytes as well as other cell types (Pennica et al., 1984; Kriegler et al., 1988; Luettiq et al., 1989). After being processed by such metalloproteinases as TNF-α -converting enzyme (TACE) between residues alanine76 and valine77, the soluble form of TNF-α of 157 amino acid residues (17 kDa) is released and mediates its biological activities through Type 1 and 2 TNF receptors (TNFR1 and TNFR2; Bazzoni and Beutler, 1996; Moss et al., 1997; Black et al., 1997). Soluble TNF-α is a homotrimer of 17-kDa cleaved monomers and transmembrane TNF-α also exists as a homotrimer of 26-kDa uncleaved monomers (Tang et al., 1996). Transmembrane TNF-α also binds to TNFR1 and TNFR2, but its biological activities are supposed to be mediated mainly through TNFR2 (Grell et al., 1995). Transmembrane TNF-α is palmitoylated at a specific cysteine residue located just at the boundary between the transmembrane and the cytoplasmic domains (Utsumi et al., 2001). In addition, serine residues of the intracellular domain of transmembrane TNF-α are phosphorylated (Pocsik et al., 1995). These kinds of post-translational modification may be important for the regulation of transmembrane TNF-α function. After releasing soluble TNF-α by TACE cleavage, the residual cytoplasmic domain of transmembrane TNF-α migrated back into the nucleus of the transmembrane TNF-α-bearing cells (Domonkos et al., 2001).

4.2. TNF-α receptors

The wide range of TNF-α activities is explained by the presence of TNFRs on almost all nucleated cells. Two distinct types of TNFRs have been identified and molecularly cloned: TNFR1 (also reffered to as TNFR55, TNFRβ, p55 or CD120a) and TNFR2 (also called TNFR75, TNFRα, p75 or CD120b), with a molecular mass of 55kDa and 75 kDa, respectively, and an equilibrum dissociation constant between the antibody and its antigen (Kd) of 500 pm and 100 pm, respectively (Fiers, 1993). Every type of cell differentially regulates the expression of the genes of TNFRs. The expression of TNFR1 gene is controlled by a non-inducible, house-keeping promoter, which doesn’t respond to separate or combinated addition of TNF-α, transforming growth factor β, interferon-α or -γ, as measured by a reporter construct in a variety of cells and is weakly induced by stimuli including TNF-α, interleukin (IL) -1, phorbol diesters and dibutyryl-cyclic adenosine monophosphate in fresh blood cells, epithelial cell lines, fibroblast cell lines and T-cells. TNFR2 is inducible expressed by up- or down-regulation of external factors. Cell lines could have 100 - 10000 copies of this receptor. The wide variability between expression of TNFR1 and TNFR2 may explain different physiological responses of cell lines (reviewed in Vandenabeele et al., 1995).

Activation of TNFRs happens by binding of TNF-α to homotrimer of TNFRs. Ligand-TNFR1 complex is rapidly internalized by clathrin-coated pits and is degraded in the lysosomes (Mosselmans et al., 1988). In comparison, TNFR2 doesn't contain tyrosine residues in its intracellular domain and, therefore is not internalized through coat pits. Treatment with selective extracellular proteolytic cleavage of TNFR2 can realise soluble TNFR2 and block its effects (Collawan et al., 1990). TNFR2 with higher affinity (Kd 100 pm) and dissociation rate (t½ 10 min) than TNFR1 (Kd 500 pm; t½>3 h) preferentially binds TNF-α at low ligand concentrations and initiate pro-inflammatory activities. However, not only level of TNF-α, but also TNFR1 to TNFR2 ratio decides how big impact of TNF-α on cell could be (Barbara et al., 1994). If TNFR2 is highly expressed, influence of TNFR1 and its cytotoxic response is reduced. Their signalling pathways are shown in Fig. 8.

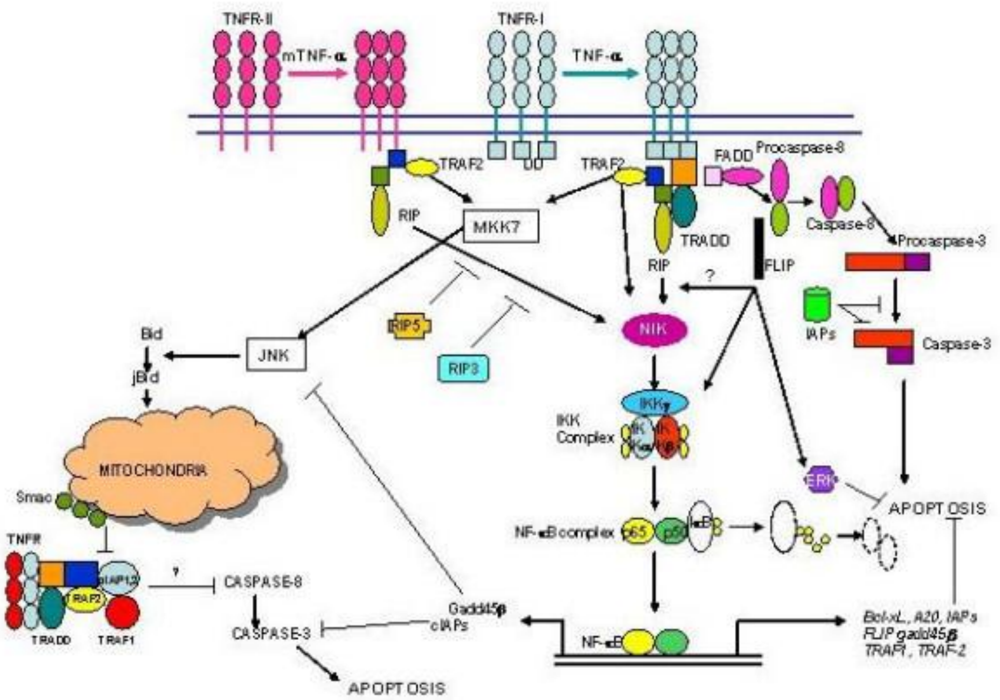


Figure 8: *Tumor necrosis factor alpha receptors and signal cascades. (borrow from Gupta et al., 2005)*

TNFR1 contents FAS-R, the "death" domain, which is connected with cytotoxic effect (Tartaglia et al., 1993). Other TNFR1 death domains are antiviral activity and induction of NOS, activation of an endosomal acidic sphinogomyelinase, which than activates nuclear factor-κB (NF-κB), and induction of IL-8 gene (Boldin et al., 1995). Activated neutral sfingomyelinase leads to activation of prolin-directed Ser/Thr protein kinase, c-Raf-1 kinase, phospholipase A_2 (PLA_2), sphingomyelinase and the transcription factor AP-1 (Wiegamnn et al., 1994; Belka et al., 1995). 5-lipooxygenase and PLA_2 result in production of arachindonic acid (AA), 5-hydroxyeicosatetraenoic acid and proinflammatory leukotrienes. Sphingomyelinase products diacyl glycerol and leads to the activation of PKC, and eventually NF-κB (Schütze et al., 1994). FAS interact with neutral sphingomyelinase, which generates ceramides. TNF-α receptor-associated proteins (TRAP-1 and TRAP-2) bind to TNFR1 in the membrane part and are strongly homological with 90 kDa HSPs (Song et al., 1994). Next two proteins, TNFR-associated factor 1 and 3 (TRAF 1 and 3) associate with TNFR1. FAS-associated death domain protein (FADD) and TNFR1-associated death domain protein (TRADD), which binds to TNFR1, activate receptor interacting protein (RIP). RIP contains N-terminal kinase domain, which activates other kinases (Stanger et al., 1995). FADD and TRADD create a scaffold permitting the recruitment of additional proteins such as the initiator of caspase, pro-caspase-8, which, when proteolytically cleaved, releases an active form of caspase-8 (Boldin et al., 1996). The active form of caspase 8 trigger pro-caspase-3, -6, -7, and other cytosolic substrates, converting these executioner pro-caspases themselves into active enzymes (Ho et al., 2005). Activation of caspase-3 results in degradation of genomic deoxyribonucleic acid (DNA). Caspase-8 inhibitory protein and inhibitor of apoptosis proteins can reduce interaction between FADD and TRADD with pro-caspase-8 (Muzio et al, 1996). In summary, TNFR1 induces a caspase-dependent apoptotic cell death. Inducible overexpression of the full-length TNFR1, without any ligand present, results in cell death and the synthesis of messenger ribonucleic acid for IL-8. At basic level of TNFR1, cell death doesn't appear (Boldin et al., 1995).

TNFR2 is constitutively phosphorylated, mainly on serine residues (Pennica et al., 1992). TNFR2 doesn't have intrinsic kinase activity, suggesting that associated molecules might mediate signal transduction. The N-terminal half of TRAF 2 is involved in the formation of homo or heterodimeric complexes and the C-terminal half in receptor interaction. TRADD and TRAF 2 interaction results not only in the activation of NF-κB but also in signalling via MAPK and JNK (Mak and Yeh, 2002). TRAF 2 binds MAPK kinases, which permits the activation of JNK, p38 SAP kinase and MAPK (Song et al., 1997). TRAF 2 is therefore critical to TNFR2-induced activation of NF-κB because TRAF 2 and RIP activate the inhibitor of NF-κB kinase (IKK), as well activating the IKK-activating kinase, NF-κB-inducing kinase (Shikama et al., 2004). Activated NF-κB translocates into the nucleus, where it binds to DNA and functions as transcriptional activator. Activated JNK subsequently activates transcription factors c-Jun, AP1 and ATF (Gupta et al., 1995). NF-κB can be activated by noncanonical way through TNFR1 and TRAF 3 in activated T-cells (McPherson et al., 2002). NF-κB switches on many genes for example: hemoxidase, MnSOD, antiapoptotic molecules (Bcl-xL, cIAPs, FLIP, Gadd45β, A20) and TNF-α itself (Gupta et al., 2005).

Recently was described full spectrum of TNF-signalling molecules. TNFRs induce ubiquitination and degradation of RIP and IKKγ via activation of specific proteins- HOIL-1, HOIP and SHARPIN (Gerlach et al., 2011). JNK activation is not always pro-proliferative, but it can drive apoptosis through the cleavage of the BH3-only protein leading to release of second mitochondrial-derived activator of caspase Smac/DIABLO and mitochondrial-mediated apoptosis (Schwabe et al., 2004). Several viruses, for example poxvirus, inhibit TNF-TNFR signalling (Chan et al., 2003).

4.3. Effects of TNF-α on the heart

The hemodynamic effects of TNF-α are characterized by decreased myocardial contractile efficiency and reduced ejection fraction, hypotension, decreased systemic vascular resistance, and biventricular dilatation (Calvin et al., 1996; Ellrodt et al., 1985; Jha et al., 1993). Before the discovery of TNF-α, several investigators suspected that sepsis-induced myocardial depression was mediated by a circulating myocardial depressant factor(s) (Clowes et al., 1983; Lefer, 1970). Parillo and colleagues (1985) demonstrated that the sera from septic patients with myocardial depression consistently depressed in vitro myocyte performance, whereas sera from septic patients without a compromised ejection fraction did not. The first experimental evidence suggesting that TNF-α mediates endotoxin-induced myocardial depression was provided by Tracey and associates (1986). They observed that TNF-α administration resulted in hypotension, metabolic acidosis, hem concentration, diffuse pulmonary infiltrates, hyperglycemia, hyperkalemia, pulmonary and gastrointestinal petechial hemorrhages, acute tubular necrosis, and death (Tracey et al., 1986). Although myocardial function was not examined, the hypotension and shock suggested myocardial depression (Fig. 9). These investigators further substantiated the link between sepsis and TNF-α by utilizing anti-TNF-α monoclonal antibodies to neutralize the circulating TNF-α and thereby prevent its adverse effects (Tracey et al., 1987). Gulick and co-workers (1989) demonstrated that TNF-α (or IL-1) inhibited cardiac myocyte adrenergic responsiveness in vitro. Similarly, TNF-α (or IL-1)-induced depression of myocardial function in an ex vivo, crystalloid-superfused papillary muscle preparation was observed by Finkel and colleagues (1992).

Because calcium homeostasis is of paramount importance to the normal myocardial contraction-relaxation cycle, several investigators have examined the effects of TNF-α on myocardial calcium handling. Indeed, coordinated and precise regulation of the oscillating intracellular calcium mediates systolic contraction, diastolic relaxation, enzymatic activity, and mitochondrial function (Meldrum et al., 1996). TNF-α-induced disruption of calcium handling may lead to dysfunctional excitation-contraction coupling and, thereby, systolic and/or diastolic dysfunction. Assessment of myocardial calcium handling can be accomplished in one of four ways: 1) the cardiac contractile state can be assessed as developed force or pressure, 2) sarcolemmal calcium handling is reflected in the action potential, 3) sarcoplasmic reticulum calcium handling is demonstrated by the calcium transient, and 4) the myofilament-regulatory complex is exhibited by the association between the calcium transient and the force of contraction (reviewed in Meldrum, 1998).

The calcium transient represents the transition from the resting state to contraction, which occurs when a small amount of calcium enters the cytosol via voltage-gated L-type calcium channels, which in turn results in a much greater release of calcium from sarcoplasmic reticulum ryanodine receptor calcium release channels. These two calcium channels have microarchitectural communication, and calcium entry through one influences the other. Yokoyama and colleagues (1993) determined that, soon after TNF-α administration, the amplitude of the calcium transient was decreased during systole. TNF-α appears to depress systolic function by disrupting calcium-induced calcium release by the sarcoplasmic reticulum. Indeed, TNF-α disrupts L-type channel induced calcium influx and thereby depresses calcium transients (Krown et al., 1995). Corroborating these findings, Oral et al. (1997) demonstrated that TNF-α's early effects on the calcium transient and systolic function were mediated by sphingosine. NO does, however, appear to mediate TNF-α-induced desensitization of myofilaments to intracellular calcium (Goldhaber et al., 1996). These findings (Gross et al., 1992; Kelly et al., 1997; Peterson et al., 1994) indicate that TNF-α-induced, sphingosine-mediated disruption of calcium-induced calcium release occurs early and that NO mediates TNF-α-induced desensitization of myofilaments to increased intracellular calcium. Although the association between massive calcium influx and myocellular ischemic injury has been established, the source of the elevated intracellular calcium remains controversial and may have important therapeutic significance. The most likely scenarios involve either ineffective sarcolemmal calcium extrusion and/or inadequate sarcoplasmic reticulum calcium sequestration (Meldrum et al., 1996). Either seems plausible because both exhibit energy-dependent kinetics, i.e., postischemia, the ATP-hungry sarcolemmal calcium-ATPse and/or sarcoplasmic reticulum calcium-ATPase would be unable to bring intracellular calcium back to the basal levels required for muscle relaxation (Meldrum et al., 1996). This, in turn, would decrease muscle shortening during a contraction, leading to both systolic and diastolic dysfunction. In addition to calcium dyshomeostasis, the mechanisms by which TNF-α causes myocardial dysfunction include direct cytotoxicity, oxidant stress, disruption of excitation-contraction coupling, and myocyte apoptosis, as well as the induction of other cardiac depressants such as IL-1 (Dinarello, 1989), IL-2 (Sobotka et al., 1990), and IL-6 (Peterson et al., 1994). Indeed, IL-1 synergistically enhances TNF-α-induced myocardial depression (Kumar et al., 1996) and cytotoxicity (Last-Barney et al., 1988). Finkel and associates (1992) demonstrated that NOS inhibition prevented the myocardial depressive effects of either TNF-α or IL-1, concluding that the negative inotropic effects were mediated by NO. LPS, TNF-α, and IL-1 each induce NOS and augment guanosine 38, 58-cyclic monophosphate, which mediates NO’s effects in other cell types (Stein et al., 1996).

NO has also been implicated in the pathogenesis of MI (Akiyama et al., 1997), hypoxia induced cardiomyocyte damage (Kitakaze et al., 1995), and autoimmune myocarditis (Ishiyama et al., 1997). Wang and Zweier (1996) observed increased NO and peroxynitrite release from the isolated rat heart after 30 min of global ischemia. Pretreatment with a NOS inhibitor resulted in a fourfold increase in the postischemic functional recovery. NO also appears to mediate the β-adrenoceptor unresponsiveness (Ungureanu-Longrois et al., 1995) that occurs during sepsis (Bensard et al., 1994). The biphasic (immediate and delayed) nature of TNF-α-induced myocardial depression suggests that TNF-α induces negative inotropic effects by at least two different mechanisms (Fig. 9; Murray et al., 1995). The early phase of TNF-α-induced functional depression occurs within minutes, whereas the delayed phase appears to require hours of TNF-α exposure (Oral et al., 1997). TNF-α may not induce high levels of NO rapidly enough to account for the early phase of myocardial depression (Oral et al., 1997).

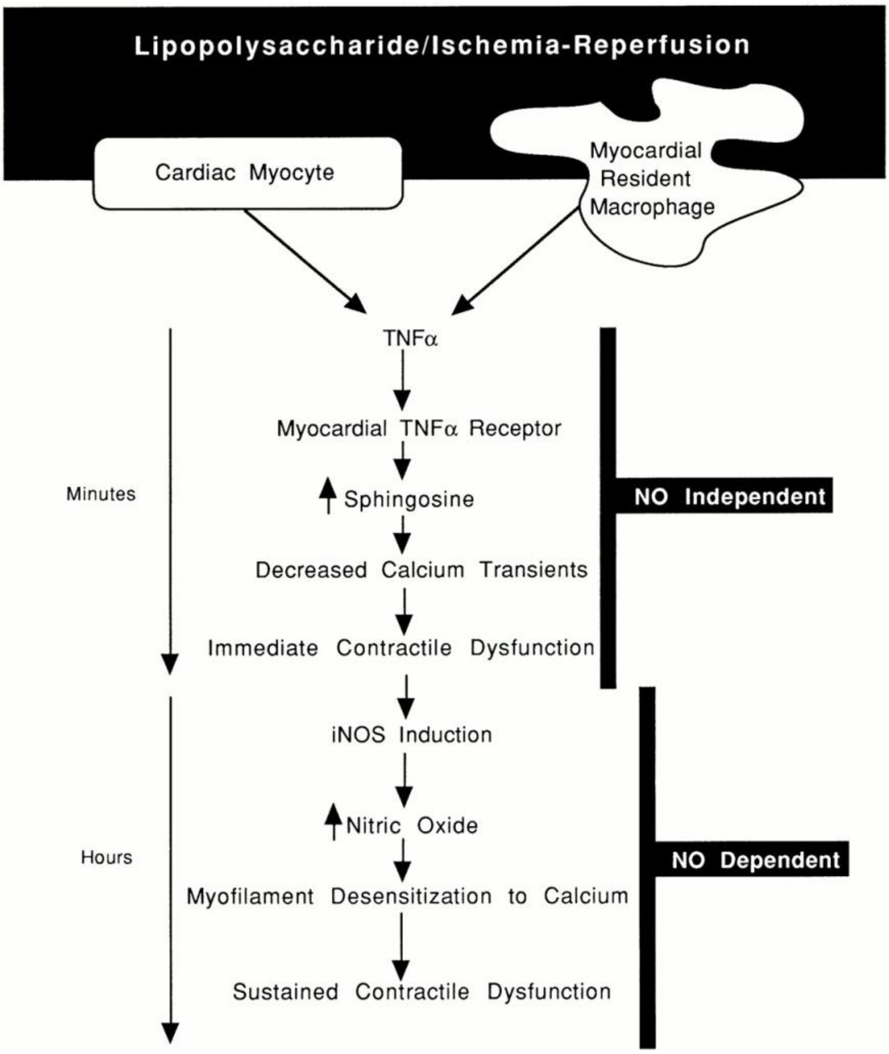


Figure 9: *Myocardial TNF-α production by both cardiac myocytes and resident macrophage contributes to 2 phases of contractile dysfunction. (Borrow from Meldrum, 1998).*

In this regard, sphingolipid metabolites are stress-induced second messengers that participate in intracellular signal transduction after TNF-α binding to the TNFR1 (Hannun et al., 1996). Two important characteristics of sphingolipid metabolites led to the hypothesis (Oral et al., 1997) that sphingosine mediates TNF-α-induced myocardial contractile dysfunction: 1) it is rapidly produced by cardiac myocytes (via sphingomyelin degeneration) after TNF-α's triggering of TNFR1 (Wiegamn et al., 1992) and 2) sphingosine decreases calcium transients by blocking the ryanodine receptor, which mediates calcium-induced calcium release from the sarcoplasmic reticulum (Sabbadini et al., 1992). These investigators (Oral et al., 1997) reported that myocardial sphingosine production occurred within minutes of TNF-α administration and temporally correlated with myocardial dysfunction and calcium dyshomeostasis in cardiac myocytes. Blockade of sphingosine production abolished TNF-α-induced contractile dysfunction, and sphingosine administration replicated TNF-α-induced contractile depression in a dose-dependent fashion.

Thus it appears likely that sphingosine mediates the early depression (NO independent) and that NO mediates the late dysfunction induced by TNF-α. Although several investigators have implicated NO in TNF-α-induced myocardial dysfunction (Goldhaber et al., 1996; Schulz et al., 1995), others have been unable to attribute all of TNF-α's depressive effects to NO (Meng et al., 1997; Yokoyama et al., 1993). In fact, it has been reported that NO can protect the myocardium during I/R injury, possibly by decreasing leukocyte mediated endothelial cell injury (Nussler and Billiar, 1993) or decreasing myocardial oxygen consumption (Sherman et al., 1997). This discrepancy may be due to differences in the quantities of NO produced during injury. The relative contribution of NO production by the calcium-dependent, constitutive form of NOS (eNOS) is at least two orders of magnitude less than the calcium-independent, cytokine-inducible form of NOS (iNOS). The low levels of NO produced by eNOS may serve a protective role, whereas the high levels produced by iNOS may be injurious (Nussler and Billiar, 1993). Thus the role of NO as a mediator of this process remains controversial; however, it is likely that TNF-α-induced myocardial depression occurs via both NO-dependent and NO-independent mechanisms (Kelly et al., 1997).

6. HYPOTHESES OF THE THESIS

1) It has been demonstrated that TNF-α via its receptor 2 (TNFR2) plays a role in the cardioprotective effects of IP (Nelson et al., 1995; Yamashita et al., 2000; Lecour et al., 2005) as well as PostC (Lacerda et al., 2009). It is also well known that CNH is associated with activation of inflammatory response (Kolar and Ostadal, 2004). With this background, our hypothesis was that TNF-α is involved in cardioprotective mechanism of CNH.

2) CNH and exercise are natural stimuli that confer sustainable cardioprotection against I/R injury (Kolar and Ostadal, 2004; Alleman et al., 2015) but it is unknown whether they can act in synergy to enhance ischemic resistance. Inflammatory response mediated by TNF-α plays a role in the infarct size-limitation by CNH (Chytilova et al., 2015) whereas exercise is associated with anti-inflammatory effects (Powers et al., 2014). Based on these facts, our hypothesis was that exercise training performed under CH affects myocardial ischemic resistance with respect to inflammatory and redox status.

7. AIMS OF THE THESIS

1) In the first part of our study, our aims were to characterize the expression of the main pro-inflammatory cytokine, TNF-α, and investigate the effect of chronic TNF-α inhibition by infliximab on cardiac ischemic tolerance, the expression of TNFR1 and TNFR2, the level of oxidative stress markers, the expression of NF-κB and its related signalling molecules in myocardium of rats adapted to CNH.

2) In the second part of our study, our aims were to determine how regular exercise training performed under conditions of CNH affects cardiac ischemic tolerance, the expression of the main markers of inflammation (TNF-α, IL-6, COX 1, COX 2, $cPLA_2$), markers of oxidative stress (iNOS, MDA) and antioxidant enzymes (MnSOD, CAT) in rats heart.

8. MATERIALS AND METHODS

This section contents methodological procedures, which were completely or at least partly performed by the author.

8.1. Animals

All experiments were performed in male Wistar rats (body weight 250-280 g, Charles River, Germany) in accordance with the Guide for the Care and Use of Laboratory Animals (published by the National Academy Press, Washington, D.C., USA).

8.2. Model of chronic normobaric hypoxia (CNH)

Rats were exposed to moderate chronic normobaric hypoxia (inspired O_2 fraction 0.1 in publication A and 0.12 in publication B) in a normobaric chamber equipped with hypoxic generators (Everest Summit, Hypoxico, NY, USA) for 3 weeks. In publication B, additional subgroup of animals (n=6) was exposed to CNH for only one week. No reoxygenation occurred during this period. The control rats were kept for the same period of time at room air. All animals were hold in a controlled environment (23°C; 12:12-h light-dark cycle; light from 5:00 AM) with free access to water and standard chow diet.

8.3. Infliximab treatment

Rats were treated weekly with a monoclonal antibody against TNF-α, infliximab (5 $mg.kg^{-1}$, i.p., Remicade; Jansen Biotech, Horsham, P.A., USA). The dose was selected from previously published pharmacokinetic study (Yang et al., 2003), and the first injection of infliximab was given one day before the start of hypoxic adaptation. Control animals got injections of saline in same volume and in same time. Normoxic and hypoxic animals were kept either at room air or in the hypoxic chamber. Corresponding treated and untreated animals were hold in the same room.

8.4. Exercise training

Rats assigned to exercise groups were habituated to forced treadmill running by increasing speed (from 25 to 30 $m.min^{-1}$) and duration (from 10 min to 60 min) of daily exercise session stepwise for 5 consecutive days. After two days of rest, the exercise protocol involved 5 days of running at 30 $m.min^{-1}$ for 60 min with a 0° inclination. Normoxic and hypoxic animals were trained either at room air or in the hypoxic chamber, respectively, during the light period. Habituation to running started after the first week of hypoxic exposure. Corresponding sedentary and trained rats were kept in the same room. The compliance of each rat with exercise training was evaluated during each session by a 5-point score: a score of 1 was given to perfectly compliant rats while a score of 5 was given to totally non-compliant ones. Mean exercise compliance score during the whole training protocol was calculated.

8.5. Infarct size and ischemic and reperfusion arrhythmias determination in open-chest rats

After anesthetization (sodium pentobarbital, 60 $mg.kg^{-1}$, i.p., Sigma Aldrich, USA), rats were ventilated (Ugo Basile, Varese, Italy) with room air at 68-70 strokes min^{-1} (tidal volume of 1.2 mL 100 g^{-1} body weight). A single-lead electrocardiogram (ECG) and blood pressure in the carotid artery were continuously recorded (Gould P23Gb; Gould, Cleveland, OH, USA) and subsequently analysed by a custom-designed software. The rectal temperature was maintained between 36.5 and 37.5°C by a heated table throughout the experiment. Hypoxic rats were anesthetized in the hypoxic chamber, and their exposure to normoxic air before the coronary artery occlusion was shorter than 40 min. Trained animals were operated immediately after the cessation of hypoxic exposure and/or the next day after the last exercise session. Left thoracotomy was performed, and a silk-braided suture 5/0 (Chirmax, Prague, Czech Republic) was placed around the left anterior descending coronary artery about 1-2 mm distal to its origin. After 10-min stabilization, regional myocardial ischemia was induced by the tightening of the suture threaded through a polyethylene tube. Ischemic period lasted 20 min with followed 3-h reperfusion induced by releasing of ligature. After 3 min of reperfusion, chest was closed, air was exhausted from thorax, and spontaneously breathing animals were maintained in deep anaesthesia. After the end of reperfusion, hearts were excised and washed with saline via aorta. The area at risk was delineated by perfusion with 5% potassium permanganate. Frozen hearts were cut into slices 1 mm thick, stained with 1% 2,3,5-triphenyltetrazolium chloride (pH 7.4; 37°C) for 30 min and fixed in formaldehyde solution. Four days later, both sides of the slices were photographed. The infarct size (IS), the size of the area at risk (AR) and the size of the LV were determined by computerized planimetric method using the software ELLIPSE (ViDiTo, Košice, Slovakia). The size of AR was normalized to LV (AR/LV), and the IS was normalized to the LV (IS/LV) and to the AR (IS/AR). The incidence and severity of ischemic arrhythmias during the 20-min ischemic insult and during the first 3 min of reperfusion were assessed according to the Lambeth Conventions. All in vivo experiments were performed by supervisor RNDr. Jan Neckář, PhD. and colleague RNDr. Petra Alánová, PhD. Photography of samples and following analysis were performed by the author.

8.6. Biochemical methods

The animals assigned to biochemical analysis were euthanized by cervical dislocation, hearts were rapidly excised, washed in cold (0°C) saline, dissected into RV, free wall of LV and the septum and weighted. All heart tissue segments were frozen in liquid nitrogen and stored at -80°C until use.

Fractionation of tissues

The samples of myocardium were crushed by pestle into power under liquid nitrogen in a ceramic bowl. The samples were homogenized by Potter homogenizer in eight volumes of ice-cold homogenization buffer (ph 7.4; 12.5 mM Tris, 250 mM sucrose, 2.5 mM EGTA, 100 mM NaF, 0.1 mM activated orthovanadate, 6 mM β-mercaptoethanol, complete protease inhibitor tablet and phospho STOP tablet). This represents homogenates. For obtaining cytosolic and the membrane fraction, homogenates were divided by centrifugal fractionation. The homogenates were centrifuged (1000xg, 10 min, 4°C). Taken supernatant were centrifuged (100000xg, 1 h, 4°C) and then centrifuged supernatant represents cytosolic franction. The pellets were re-homogenized by Potter homogenizer in 500 µl of the homogenization buffer containing 1% Triton X-100 and incubated for 30 min on ice with every 5 min vortexing. The solubilised pellets were centrifuged (100000xg, 1h, 4°C). The obtaining supernatant represents the membrane fraction. Homogenates and both fractions were stored at -80°C until use. These samples of homogenates and cytosolic and particulate fractions were used for immunoblotting and the determinations of cytokines, monocyte chemoattractant protein (MCP-1) and 3-nitrotyrosine (3-NT).

Immunoblotting

LV samples were mixed with samples buffer (Bio Rad, Hercules, CA, USA), boiled for 5 min and centrifuge at low speed. Proteins from the samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using 10-15% gel and transferred onto polyvinylidene difluoride membranes (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, membranes were washed and probed at 4°C with following primary antibodies: anti-TNFR1 (Santa Cruz Biotechnology, Dallas, Cambridge, MA, USA; sc-1070, 1:1000), anti-TNFR2 (Santa Cruz Biotechnology; sc-7862, 1:1000), anti-NF-κB p65 (Santa Cruz Biotechnology; sc-372, 1:500), anti-manganese SOD (MnSOD; Sigma Aldrich, Prague, Czech Republic; S5069, 1:250), anti-heme oxygenase (HO-1; Abcam, Cambridge, MA, USA; ab13243 , 1:1000), anti-aldehyde dehydrogenase (ALDH-2; Santa Cruz Biotechnology; sc-48837, 1:1000), anti-COX-1 (Santa Cruz Biotechnology; sc-1752, 1:1000, anti-COX-2 (Santa Cruz Biotechnology; sc-1747, 1:1000), anti-iNOS (BD Bioscience, San Jose, CA, USA; 610432, 1:500), anti-catalase (CAT; Abcam; ab16731, 1:2000), anti-cPLA$_2$α (Cell Signaling, Danvers, MA, USA; 2832S, 1:2000), anti-p-cPLA$_2$α (Cell Signaling; 2831S, 1:2000), anti-extracellular signal-regulated kinase 1/2 (ERK1/2; Cell Signaling; 4695S, 1:2000), anti-p-ERK1/2 (Cell Signaling; 4377S, 1:2000), anti-p38 (Cell Signaling; 9212S, 1:2000), anti-p-p38 (Cell Signaling; 9215S, 1:2000) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology; sc-25778, 1:500). After incubation overnight (4°C) or 1.5 h (room temperature), respectively, the membranes were washed and incubated for 1 h at room temperature with anti-rabbit (Bio Rad; 170-6515), anti-mouse (Thermo Fisher Scientific, Prague, Czech Republic) and anti-goat (Sigma Aldrich; A8919), respectively, horseradish peroxidise-labelled secondary antibodies. Bands were visualised by enhanced chemiluminescence on the LAS system or on the medical X-ray films (Agfa, Berlin, Germany). Image J (Java Technology, Cupertino, CA, USA) software was used for quantification of the relative abundance of proteins. To ensure the specificity of immunoreactive proteins, prestained molecular weight protein standards (Bio Rad) were used. The samples from each experimental group were run on the same gel and quantified on the same membrane. Neither hypoxia nor exercise affected the expression of GAPDH, which was used as aloading control.

Analysis of malondialdehyde (MDA)

The LV tissue samples were crushed by pestle into a powder under liquid nitrogen in a ceramic bowl. Then 500 μl of the homogenization buffer (25 mmol/l Tris-HCl and 0,1% Triton X-100) was added into the bowel. The power with the buffer was mixed up and transferred into new Eppendorf tube. The samples were homogenized by UV homogenizer and centrifuged (1000 xg, 10 min, 4°C). Supernatant (100 μl) was taken for the determination of MDA concentration, and the rest of supernatant was used for the determination of total protein by Bradford's method. After adding 20 μl of NaOH (6 mol/l) and vortexing, the samples were incubated at 60°C for 30 min followed by 5 min cooling at – 20°C, deproteinized by 50 μl of HClO4 (35% v/v) and centrifuged (10000xg 5 min, 4°C). Supernatant (100 μl) was taken into special dark tubes, then 10 μl of 2,4-dinitrophenylhydrazine (5 mmol/l) was added and closed with cover. The samples were incubated in the dark for 10 min, and analyzed by an high-performance liquid chromatography system (Shimadzu, Japan; column EC Nucleosil 100-5 C18; 4,6 mm x 125 mm; flow 1.0 ml/min; sampling volume 30-100 μl) with the UV detection set on 310 nm. Concentration of MDA was normalized to total protein.

Cytokines, MCP-1 and 3-NT assays

Detection of TNF-α, IL-6, IL-10, MCP-1 and 3-NT was assessed. For measurement of TNF-α, IL-6 and IL-10 in homogenates or in cytosolic and membrane fractions, respectively, were used the DuoSet ELISA capture method (eBioscience, Vienna, Austria; TNF-α: BMS622, IL-6: BMS625, IL-10: BMS629). Protein levels of MCP-1 were determined using rat MCP-1 ELISA kit (BD Biosciences; 555130). Competitive ELISA kit (Cayman, Neratovice, Czech Republic; 489542) was used to detect oxidative stress marker, 3-NT. These assays were performed on samples from different experimental groups according to the protocols described by the manufacturer.

8.7. Statistical analysis

Normally distributed variables are expressed as mean ± SEM. One-way ANOVA and subsequent Tukey's multiple comparison tests were used to examine differences between the groups. Not normally distributed data are expressed as median ± interquartil range. Differences in the number of premature ventricular complexes between the groups were compared by the Kruskal-Wallis non-parametric test. The incidence if ventricular tachycardia and fibrillation was examined by Fisher's exact test. Differences were assumed statistically significant when $P < 0.05$. Statistical analyses were performed using GraphPad Prism 6.01 (Graphpad Software Inc., CA, USA).

9. RESULTS

9.1. The role of TNF-α in cardioprotection afforded by chronic normobaric hypoxia (Publication A)

Body weight and hematocrit

Adaptation of rats to CNH caused retardation of body growth, pronounced hypertrophy of the RV and mild hypertrophy of the LV as compared to age matched normoxic controls. The hematocrit increased to 66.0±1.8% in CNH rats as compared to 44.6±0.4% in normoxic animals. Treatment with infliximab had no effect on heart weight parameters but reduced hematocrit level to 62.0±1.4% in CNH rats (Table 2).

Table 2: *Body and heart weight parameters and hematocrit in untreated and infliximab-treated rats adapted to chronic normobaric hypoxia (CNH) and normoxic (Norm) controls.*

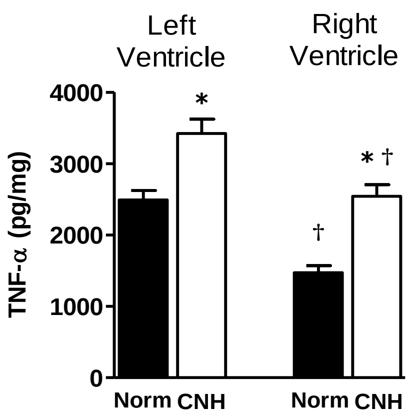
Group	n	BW (g)	RV/BW (mg/g)	LV/BW (mg/g)	HW/BW (mg/g)	RV/(LV+S)	Hematocrit (%)
Norm	6	443 ± 9	0.54 ± 0.03	1.53 ± 0.01	2.51 ± 0.03	0.27 ± 0.01	44.6 ± 0.4
Norm + Infliximab	8	440 ± 11	0.49 ± 0.01	1.46 ± 0.04	2.36 ± 0.07	0.26 ± 0.01	45.6 ± 0.5
CNH	6	342 ± 12*	1.42 ± 0.12*	1.78 ± 0.04*	3.67 ± 0.11*	0.63 ± 0.06*	66.0 ± 1.8*
CNH + Infliximab	8	349 ± 8*	1.28 ± 0.03*	1.74 ± 0.03*	3.48 ± 0.05*	0.58 ± 0.02*	62.0 ± 1.4*#

BW, Body weight; RV/BW, relative weight of right ventricle; LV/BW, relative weight of left ventricle; HW/BW, relative heart weight; RV/(LV+S), right-to-left ventricular weight ratio. Values are means SEM; *$p<0.05$ vs. corresponding normoxic group; #$p<0.05$ vs. corresponding untreated group.

TNF-α and IL-10 levels in cardiac tissue

In normoxic animals, the total concentration of TNF-α and IL-10 in myocardial homogenates were lower in RV as compared to LV (by 41 and 27%, respectively; Fig. 11A,B). CNH increased TNF-α in LV (by about 40%) and RV (by about 80%); however, this level remained lower in RV compared to LV of hypoxic rats (Fig. 11A). CNH reduced IL-10 levels in LV by 24% but had no effect on the IL-10 levels in RV (Fig. 11B). Previously published data have indicated that the IL-10/TNF-α ratio is an important determinant of myocardial inflammation (Khaper et al., 2010). In both normoxic and hypoxic hearts, the ratio was significantly higher in RV (Fig. 11C). CNH markedly reduced the IL-10/TNF-α ratio in both LV and RV from 1.37±0.06 and 1.71±0.17, respectively, in normoxic animals to 0.76±0.03 and 1.06±0.05, respectively, in hypoxic animals. These results indicate a pro-inflammatory response caused by CNH.

A



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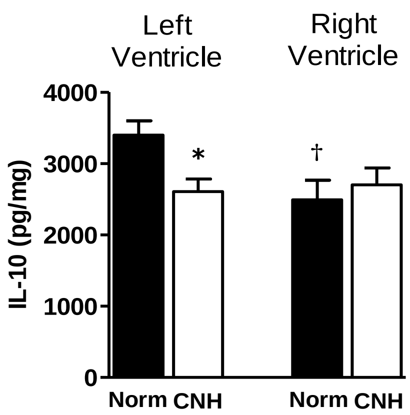

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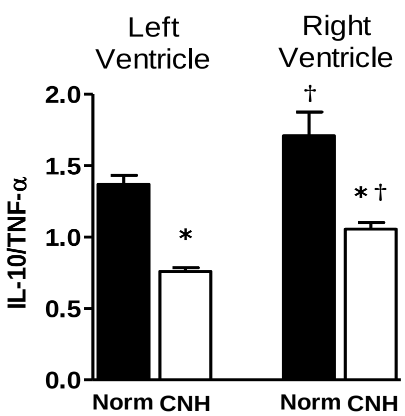


Figure 11: *Myocardial levels of (A) TNF-α, (B) IL-10 and (C) IL-10/TNF-α ratio from homogenates of left and right ventricles of rats adapted to chronic normobaric hypoxia (CNH) and normoxic controls (Norm). Values are means SEM from 6 to 8 hearts in each group;* $^{*}p<0.05$ *vs. corresponding normoxic group;* $^{\dagger}p<0.05$ *vs. left ventricle.*

Accumulating evidence suggests that not only secreted (cytosolic) but also transmembrane TNF-α precursor could be involved in the pro-inflammatory response (Horiuchi et al., 2010). Therefore, in a separate set of experiments, TNF-α concentration was analysed in the cytosolic and membrane fractions of LV collected from infliximab-treated and untreated normoxic and hypoxic rats. Particulate fractions of all experimental groups contained approximately three times more TNF-α as compared to the corresponding cytosolic fractions. CHN equally increased TNF-α level in both fractions which was completely inhibited by chronic infliximab treatment (Fig. 12A,B).

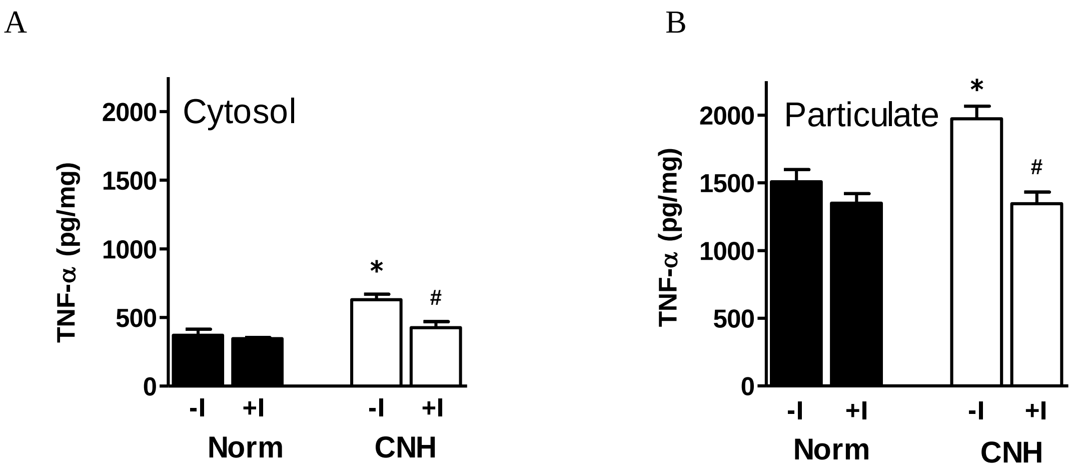


Figure 12: *The effect of infliximab (I) on myocardial levels of TNF-α in (A) cytosolic and (B) particulate fractions of left ventricle from rats adapted to chronic normobaric hypoxia (CNH) and normoxic controls (Norm). Values are means SEM from 6 to 8 hearts in each group;* $^{*}p<0.05$ *vs. corresponding normoxic group;* $^{\#}p<0.05$ *vs. corresponding untreated group.*

Cardiac ischemic tolerance

The mean normalized AR (AR/LV) was 35–41% and did not differ among groups. The infarct size reached 50.8±4.3% of the AR in the normoxic group. CNH reduced myocardial infarction to 35.5±2.4%. Chronic administration of infliximab had no effect on infarct size in normoxic rats (53.0±3.9%), but blunted the infarct size-limiting effect of CNH (44.9±2.0%; Fig. 13A). Neither CNH nor infliximab significantly affected the total number of ischemic arrhythmias (Fig. 13B). At the start of reperfusion, infliximab almost doubled the number of arrhythmias in normoxic rats from 72±22 in untreated group to 134±24, but this effect was not statistically significant due to high variability within the groups. CNH markedly reduced the total number of reperufsion arrhythmias in both untreated (23±6; p=0.083) and infliximab-treated (36±7; p<0.05) groups when compared to the corresponding normoxic animals (Fig. 13C).

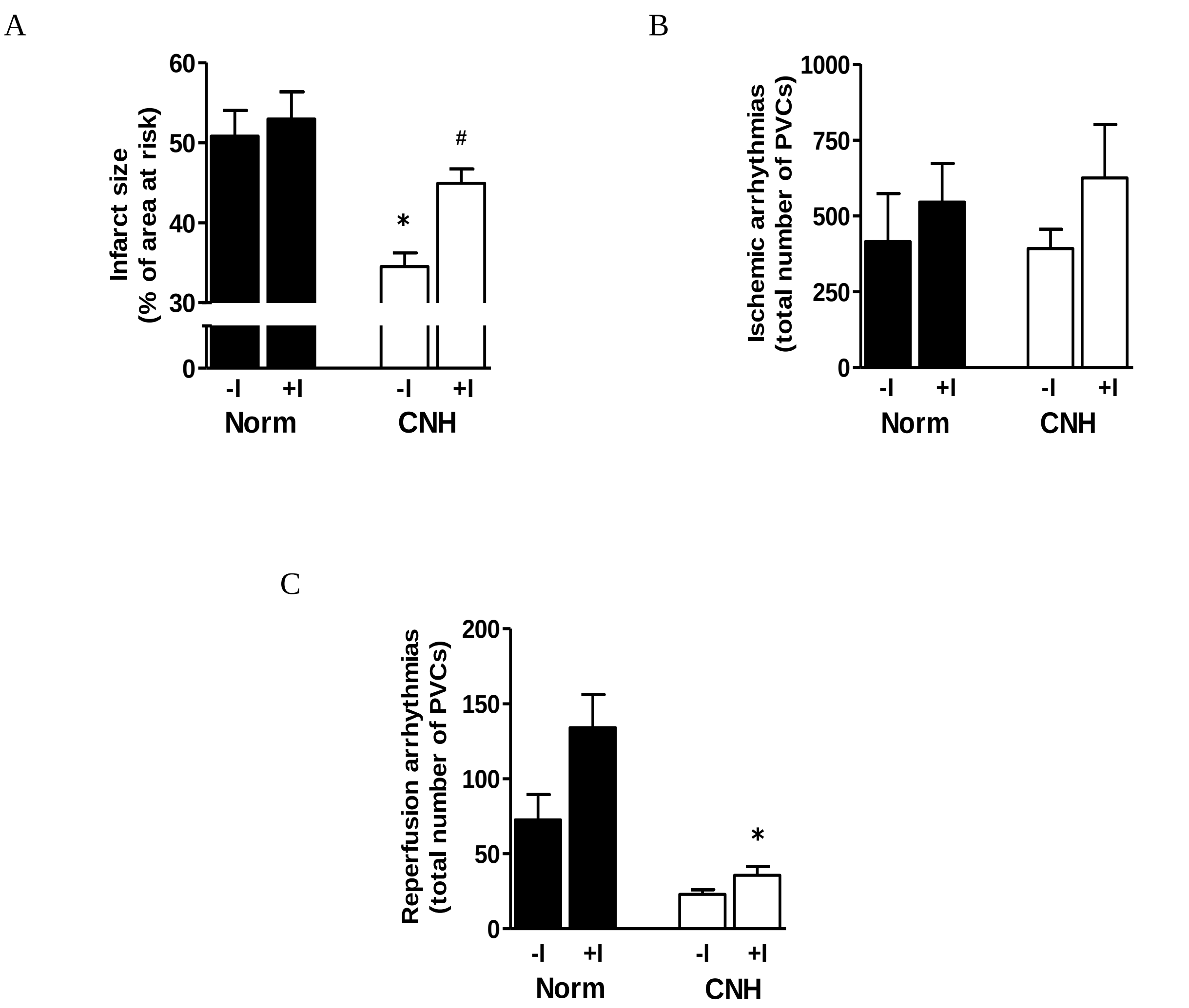


Figure 13*: The effect of infliximab (I) on (A) myocardial infarct size, (B) the total number of premature ventricular complexes (PVCs) during 20 min of ischemia and (C) the number of PVCs during the first 3 min of reperfusion in rats adapted to chronic normobaric hypoxia (CNH) and normoxic controls (Norm). Values are means SEM from 8 to 11 hearts in each group;* $^{*}p<0.05$ *vs. corresponding normoxic group;* $^{\#}p<0.05$ *vs. corresponding untreated group.*

Effect of infliximab on the expression of TNF-α receptors

Adaptation to CNH did not change the expression of TNFR1 in LV myocardium but increased the protein level of TNFR2 by 135% that was completely inhibited by infliximab treatment (Fig. 14A,B). Infliximab had no effect on protein expression of TNF-α receptors in LV of normoxic rats.

A

B

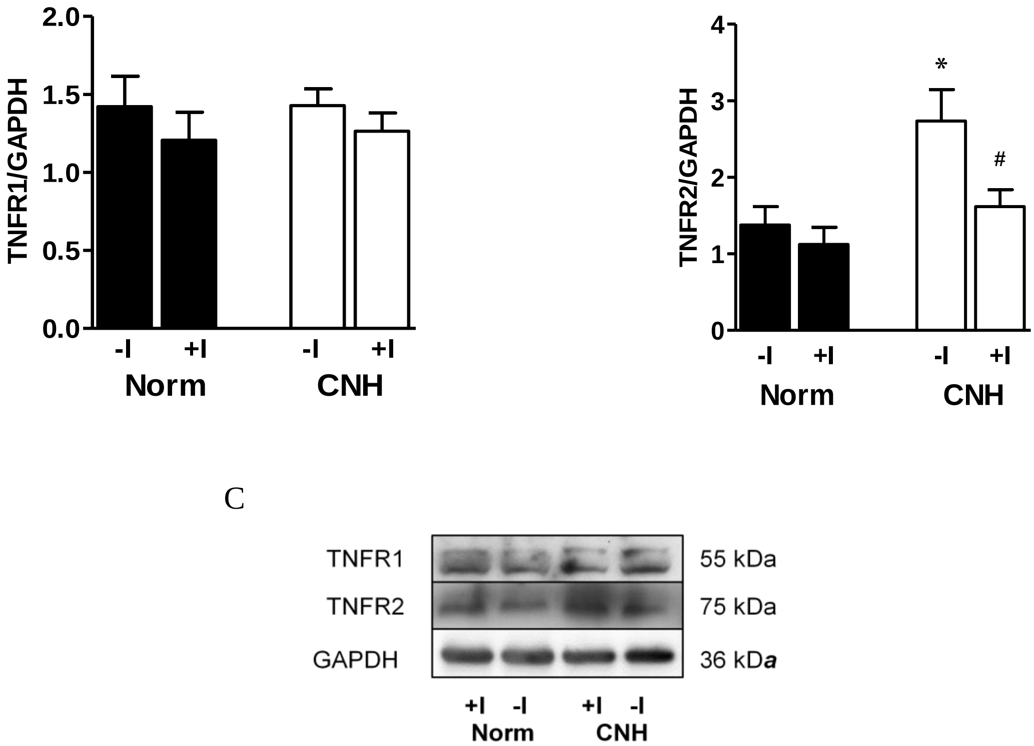


Figure 14: *The effect of infliximab (I) on myocardial levels of TNF-α (A) receptor 1 (TNFR1) and (B) receptor 2 (TNFR2) in left ventricle of rats adapted to chronic normobaric hypoxia (CNH) and normoxic controls (Norm). (C) Representative Western blots of TNFR1 and TNFR2 are shown; GAPDH was used as a loading control. Values are means SEM from 6 to 8 hearts in each group;* $^{*}p<0.05$ *vs. corresponding normoxic group;* $^{\#}p<0.05$ *vs. corresponding untreated group.*

Expression of NF-κB and cardioprotective signalling molecules

In LV of rat hearts adapted to CNH, increased TNF-α level and the expression of TNFR2 was accompanied by elevated expression of NF-κB (by 53%), which was also abolished by infliximab; no effect of treatment was observed in normoxic hearts (Fig. 15A). CNH increased expression of iNOS and COX-2 (by 162 and 46%, respectively; Fig. 15C,D). Chronic infliximab treatment had no significant effect on iNOS and COX-2 in both normoxic and CNH hearts. Nevertheless, the trend of decreasing iNOS expression in CNH hearts treated by infliximab was apparent ($p=0.097$; Fig. 15C). Neither CNH nor infliximab significantly affected myocardial concentration of MCP-1 and expression of ALDH-2 and HO-1 (Fig. 15B,E,F).

Expression of MnSOD and oxidative stress markers

CNH increased the myocardial expression of mitochondrial MnSOD and the concentrations of oxidative stress markers, MDA and 3-nitrotyrosine by 64–72% compared to the normoxic values. Chronic infliximab treatment completely eliminated these effects of CNH without affecting MnSOD and oxidative stress markers in normoxic controls (Fig. 16A–C).

The author of the thesis analysed experiments on infarct size and ventricular arrhythmias determination, prepared cytosolic and particulate fractions and homogenates for biochemical analysis and performed determination of cytokines, signalling molecules and markers of oxidative and nitrosative stress. The whole experiments in vivo were done by supervisor RNDr. Jan Neckář, PhD. in cooperation with colleague RNDr. Petra Alánová, PhD.

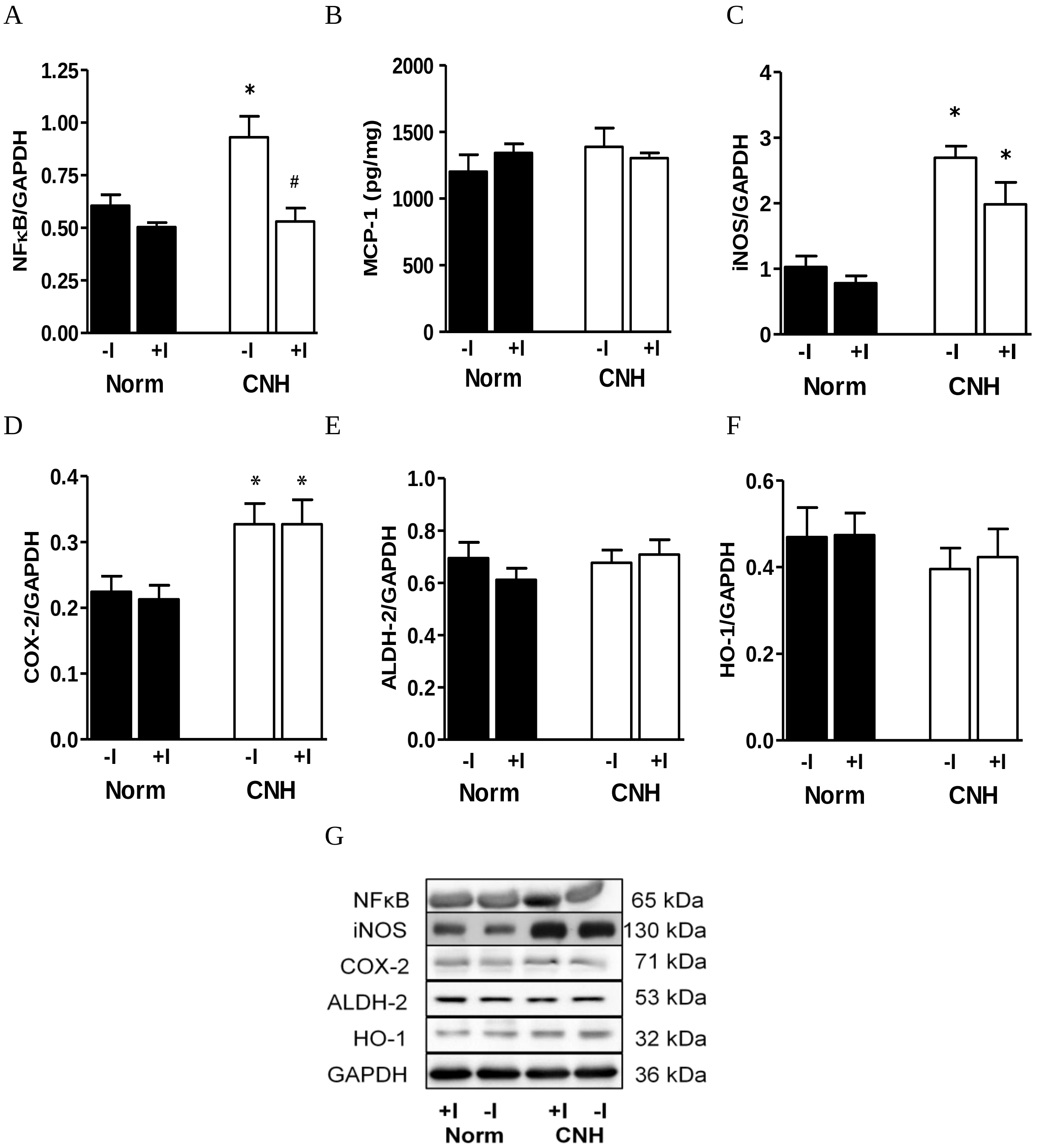


Figure 15: *The effect of infliximab (I) on myocardial level of (A) nuclear factor κB (NF-κB), concentration of (B) monocyte chemoattractant protein-1 (MCP-1), and levels of (C) inducible nitric oxide synthase (iNOS), (D) cyclooxygenase 2 (COX-2), (E) aldehyde dehydrogenase 2 (ALDH-2) and (F) haeme oxygenase 1 (HO-1) in left ventricle of rats adapted to chronic normobaric hypoxia (CNH) and normoxic controls (Norm). (G) Representative Western blots of the analysed proteins are shown; GAPDH was used as a loading control. Values are means SEM from 6 to 8 hearts in each group; *p<0.05 vs. corresponding normoxic group;* $^{\#}$*p<0.05 vs. corresponding untreated group.*

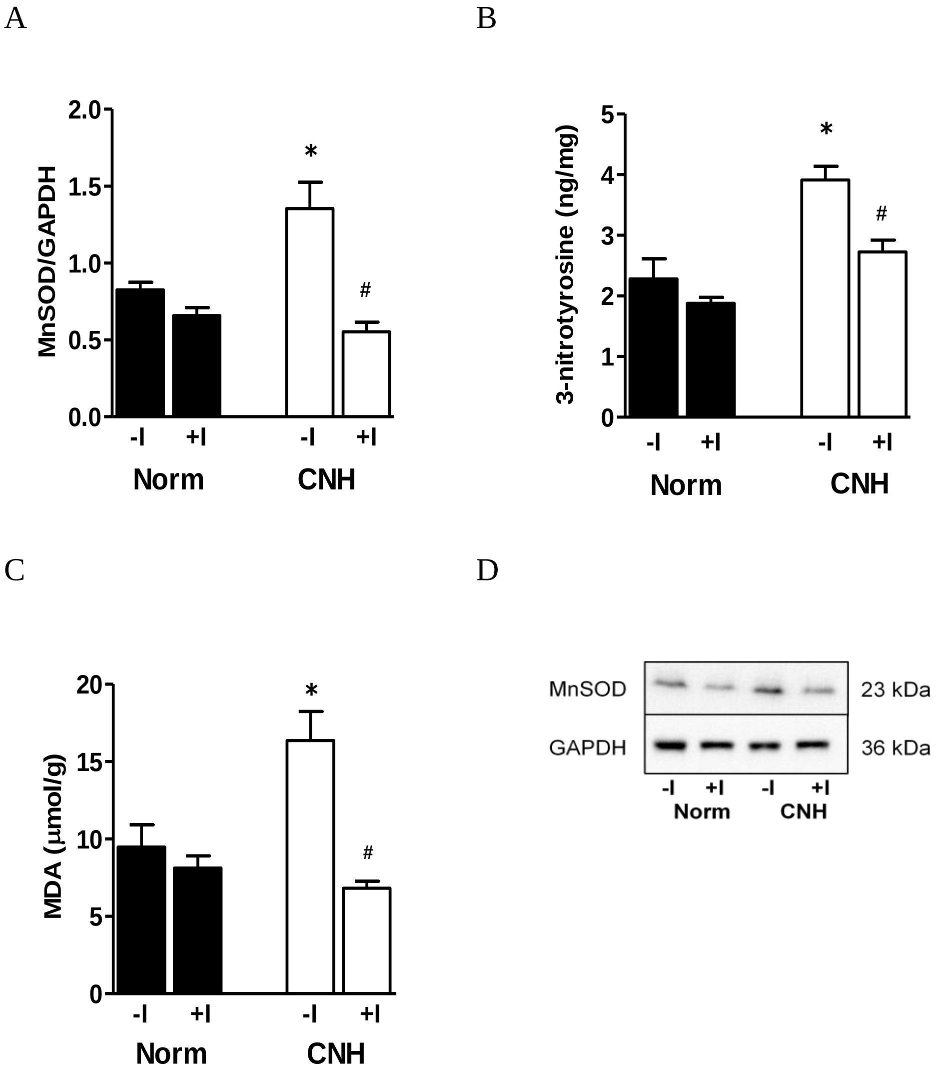


Figure 16: *The effect of infliximab (I) on myocardial level of (A) mitochondrial manganese superoxide dismutase (MnSOD) and concentrations of (B) malondialdehyde (MDA) and (C) 3-nitrotyrosine in left ventricle of rats adapted to chronic normobaric hypoxia (CNH) and normoxic controls (Norm). (D) Representative Western blot of MnSOD is shown; GAPDH was used as a loading control. Values are means SEM from 6 to 8 hearts in each group;* $^{*}p<0.05$ *vs. corresponding normoxic group;* $^{\#}p<0.05$ *vs. corresponding untreated group.*

9.2. The effect of regular exercise training under hypoxic conditions on myocardial ischemic tolerance (Publication B)

Body and heart weight and hematocrit

Adaptation of rats to CNH did not significantly affect body weight, while exercise training caused growth retardation, which was more pronounced in animals trained under hypoxic conditions. No significant differences in LV weight were observed among the groups, except for the hypoxic exercised rats, which showed increased LV weight normalized to body weight. CNH led to RV hypertrophy and increased hematocrit. These variables were not affected by exercise training (Table 3).

Table 3: *Body and heart weight parameters and hematocrit in sedentary and exercise-trained rats adapted to chronic normobaric hypoxia (CNH) and normoxic (Norm) controls.*

Group	n	BW (g)	LVW (mg)	LV/BW (mg/g)	RVW (mg)	RV/BW (mg/g)	Hematocrit (%)
Norm sedentary	8	422 ± 9	538 ± 20	1.275 ± 0.038	229 ± 6	0.542 ± 0.008	45.7 ± 0.8
Norm trained	9	380 ± 7#	528 ± 20	1.391 ± 0.044	205 ± 5	0.541 ± 0.014	45.2 ± 0.8
CNH sedentary	8	397 ± 6	506 ± 22	1.271 ± 0.039	341 ± 15*	0.857 ± 0.033*	53.1 ± 1.3*
CNH trained	8	330 ± 3*#	474 ± 12	1.436 ± 0.031*	288 ± 7*#	0.875 ± 0.027*	55.9 ± 0.8*

BW, Body weight; LVW, left ventricle weight; LV/BW, relative weight of left ventricle; RVW, right ventricle weight; RV/BW, relative weight of right ventricle. Values are means SEM; *p<0.05 vs. corresponding normoxic group; #p<0.05 vs. corresponding sedentary group.

Myocardial infarct size and arrhythmias

The mean normalized AR (AR/LV) was 39–43% and did not differ among the groups. The infarct size reached 54.1±4.0% of the AR in the normoxic group and exercise training decreased it to 44.3±2.7%. CNH reduced infarct size to 36.7±3.3% but exercise at hypoxia did not provide any additive protection (37.4±3.7%; Fig. 17).

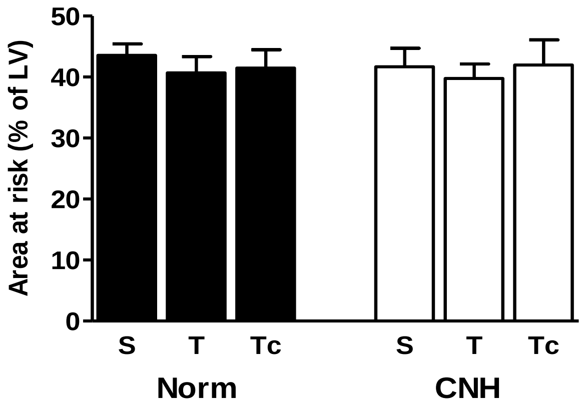
Neither CNH nor exercise training significantly affected the total number of ischemic ventricular arrhythmias (Fig. 18A) and the total duration of tachyarrythmias (tachycardia and reversible fibrillation; Fig. 18B). However, animals trained at hypoxia were more susceptible to ischemic arrhythmias than their normoxic counterparts. Sustained fibrillation occurred in 18–32% of rats, except for the sedentary hypoxic group which exhibited only reversible fibrillation; the differences among groups did not reach statistical significance (Fig. 18C). CNH reduced the total number of arrhythmias occurring at the beginning of reperfusion but exercise abolished this effect (Fig. 18D).

The mean exercise compliance score of normoxic and chronically hypoxic animals was 1.29 and 2.06, respectively. To verify that the somewhat worse compliance of rats exercising at hypoxia compared to those trained at room air did not affect myocardial ischemic tolerance, we selected well-compliant animals (score of 1.0–1.5) from both groups. The mean score was 1.23 and 1.24 in selected normoxic and CNH subgroups, respectively. Fig. 17 and Fig. 18 show that this selection had no significant effect on infarct size and the susceptibility to arrhythmias.

IL-6, TNF-α and its receptors

Adaptation to CNH for 3 weeks increased myocardial levels of TNF-α and IL-6 by 53% and 88%, respectively, compared to the normoxic sedentary group. No increase was absent when TNF-α was measured after the first week of the hypoxic exposure (93% of normoxic level). Exercise training had no effect on these cytokines in the hearts of normoxic rats but it significantly attenuated their increase induced by CNH (Fig. 19A,B). CNH had no effect on the myocardial protein level of TNFR1 while significantly increasing TNFR2 level (by 102%). Exercise training of normoxic rats affected neither TNFR1 nor TNFR2 but it prevented the increase in TNFR2 level in the group adapted to CNH (Fig. 19C,D).

A



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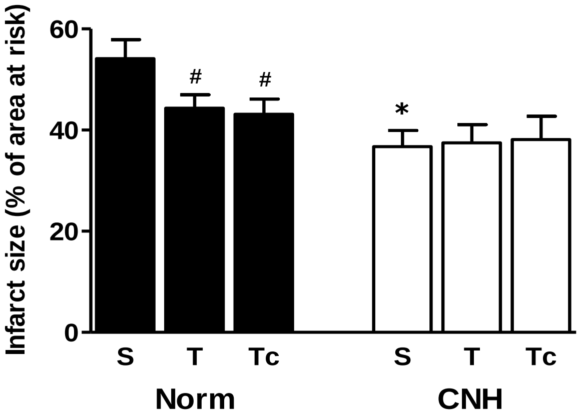

Figure 17: *(A) Myocardial area at risk and (B) infarct size induced by coronary artery occlusion and reperfusion in sedentary (S) and exercise-trained (T) rats adapted to chronic normobaric hypoxia (CNH) and normoxic (Norm) controls. Tc denotes the subgroups well-compliant to exercise training. Values are means SEM from 7–15 hearts in each group;* **p<0.05 vs. corresponding normoxic group;* $^{\#}$*p<0.05 vs. corresponding sedentary group.*

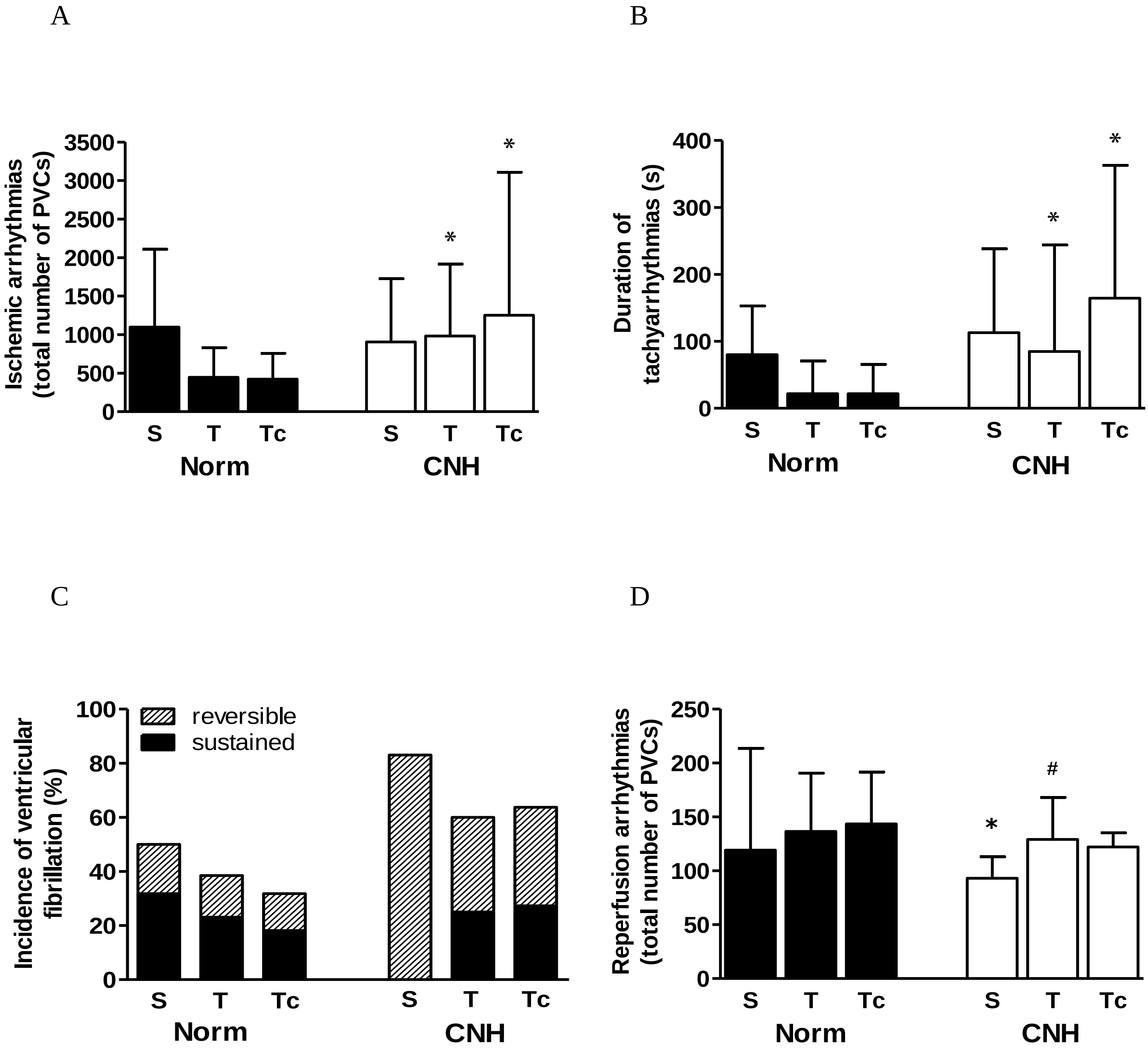

Figure 18: *(A) The total number of premature ventricular complexes (PVCs), (B) total duration of tachyarrhythmias and (C) the incidence of reversible/sustained ventricular fibrillation during coronary artery occlusion, and (D) total number of PVCs during the first 3 min of reperfusion in sedentary (S) and exercise-trained (T) rats adapted to chronic normobaric hypoxia (CNH) and normoxic (Norm) controls. Tc denotes the subgroups well-compliant to exercise training. Values (graphs A, B and D) are shown as median with interquartil range from 7–15 hearts in each group; $^{*}p<0.05$ vs. corresponding normoxic group; $^{\#}p < 0.05$ vs. corresponding sedentary group.*

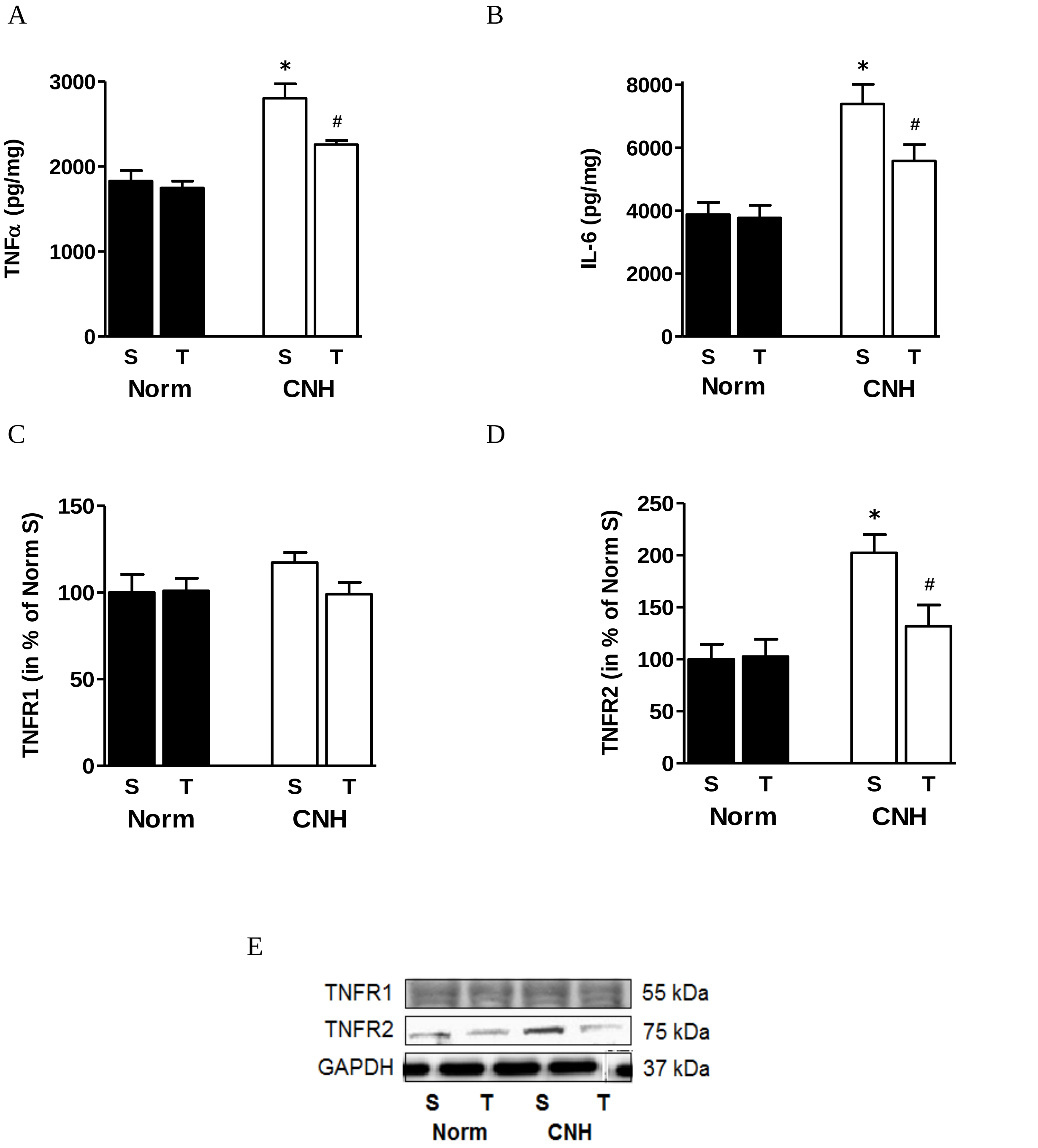

Figure 19: *Myocardial levels of (A) tumour necrosis factor-α (TNF-α), (B) interleukin-6 (IL-6), TNF-α (C) receptor 1 (TNFR1) and (D) receptor 2 (TNFR2) in left ventricle of sedentary (S) and exercise-trained (T) rats adapted to chronic normobaric hypoxia (CNH) and normoxic (Norm) controls. (E) Representative Western blots of TNF-α receptor 1 and 2 are shown; GAPDH was used as a loading control. Values are means SEM from 8 hearts in each group;* $^{*}p<0.05$ *vs. corresponding normoxic group;* $^{\#}p<0.05$ *vs. corresponding sedentary group.*

NF-κB and relating signalling

The expression of transcription factor NF-κB was increased by CNH by 71%. This increase was reduced by exercise training which had no effect in normoxic rats. Nevertheless, NF-κB level still remained significantly higher in rats exercising at hypoxia compared to their normoxic counterparts (Fig. 20A). CNH increased the expression of iNOS (by 63%) which was not significantly affected by exercise (Fig. 20B). Both $cPLA_2\alpha$ and its phosphorylated form were upregulated by CNH by 13% and 26%, respectively. These increases were abolished by exercise training which had no effect in the normoxic group (Fig. 20C,D). Neither CNH nor exercise affected COX-1 level, while COX-2 level was increased by 43% in the CNH group, the effect being attenuated by exercise (Fig. 20E,F).

MDA and antioxidant enzymes

Myocardial MDA concentration increased by 76% and the expression of MnSOD and CAT rose by 75% and 24%, respectively, in the hearts of rats adapted to CNH for 3 weeks. MnSOD measured after the first week of the hypoxic exposure remained unaffected, reaching 101% of normoxic value. Exercise training had no effect in the normoxic animals and it only tended to attenuate the CNH-induced increases of MDA, MnSOD and CAT without reaching statistical significance (Fig. 21A,B,C). Neither CNH nor exercise affected the expression of CS which is commonly used as a marker of mitochondrial mass (Fig. 21D)

The author of the thesis participated on analyses of experiments on infarct size and ventricular arrhythmias determination, prepared homogenates for biochemical analysis and performed determination of cytokines, signalling molecules and markers of oxidative and nitrosative stress. The whole experiments in vivo were done by supervisor RNDr. Jan Neckář, PhD. in cooperation with colleague RNDr. Petra Alánová, PhD.

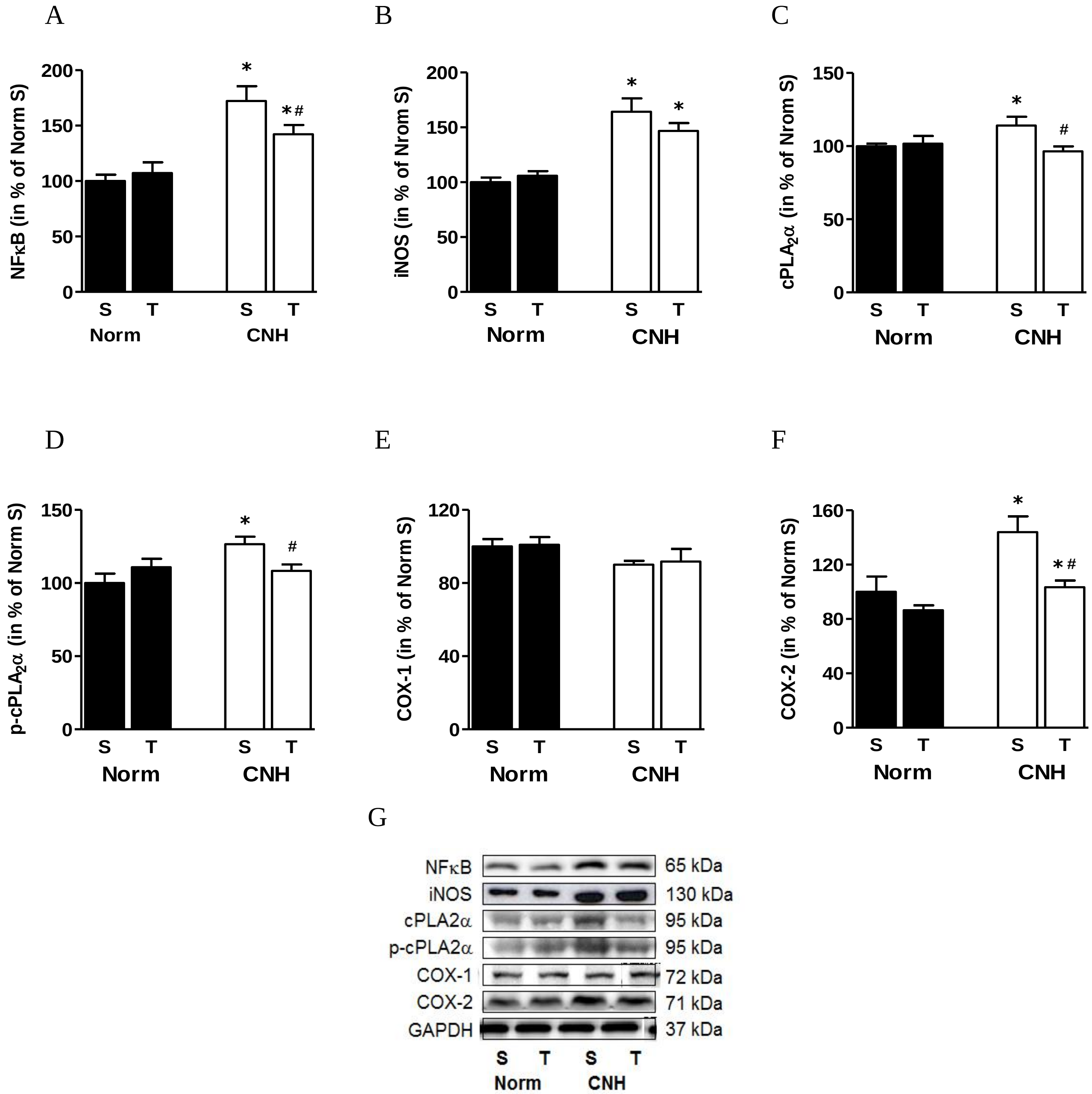

Figure 20: *Myocardial levels of (A) nuclear factor-κB (NF-κB), (B) inducible nitric oxide synthase (iNOS), (C) cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$), (D) phosphorylated form of $cPLA_2\alpha$ ($p\text{-}cPLA_2\alpha$), (E) cyclooxygenase-1 (COX-1) and (F) cyclooxygenase-2 (COX-2) in left ventricle of sedentary (S) and exercise-trained (T) rats adapted to chronic normobaric hypoxia (CNH) and normoxic (Norm) controls. (G) Representative Western blots of the analysed proteins are shown; GAPDH was used as a loading control. Values are means SEM from 8 hearts in each group;* $^{*}p<0.05$ *vs. corresponding normoxic group;* $^{\#}p<0.05$ *vs. corresponding sedentary group.*

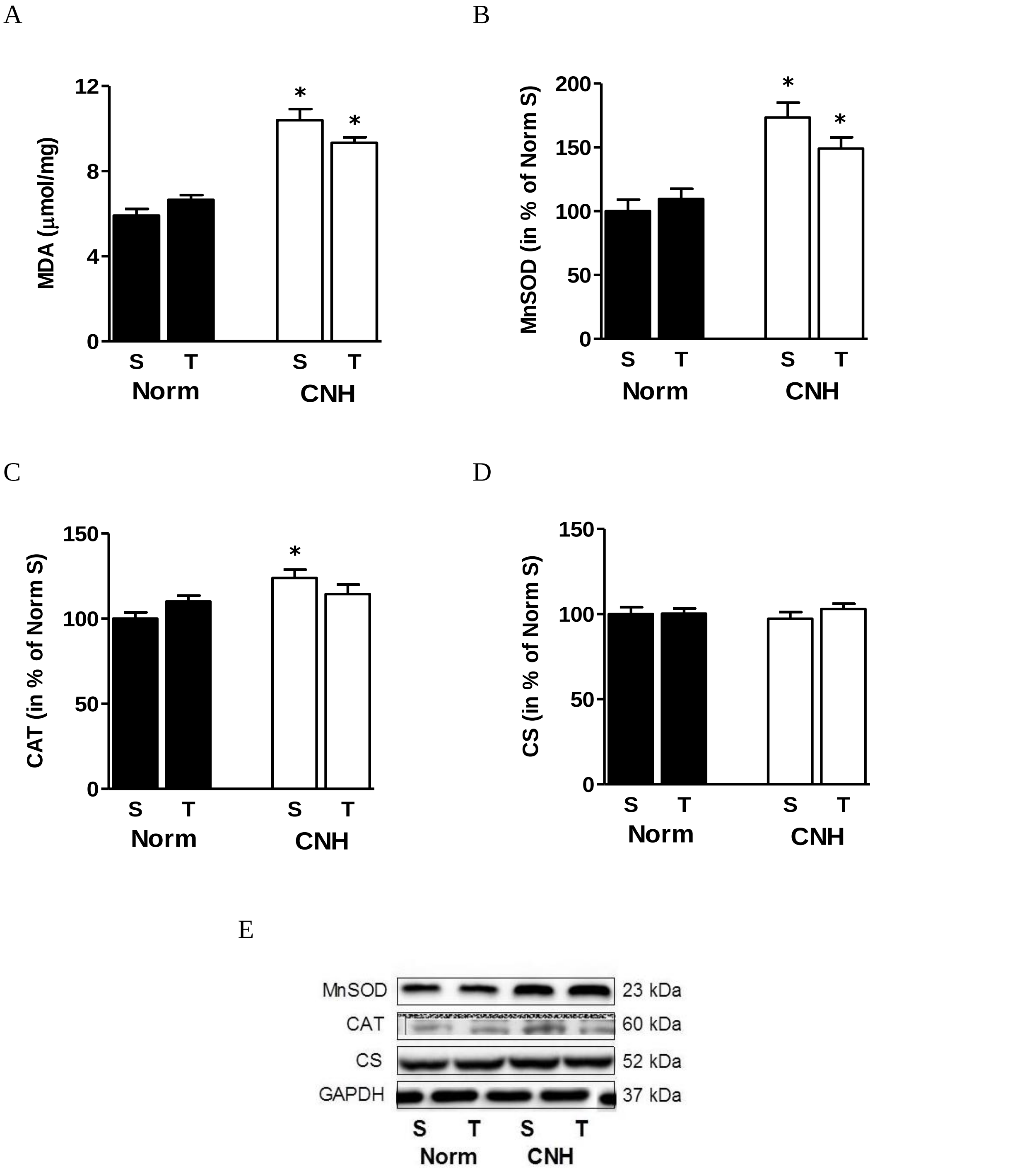

Figure 21: *(A) Concentration of malondialdehyde (MDA) and myocardial levels of (B) manganese superoxide dismutase (MnSOD), (C) catalase (CAT) and (D), citrate synthase (CS) in left ventricle of sedentary (S) and exercise-trained (T) rats adapted to chronic normobaric hypoxia (CNH) and normoxic (Norm) controls. (E) Representative Western blots of the analysed proteins are shown; GAPDH was used as a loading control. Values are means SEM from 8 hearts in each group;* $^{*}p<0.05$ *vs. corresponding normoxic group.*

9. DISSCUSION

The role of TNF-α in cardioprotection afforded by chronic normobaric hypoxia (Publication A)

We have confirmed our first hypothesis that TNF-α is involved in cardioprotective mechanism of CH.

The main finding of the present study is that adaptation to CNH improved cardiac ischemic tolerance in rats that was accompanied by increased myocardial concentration of proinflammatory cytokine TNF-α and its receptor TNFR2. Chronic treatment with TNF-α inhibitor infliximab during adaptation attenuated the infarct size-limiting effect of CNH. CNH increased myocardial oxidative stress and induced overexpression of transcription factor NF-κB and MnSOD that were abolished by infliximab treatment.

Adaptation to CH represents the protective phenomenon that improves cardiac ischemic tolerance with a similar efficiency as different forms of acute conditioning (pre-, per- and post-conditioning). However, as compared to the fast activation of protection by conditioning, the development of ischemia-tolerant phenotype of chronically hypoxic hearts needs more time – from several days to weeks (Asemu et al., 2000; Zhang et al., 2000; Neckar et al., 2013). Moreover, the cardioprotection afforded by CH persists for weeks (Neckar et al., 2004; Fitzpatrick et al. 2005), that is much longer than short-lived effects of conditioning. Therefore, the improved ischemic tolerance of CH hearts can be considered as a form of sustained cardioprotection (Peart and Headrick, 2008). However, its underlying mechanism has not been fully elucidated (Ostadal and Kolar, 2007). In the present study, we indicate for the first time a role of TNF-α signalling in the cardioprotective mechanism of CNH.

We demonstrated that CNH markedly increased TNF-α level in both RV and LV. This finding is in agreement with the increased expression of TNF-α and pro-inflammatory genes in hearts of chronically hypoxic adult rats or foetal guinea-pigs (Chen et al., 2007; Oh et al., 2008; Klusonova et al., 2009). Interestingly, Smith et al. (2001) showed that knockout TNF-α/ mice exhibited lower pulmonary hypertension and RV hypertrophy upon adaptation to CH than wild-type animals. Although we did not detect a significant effect of TNF-α inhibition on RV hypertrophy, the erythropoietic response to CNH was attenuated in infliximab-treated rats, as indicated by a smaller increase in hematocrit. Altogether, these results suggest that

TNF-α plays a role not only in the induction of the improved cardiac ischemic tolerance but also in other adaptive responses of the organism to CH.

TNF-α is generated as a precursor called transmembrane TNF-α, a 26-kDa protein. This form is subsequently cleaved by TNF-α-converting enzyme to the secreted (soluble) and active form of TNF-α (17 kDa) that mediates its biological action through its receptors, TNFR1 and TNFR2. The activation of TNF-α receptor-specific response was shown as an important event in cardiac ischemic tolerance. While an excessive TNF-α expression and subsequent TNFR1 activation are deleterious, a lower TNF-α concentration and TNFR2 activation are protective (Flaherty et al., 2008; Lacerda et al., 2009; Schulz and Heusch, 2009; Katare et al., 2010). Previously, Ramirez et al. (2012) observed decreased gene expression of TNFR1 in CH rat hearts. Our results showed increased expression of TNFR2 but not TNFR1 in LV of rats adapted to CNH. Moreover, chronic treatment by TNF-α inhibitor infliximab abolished the increased TNFR2 level and blunted infarct size-limiting effect of CNH. These data suggest that adaptation to CNH improved cardiac ischemic tolerance in rat hearts by activation of protective TNFR2 signalling but had no effect on detrimental signalling mediated by TNFR1.

Not only secreted TNF-α but also its transmembrane form exerts various biological actions that modulate the local inflammation and contribute to physiological as well as pathophysiological responses (Horiuchi et al., 2010). Transmembrane TNF-α mediates its biological activities mainly through TNFR2 (Grell et al., 1995) which is the key receptor for the beneficial role of TNF-α in cardiac I/R injury. With this background, we analysed the expression of TNF-α in both cytosolic and particulate (membrane) fractions of LV collected from normoxic and CH rats. CNH increased the TNF-α level equally in both subcellular fractions, and infliximab treatment abolished these effects. Therefore, our results do not allow to suggest whether the membrane-bound TNF-α precursor or the secreted form of TNF-α primarily contributes to the cardioprotective phenotype of CNH rats. However, we cannot exclude their specific role in the progression of myocardial remodelling due to CH as was suggested earlier based on the responses of transgenic mice overexpressing a mutated non-cleavable transmembrane TNF-α or secreted form of TNF-α (Diwan et al., 2004).

CH induces expression of more than 20 transcription factors. NF-κB is one of the most important transcription factors that play the pivotal role in regulating both beneficial and detrimental processes (Cummins and Taylor, 2005). NF-κB signalling constitutes the complex of anti-inflammatory and proinflammatory signals, including cytokines (Diwan et al., 2004;

Taylor and Cummins, 2009). As was shown earlier in cell lines, unlike TNFR1, TNFR2 stimulation via transmembrane TNF-α can induce long-lasting activation of NF-κB and NF-κB-associated signalling (reviewed in Naude et al., 2011). The activation of NF-κB has been demonstrated in hearts subjected to a delayed preconditioning (Morgan et al., 1999; Xuan et al., 1999; Qiao et al., 2013). Similarly, in the present study, the expression of NF-κB was markedly elevated in ischemia-tolerant CNH hearts and infliximab treatment abolished both NF-κB overexpression and cardioprotection. These findings suggest a close relationship between TNF-α, TNRF2, NF-κB and the cardioprotective phenotype afforded by CNH.

In the present study, CNH had no effect on myocardial level of MCP-1 and the expression of ALDH-2 and HO-1. Although these molecules were earlier described as protective against myocardial I/R injury (Hangaishi et al., 2000; Martire et al., 2003, Chen et al., 2008), it seems unlikely that they play a major role in cardiac ischemic tolerance afforded by CNH. As compared with the above-mentioned molecules, CNH increased expression of iNOS and COX-2. However, chronic infliximab treatment had no effect on COX-2 level and only slightly reduced iNOS expression in CNH rat hearts. Similarly, the blockade of TNF-α signalling by other TNF-α inhibitor etanercept did not prevent the increased expression of iNOS in RV of chronically hypoxic juvenile rats (Dunlop et al., 2014). These findings suggest that CNH-induced cardiac overexpression of iNOS and COX-2, respectively, is related to TNF-α-mediated cell signalling. As shown previously, iNOS and COX-2 were revealed as important protective mediators/effectors of the late phase of preconditioning (Bolli, 2000). Therefore, we cannot exclude that increased LV expression of these molecules contributes to protective cardiac phenotype conferred by chronic hypoxia.

In the present study, CNH led to lipid peroxidation and protein nitrosylation as indicated by increased myocardial levels of MDA and nitrotyrosine respectively. This is in line with our previous observation of a homogeneously increased immunofluorescent staining of nitrosylated proteins in LV myocardium of chronically hypoxic rats (Hlavackova et al., 2010). The surge of both markers induced by CNH appears to be linked to TNF-α as it was completely abolished by infliximab.

It has been suggested that ROS play an important role in the cell survival and death triggered by TNF-α signalling. The main sources of TNF-α-induced ROS generation are mitochondria (Kim et al., 2010) where MnSOD is the dominant antioxidative enzyme. Previous reports showed that TNF-α increased MnSOD expression and activity in a delayed form of preconditioning and TNF-α antibodies blocked cardioprotection (Nelson et al., 1995;

Yamashita et al., 1999, 2000). Similarly, in the present study, chronic infliximab treatment abolished the CNH-induced increase of myocardial expression of MnSOD. As shown previously, the improved cardiac ischemic tolerance conferred by adaptation to CH was associated with the increased expression of MnSOD but not cytosolic CuZnSOD (Guo et al., 2009; Neckar et al., 2013). Furthermore, Balkova et al. (2011) demonstrated that MnSOD expression and activity in myocardial mitochondria negatively correlated with infarct size in rats adapted to a cardioprotective regimen of chronic intermittent hypoxia. Therefore, the increase of MnSOD expression likely represents the key cardioprotective action during adaptation to CNH that is dependent on TNF-α induced ROS generation.

The effect of regular exercise training under hypoxic conditions on myocardial ischemic tolerance (Publication B)

We have confirmed our second hypothesis that both cardioprotective phenomena-CNH and exercise increased cardiac ischemic tolerance, but their synergy didn’t have additional effect.

The present study was designed to determine whether a combination of two well-established forms of sustainable cardioprotection induced by CH and exercise training can result in the amplification of ischemia-resistant cardiac phenotype. Our data are in line with a number of earlier reports showing that these adaptive interventions acting separately to reduce myocardial infarct size induced by acute I/R insult. The novel finding is that rats subjected to regular exercise during continuous exposure to hypoxic atmosphere exhibited the same infarct-sparing effect as their sedentary counterparts. CNH led to proinflammatory response, increased myocardial expression of several related potentially protective mediators and antioxidant enzymes while none of these effects were observed in the rats exercising at room air. On the other hand, exercise in hypoxia abolished or significantly attenuated most of the CNH-induced responses related to inflammation, including the increased TNF-α and IL-6 levels and the overexpression of TNFR2, NF-κB, $cPLA_2\alpha$ and COX-2, without significantly affecting the upregulation of iNOS and antioxidant enzyme MnSOD.

We reported in our first publication (Chytilova et al., 2015) that the treatment of rats with antibodies against TNF-α during adaptation to CNH suppressed the infarct size-limiting effect and eliminated the CNH-induced increases in myocardial levels of TNF-α, its receptor TNFR2, NF-κB and MnSOD. These results led us to conclude that TNF-α is involved in the protective mechanism of CNH, its effect being possibly mediated by TNFR2 and the NF-κB-dependent activation of redox signalling with increased antioxidant defence (Chytilova et al., 2015). TNF-α is a key cytokine which plays an essential role in the initiation of inflammatory response. While excessive levels of TNF-α have detrimental actions on the heart mediated by TNFR1 (which was not affected in our study), the activation of TNFR2 by low levels of this cytokine is protective (Schulz et al., 1995). Several studies have demonstrated that TNF-α can also induce various forms of conditioning (Nelson et al., 1995; Yamashita et al., 2000; Lecour et al., 1995).

Regarding the involvement of cytokines in exercise-induced cardioprotection, the available data are scarce and conflicting. Serra et al. (2010) did not observed any effect of regular exercise training itself on the myocardial levels of TNF-α and IL-6 in rats. On the other hand, TNF-α neutralisation blunted the protection induced by a single exercise session, likely *via* the prevention of antioxidant response (Yamashita et al., 1999). Regarding IL-6, a recent report indicated that this myokine released from skeletal muscles mediated cardioprotective effects of exercise in mice. Exercise did not affect myocardial IL-6 level but it upregulated its receptor and activated IL-6 signalling pathways (McGinnis et al., 2015). Thus, the absence of any effect of exercise alone on myocardial cytokines in our study does not necessarily mean that they are not involved in the induction of protected cardiac phenotype.

Exercise has been shown to reduce sympathetic activation and stimulation of myocardial adrenoceptors associated with the adaptation to CH (Favret et al., 2001) which plays an important role in the cardioprotection conferred by hypoxic conditioning (Mallet et al., 2006). Interestingly, exercise training completely abolished the increase of myocardial TNF-α and IL-6 levels caused by the sustained pharmacological stimulation of β-adrenoceptors (Serra et al., 2010). Given our finding that TNF-α plays a role in the induction of the ischemia-resistant phenotype of CNH hearts (Chytilova et al., 1995), its blunted response to hypoxia in exercised rats may be expected to attenuate the protective effect. However, here we show that exercise training abolished only the CNH-induced suppression of early reperfusion arrhythmias, whereas the infarct-sparing effect remained unaffected. This can be possibly explained by another protective mechanism activated by exercise that just compensated for the blunted TNF-α signalling. The absence of any influence of exercise training alone on the potentially protective molecules detected in our study seems to support this view. Nevertheless, it should be noted that NF-κB and iNOS upregulated by CNH remained significantly higher in exercised hearts compared to their normoxic counterparts, and COX-2 also exhibited similar expression pattern. Both iNOS and COX-2 have been shown to play a role in delayed forms of cardioprotection (Becker, 2004). Thus, their levels might be still sufficient to maintain ischemia-resistant cardiac phenotype in the present combined CNH/exercise setting.

It can be assumed that TNF-α increase occurred already during the first week of CNH exposure when the animals did not exercise and this initial response was able to induce the persisting cardioprotected state. Indeed, it has been shown that TNF-α can result in the long-lasting activation of NF-κB and its downstream targets (Naude et al., 2011). However, our observation of unchanged levels of TNF-α and MnSOD after one week of hypoxia seems to rule out this possibility. Accordingly, previously we did not detect any reduction of infarct size during the first week of exposure to CNH (Suematsu et al., 2003).

Inflammation and oxidative stress are mutually related. Specifically, TNF-α stimulates ROS production while ROS can promote the TNF-α-induced inflammatory cascade (Kaur et al., 2009; Murphy et al., 1992; Roberge et al., 2014). It has been shown that mitochondria are the principal source of ROS formation in the TNF-α pathway (Suematsu et al., 2003). Signalling *via* ROS-dependent pathways appears to play a key role in cardioprotection against I/R insult conferred by various stimuli including CH (Kolar and Ostadal, 2007) and exercise training (Akita et al., 2007) as indicated by the elimination of their infarct-sparing effects by antioxidant treatments during hypoxic exposure and training sessions, respectively. Numerous but not all studies demonstrated the increased myocardial capacity of antioxidant defence systems induced by CH or exercise as a prerequisite for their salutary effects against I/R injury. The enhanced expression of MnSOD and CAT in hearts of CNH rats in the present study is in line with these results. Excess formation of ROS without adequate activation of cellular antioxidants caused by a brief periodic interruption of hypoxic exposure may result in a disturbed redox balance and a loss of protection (Neckar et al., 2013; Kasparova et al., 2015). Regarding exercise training, we did not detect any significant effect on MDA and antioxidant enzymes in ventricular homogenate in agreement with a number of reports summarized in a recent review (Powers et al., 2014). Nevertheless, it has been suggested that cardioprotection induced by a longer duration of exercise is mediated, at least in part, by MnSOD (Frasier et al., 2011; Powers et al., 2014), the primary mitochondrial antioxidant enzyme. Indeed, some studies detected the increased level and activity of this enzyme following exercise in myocardial mitochondrial fraction (Somani et al., 1995; Kavazis et al., 2008). Although we cannot exclude that exercise led to the upregulation of MnSOD in mitochondria also in our present study, the unchanged level of CS reflecting mitochondrial mass makes this an unlikely possibility.

10. CONCLUSIONS

1) TNF-α is involved in the cardioprotective mechanism afforded by CNH. TNF-α contributes to the improved cardiac ischemic tolerance of CNH rats possibly via its receptor TNFR2 and the NF-κB-dependent activation of protective redox signalling with increased antioxidant defence.

2) Regular exercise training of rats during their adaptation to CNH conferred the same infarct size-limiting effect as CNH alone, despite markedly attenuating the CNH-induced increase in myocardial inflammatory response and related cardioprotective signalling. The maintenance of ischemia-resistant cardiac phenotype in CNH combined with exercise can be possibly attributed to the persisting activity of NF-κB/iNOS pathway and increased myocardial antioxidant defense capacity.

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12. SUPPLEMENTS

Thesis is based on these publications with IF:

Publication A: A. Chytilova, G.H. Borchert, P. Mandikova-Alanova, A.H. Khan, J.D. Imig, F. Kolar, J. Neckar: TNF-α contributes to improved cardiac ischemic tolerance in rats adapted to chronic continuous hypoxia. Acta Physiol 214(1): 97 - 108, 2015. IF= 4.066

Publication B: P. Alanova, A. Chytilova, J. Neckar, J. Hrdlicka, P. Micova, K. Holzerova, M. Hlavackova, K. Machackova, F. Papousek, J. Vasinová, D. Benak, O. Novakova, F. Kolar: Myocardial ischemic tolerance in rats subjected to endurance exercise training during adaptation to chronic hypoxia. (under revision in Journal of Applied Physiology) IF= 3.004

Other publications with IF:

T. Ravingerova, S. Čarnicka, V. Ledvenyiova, E. Barlaka, E. Galatou, A. Chytilova, P. Mandíkova, M. Namcekova, A. Adameova, F. Kolar, A. Lazou: Upregulation of genes involved in cardiac metabolism enhances myocardial resistance to ischemia/reperfusion in the rat heart. Physiol Res 62 (Suppl. 1): S151 - S163, 2013. IF= 1.618

M. Milerova, Z. Drahota, A. Chytilova, K. Tauchmannova, J. Houstek, B. Ostadal: Sex difference in the sensitivity of cardiac mitochondrial permeability transition pore to calcium load. Mol Cell Biochem 412(1-2):147-54, 2016. IF= 2.613

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P. Micova, K. Hahnova, M. Hlavackova, B. Elsnicova, A. Chytilova, K. Holzerova, J. Zurmanova, J. Neckar, F. Kolar, O. Novakova, J. Novotny: Chronic intermittent hypoxia affects the cytosolic phospholipase $A_2\alpha$/cyclooxygenase 2 pathway via β2-adrenoceptor-mediated ERK/p38 stimulation. (under revision in Molecular and Cellular Biochemistry) IF= 2.613

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Tumour necrosis factor-α contributes to improved cardiac ischaemic tolerance in rats adapted to chronic continuous hypoxia

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Abstract

Aim: It has been demonstrated that tumour necrosis factor-alpha (TNF-α) *via* its receptor 2 (TNFR2) plays a role in the cardioprotective effects of preconditioning. It is also well known that chronic hypoxia is associated with activation of inflammatory response. With this background, we hypothesized that TNF-α signalling may contribute to the improved ischaemic tolerance of chronically hypoxic hearts.

Methods: Adult male Wistar rats were kept either at room air (normoxic controls) or at continuous normobaric hypoxia (CNH; inspired O_2 fraction 0.1) for 3 weeks; subgroups of animals were treated with infliximab (monoclonal antibody against TNF-α; 5 mg kg^{-1}, i.p., once a week). Myocardial levels of oxidative stress markers and the expression of selected signalling molecules were analysed. Infarct size (tetrazolium staining) was assessed in open-chest rats subjected to acute coronary artery occlusion/reperfusion.

Results: CNH increased myocardial TNF-α level and expression of TNFR2; this response was abolished by infliximab treatment. CNH reduced myocardial infarct size from $50.8 \pm 4.3\%$ of the area at risk in normoxic animals to $35.5 \pm 2.4\%$. Infliximab abolished the protective effect of CNH ($44.9 \pm 2.0\%$). CNH increased the levels of oxidative stress markers (3-nitrotyrosine and malondialdehyde), the expression of nuclear factor κB and manganese superoxide dismutase, while these effects were absent in infliximab-treated animals. CNH-elevated levels of inducible nitric oxide synthase and cyclooxygenase 2 were not affected by infliximab.

Conclusion: TNF-α plays a role in the induction of ischaemia-resistant cardiac phenotype of CNH rats, possibly *via* the activation of protective redox signalling.

Keywords chronic hypoxia, ischaemia/reperfusion injury, reactive oxygen species, tumour necrosis factor-α.

It is well known that long-term adaptation to chronic hypoxia protects mammals against lethal myocardial injury caused by acute ischaemia/reperfusion (I/R) insult. As compared to short-lived cardioprotective phenomena (preconditioning and post-conditioning), chronic hypoxia represents another form of protection that persists for weeks after the cessation of stimulus (Neckář *et al.* 2004, Fitzpatrick *et al.* 2005). A large

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body of research suggests that both short-lived preconditioning and long-lasting effects of chronic hypoxia utilize essentially similar endogenous pools of protective pathways. Nevertheless, chronic hypoxia not only activates these signalling pathways but also affects the expression of their components and other proteins associated with energy maintenance and oxygen homoeostasis (Oštádal & Kolář 2007).

It is generally accepted that reactive oxygen species (ROS) may exert both deleterious (cell death) and beneficial (activation of protective signalling) actions in ischaemic and reperfused myocardium (Penna *et al.* 2009). Adaptation to chronic hypoxia leads to increased ROS formation, which is important for the induction of a protective cardiac phenotype (Kolář *et al.* 2007, Hlaváčková *et al.* 2010). ROS-dependent signalling can increase myocardial capacity of antioxidant defence systems in chronically hypoxic hearts before I/R insult, thereby preventing excess oxidative stress and reducing tissue injury (Guo *et al.* 2009, Balková *et al.* 2011, Neckář *et al.* 2013). A growing body of evidence suggests the close relationship between ROS and inflammation that is mediated by a variety of signalling molecules including cytokines (Khaper *et al.* 2010). It has been shown that both ROS and pro-inflammatory cytokines contribute to physiological and pathophysiological cardiovascular events including adaptation to stress, myocardial remodelling and development of heart failure. It is suggested that the balance between pro-oxidant and pro-inflammatory mediators with antioxidant and anti-inflammatory mediators determine the overall response of either protection or damage (Khaper *et al.* 2010).

Tumour necrosis factor-α (TNF-α) represents a key pro-inflammatory cytokine playing a central role in initiating and sustaining inflammation. TNF-α is involved in pathophysiology of many cardiovascular diseases including myocardial infarction, heart failure, hypertension and atherosclerosis (Kleinbongard *et al.* 2011). In relation to acute I/R, it has been shown that TNF-α not only exerts detrimental actions on the heart, but also activates intracellular signalling pathways that improve cardiac ischaemic tolerance. Indeed, TNF-α activates acute and late preconditioning (Nelson *et al.* 1995, Li *et al.* 1999, Yamashita *et al.* 2000, Lecour *et al.* 2005, Skyschally *et al.* 2007) as well as post-conditioning (Lacerda *et al.* 2009, 2012). It has been proposed that a slight increase in TNF-α level provides protection as compared to detrimental effects at high levels (Sack 2002, Lecour & James 2011). This concept of dose-dependent action is in accord with earlier described cardioprotective roles for ROS (Penna *et al.* 2009). There is only limited knowledge about the role of TNF-α in myocardium adaptation to chronic hypoxia. It has been suggested that TNF-α could be involved in the adaptation of both left (LV) and right ventricles (RV) to hypoxia (Smith *et al.* 2001, Chen *et al.* 2007).

It is known that hypoxia as well as TNF-α or ROS activate the transcriptional factor nuclear factor-kappa B (NF-κB; Dhingra *et al.* 2009, Fitzpatrick *et al.* 2011). NF-κB affects cardiovascular functions by regulating expression of genes associated with oxidative and nitrosative stress, cell survival and inflammation, including cytokines and chemokines (Van der Heiden *et al.* 2010). For example, NF-κB can increase the expression of inducible nitric oxide synthase (iNOS), manganese superoxide dismutase (MnSOD) and activate chemokine monocyte chemoattractant protein-1 (MCP-1; Martin *et al.* 1997, Xuan *et al.* 1999, Thapa *et al.* 2011). Besides other actions, these molecules can also be involved in the protective signalling that limits the deleterious effects of acute myocardial I/R (Morgan *et al.* 1999, Xuan *et al.* 1999, 2000, Martire *et al.* 2003, Morimoto *et al.* 2008, Yamashita *et al.* 1999).

The detail analysis of TNF-α expression in chronically hypoxic rat hearts and its role in cardiac ischaemic tolerance has not been examined yet. Therefore, the aim of this study was to characterize the expression of the main pro-inflammatory cytokine, TNF-α, in LV and RV of rats adapted to chronic hypoxia. The effect of chronic TNF-α inhibition by infliximab on myocardial infarction, the expression of TNF-α receptor R1 (TNFR1) and R2 (TNFR2), the level of oxidative stress markers, and the expression of NF-κB and its related signalling molecules were investigated.

Material and methods

Animals

Adult male Wistar rats (250–280 g, Charles River, Germany) were exposed to moderate continuous normobaric hypoxia (CNH; inspired O_2 fraction 0.1) in a normobaric chamber equipped with hypoxic generators (Everest Summit, Hypoxico, NY, USA) for 3 weeks. No reoxygenation occurred during this period. The control rats were kept for the same period of time at room air. All animals were housed in a controlled environment (23 °C; 12 : 12-h light–dark cycle; light from 5:00 AM) with free access to water and standard chow diet. Separate groups of CNH and normoxic rats were treated weekly with a monoclonal antibody against TNF-α, infliximab (5 mg kg^{-1}, i.p., Remicade; Janssen Biotech, Horsham, PA, USA). The dose was selected from previously published pharmacokinetic study (Yang *et al.* 2003), and the first injection of infliximab was given one day before the start of hypoxic adaptation. At the end of 3-week period, haematocrit was measured in the tail blood.

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The animals assigned to biochemical analyses were killed by cervical dislocation immediately after the cessation of hypoxic exposure. Intact hypoxic and normoxic hearts (without I/R) were rapidly excised, washed in cold (0 °C) saline and dissected into RV, free wall of LV and the septum. All heart tissue segments were weighed; LV and RV were frozen in liquid nitrogen and stored at −80 °C until use. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (published by the National Academy of Science, National Academy Press, Washington, DC, USA). Experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology, The Czech Academy of Sciences.

Myocardial ischaemia/reperfusion

Animals were subjected to acute I/R as described previously (Neckář *et al.* 2002). Anesthetized (sodium pentobarbital, 60 mg kg^{-1} i.p.) rats were ventilated (Ugo Basile, Varese, Italy) with room air at 68–70 strokes min^{-1} (tidal volume of 1.2 mL 100 g^{-1} body wt). A single-lead electrocardiogram (ECG) and blood pressure in the carotid artery were continuously recorded (Gould P23Gb; Gould, Cleveland, OH, USA) and subsequently analysed by a custom-designed software. The rectal temperature was maintained between 36.5 and 37.5 °C by a heated table throughout the experiment. Hypoxic rats were anesthetized in the hypoxic chamber, and their exposure to normoxic air before the coronary artery occlusion was shorter than 40 min. This short reoxygenation has no effect on cardiac ischaemic tolerance as we showed earlier (Neckář *et al.* 2013).

Left thoracotomy was performed, and a silk-braided suture 5/0 (Chirmax, Prague, Czech Republic) was placed around the left anterior descending coronary artery about 1–2 mm distal to its origin. After 10-min stabilization, regional myocardial ischaemia was induced by the tightening of the suture threaded through a polyethylene tube. After a 20-min occlusion period, the ligature was released and reperfusion of previously ischaemic tissue continued. After 3 min of reperfusion, chest was closed, air was exhausted from thorax, and spontaneously breathing animals were maintained in deep anaesthesia following 3 h.

Infarct size determination

Hearts were excised and washed with saline *via* aorta. The area at risk was delineated by perfusion with 5% potassium permanganate as described earlier (Neckář *et al.* 2002). Frozen hearts were cut into slices 1 mm thick, stained with 1% 2,3,5-triphenyltetrazolium chloride (pH 7.4; 37 °C) for 30 min and fixed in formaldehyde solution. Four days later, both sides of the slices were photographed. The infarct size (IS), the size of the area at risk (AR) and the size of the LV were determined by computerized planimetric method using the software Ellipse (ViDiTo, Košice, Slovakia). The size of AR was normalized to LV (AR/LV), and the IS was normalized to the LV (IS/LV) and to the AR (IS/AR). The incidence and severity of ischaemic arrhythmias during the 20-min ischaemic insult and during the first 3 min of reperfusion were assessed according to the Lambeth Conventions as previously described (Asemu *et al.* 2000).

Fractionation of tissues

The samples of myocardium were crushed by pestle into small pieces with liquid nitrogen in a ceramic bowl. The samples were homogenized by Potter homogenizer in the homogenization buffer (mmol L^{-1}: 2.5 Tris, 2.5 EGTA, 100 NaF, 250 saccharose, 0.1 activated orthovanadate, 6 mercaptoethanol, complete protease inhibitor cocktail tablet) and were centrifuged at 1000 ***g*** for 10 min in 4 °C. The supernatants were transferred into new tubes and were centrifuged at 100 000 ***g*** for 1 h in 4 °C. Thereafter, we obtained protein from supernatant, representing the cytosolic fraction. The pellets were re-homogenized in homogenization buffer containing 1% Triton X-100 and incubated for 30 min on ice. The solubilized pellets were centrifuged at 100 000 ***g*** for 1 h in 4 °C. The supernatant of this centrifugation represents the membrane fraction. Both fractions were stored at −80 °C until use.

Cytokines, MCP-1 and 3-nitrotyrosine assays

For measurement of TNF-α and IL-10, we used the DuoSet ELISA capture method (eBioscience, Vienna, Austria). Protein levels of MCP-1 were determined using rat MCP-1 ELISA kit (BD Biosciences, San Jose, CA, USA). Non-competitive ELISA kit (Cayman, Neratovice, Czech Republic) was used to detect oxidative stress marker, 3-nitrotyrosine (3-NT). These assays were performed on homogenized samples from different experimental groups according to the standards described by the manufacturers. The results are expressed *per* mg of total protein.

Malondialdehyde (MDA) assay

The samples of LV for determination of lipid peroxidation marker MDA were pulverized into a powder under liquid nitrogen. After adding 500 μL of the homogenization buffer (25 mmol L^{-1} Tris–HCl and 0.1% Triton X-100), samples were homogenized and centrifuged (1000 ***g***, 10 min, 4 °C). Supernatant (100 μL) was

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taken for the determination of MDA concentration. After adding 20 μL of NaOH (6 mol L^{-1}) and vortexing, the samples were kept at 60 °C for 30 min followed by 5-min cooling at −20 °C, deproteinized by 50 μL of HClO4 (35% v/v) and centrifuged (10 000 *g*, 5 min, 4 °C). Supernatant (100 μL) was mixed with 10 μL of 2,4-dinitrophenylhydrazine (5 mmol L^{-1}), kept in the dark for 10 min, and analysed by an HPLC system (Shimadzu, Japan; column EC Nucleosil 100–5 C18; 4.6 mm × 125 mm; flow 1.0 mL min^{-1}; sampling volume 30–100 μL) with the UV detection set on 310 nm. Concentration of MDA was normalized to total protein contents.

Western Blot analysis

Detergent-treated extracts of LV homogenate were prepared as described earlier (Borchert *et al.* 2011). Proteins were separated by SDS-PAGE electrophoresis (10 or 15% gels) and transferred to nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany). After blocking with 5% dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 min at room temperature, membranes were washed and probed with rabbit anti-TNFR2 (Santa Cruz Biotechnology, Dallas, TX, USA; 1 : 1000), anti-MnSOD (Sigma-Aldrich, Prague, Czech Republic; 1 : 250), anti-NF-κB p65 (Santa Cruz Biotechnology; 1 : 500), anti-haeme oxygenase 1 (HO-1; Santa Cruz Biotechnology; 1 : 1000), anti-GAPDH (Santa Cruz Biotechnology; 1 : 500), goat anti-TNFR1 (Santa Cruz Biotechnology; 1 : 1000), anti-aldehyde dehydrogenase 2 (ALDH-2; Santa Cruz Biotechnology; 1 : 1000), anti-cyclooxygenase 2 (COX-2; Abcam, Cambridge, MA, USA; 1 : 1000) and mouse anti-iNOS (BD Biosciences; 1 : 500) antibodies at 4 °C over night. The membranes were washed and incubated with anti-rabbit (Bio-Rad, Prague, Czech Republic; 1 : 5000), anti-mouse and anti-goat (Sigma-Aldrich; 1 : 5000 in TTBS), respectively, HRP-labelled secondary antibodies for 60 min at room temperature. Bands were visualized by enhanced chemiluminescence on the LAS system or on the medical X-ray films (Agfa, Berlin, Germany). ImageJ software (Java Technology, Cupertino, CA, USA) was used for quantification of the relative abundance of proteins. To ensure the specificity of immunoreactive proteins, pre-stained molecular weight protein standards (Bio-Rad) were used. The samples from each experimental group were run on the same gel and quantified on the same membrane. Hypoxia did not affect the expression of GAPDH, which was used as a loading control.

Statistical analysis

The results are expressed as means ± SEM. One-way anova or anova for repeated measurements and subsequent Student–Newman–Keuls test were used for comparison of differences in normally distributed variables between groups. Differences in the number of PVCs between the groups were compared by the Kruskal–Wallis nonparametric test. Differences were assumed statistically significant when $P < 0.05$.

Results

Body weight and haematocrit

Adaptation of rats to CNH caused retardation of body growth, pronounced hypertrophy of the RV and mild hypertrophy of the LV as compared to age-matched normoxic controls. The haematocrit increased to 66.0 ± 1.8% ($P < 0.05$) in CNH rats as compared to 44.6 ± 0.4% in normoxic animals. Treatment with infliximab had no effect on heart weight parameters but reduced haematocrit level to 62.0 ± 1.4% in CNH rats ($P < 0.05$; Table 1).

TNF-α and IL-10 levels in cardiac tissue

In normoxic animals, the total concentration of TNF-α and IL-10 in myocardial homogenates were lower in

Table 1 Body and heart weight parameters and haematocrit in untreated and infliximab-treated rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls

Group	*n*	BW (g)	RV/BW (mg g^{-1})	LV/BW (mg g^{-1})	HW/BW (mg g^{-1})	RV/(LV+S)	Haematocrit (%)
Normoxia	6	443 ± 9	0.54 ± 0.03	1.53 ± 0.01	2.51 ± 0.03	0.27 ± 0.01	44.6 ± 0.4
Normoxia + infliximab	8	440 ± 11	0.49 ± 0.01	1.46 ± 0.04	2.36 ± 0.07	0.26 ± 0.01	45.6 ± 0.5
CNH	6	342 ± 12*	1.42 ± 0.12*	1.78 ± 0.04*	3.67 ± 0.11*	0.63 ± 0.06*	66.0 ± 1.8*
CNH + infliximab	8	349 ± 8*	1.28 ± 0.03*	1.74 ± 0.03*	3.48 ± 0.05*	0.58 ± 0.02*	62.0 ± 1.4*,#

BW, Body weight; RV/BW, relative weight of right ventricle; LV/BW, relative weight of left ventricle; HW/BW, relative heart weight; RV/(LV+S), right-to-left ventricular weight ratio. Values are means ± SEM; *$P < 0.05$ vs. corresponding normoxic group; #$P < 0.05$ vs. corresponding untreated group.

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RV as compared to LV (by 41 and 27%, respectively; $P < 0.05$; Fig. 1a,b). CNH increased TNF-α in LV (by about 40%) and RV (by about 80%); however, this level remained lower in RV compared to LV of hypoxic rats (Fig. 1a). CNH reduced IL-10 levels in LV by 24% ($P < 0.05$) but had no effect on the IL-10 levels in RV (Fig. 1b).

Previously published data have indicated that the IL-10/TNF-α ratio is an important determinant of myocardial inflammation (Khaper *et al.* 2010). In both normoxic and hypoxic hearts, the ratio was significantly higher in RV (Fig. 1c). CNH markedly reduced the IL-10/TNF-α ratio in both LV and RV from 1.37 ± 0.06 and 1.71 ± 0.17, respectively, in normoxic animals to 0.76 ± 0.03 and 1.06 ± 0.05, respectively, in hypoxic animals ($P < 0.05$). These results indicate a pro-inflammatory response caused by CNH.

Accumulating evidence suggests that not only secreted (cytosolic) but also transmembrane TNF-α precursor could be involved in the pro-inflammatory response (Horiuchi *et al.* 2010). Therefore, in a separate set of experiments, TNF-α concentration was analysed in the cytosolic and membrane fractions of LV collected from infliximab-treated and untreated normoxic and hypoxic rats. Particulate fractions of all experimental groups contained approximately three times more TNF-α as compared to the corresponding cytosolic fractions. CHN equally increased TNF-α level in both fractions which was completely inhibited by chronic infliximab treatment (Fig. 2a,b).

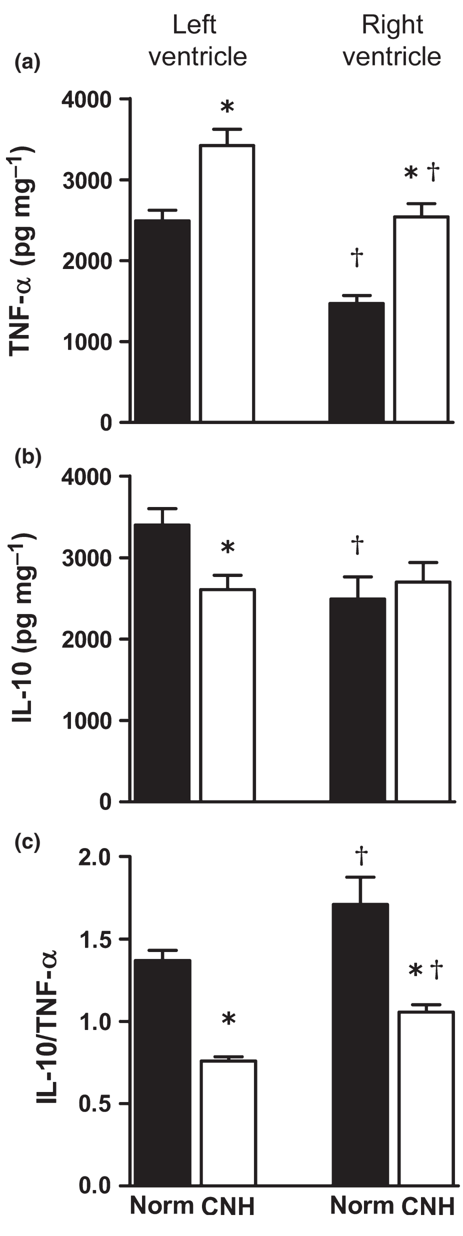

Figure 1 Myocardial levels of (a) TNF-α, (b) IL-10 and (c) IL-10/TNF-α ratio from homogenates of left and right ventricles of rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (Norm). Values are means ± SEM from 6 to 8 hearts in each group; $^*P < 0.05$ vs. corresponding normoxic group; $^\dagger P < 0.05$ vs. left ventricle.

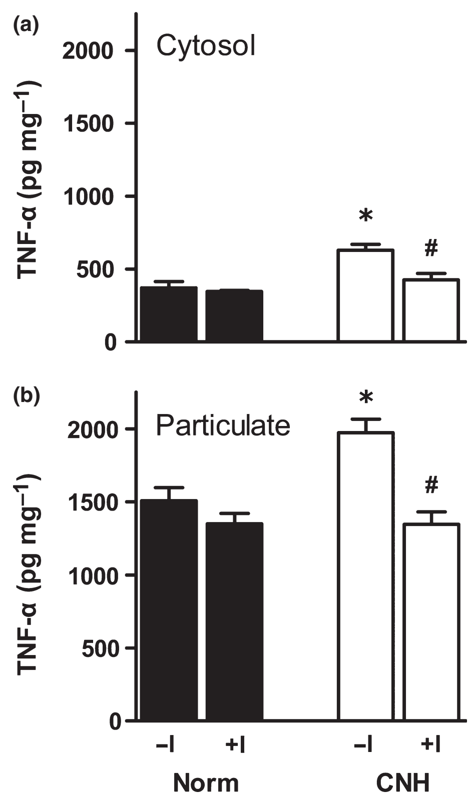

Figure 2 The effect of infliximab (I) on myocardial levels of TNF-α in (a) cytosolic and (b) particulate fractions of left ventricle from rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (Norm). Values are means ± SEM from 6 to 8 hearts in each group; $^*P < 0.05$ vs. corresponding normoxic group; $^\#P < 0.05$ vs. corresponding untreated group.

Table 2 Heart rate and mean arterial blood pressure after stabilization (Baseline), at the end of 20-min coronary artery occlusion (Ischaemia) and at the end of 3-h reperfusion in untreated and infliximab-treated rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls

	Baseline	Ischaemia 20 min	Reperfusion 3 h
Heart rate (beats min^{-1})			
Normoxia	396 ± 12	402 ± 15	350 ± 6§,¶
Normoxia + infliximab	398 ± 13	407 ± 11	388 ± 10$^{\#}$
CNH	390 ± 7	386 ± 6	363 ± 8
CNH + infliximab	400 ± 6	402 ± 6	391 ± 8
Blood pressure (mmHg)			
Normoxia	101 ± 6	105 ± 7	88 ± 6
Normoxia + infliximab	108 ± 7	114 ± 7	105 ± 4$^{\#}$
CNH	126 ± 2*	127 ± 6	128 ± 2*
CNH + infliximab	129 ± 4*	123 ± 9	115 ± 5

Values are means ± SEM; *$P < 0.05$ vs. normoxia; $^{§}P < 0.05$ vs. baseline; $^{¶}P < 0.05$ vs. ischaemia; $^{\#}P < 0.05$ vs. corresponding untreated group.

Cardiac ischaemic tolerance

CNH slightly but significantly increased the baseline values of mean arterial pressure (MAP; Table 2). Neither CNH nor infliximab affected heart rate (HR) before ischaemia and at the end of ischaemia. At the end of reperfusion, HR was decreased in normoxic rats as compared to baseline and ischaemic values ($P < 0.05$). Both MAP and HR were significantly decreased in normoxic controls as compared to corresponding infliximab-treated group, and MAP was also lower compared to untreated CNH rats ($P < 0.05$; Table 2).

The mean normalized AR (AR/LV) was 35–41% and did not differ among groups. The IS reached 50.8 ± 4.3% of the AR in the normoxic group. CNH reduced myocardial infarction to 35.5 ± 2.4% ($P < 0.05$). Chronic administration of infliximab had no effect on infarct size in normoxic rats (53.0 ± 3.9%), but blunted the infarct size-limiting effect of CNH (44.9 ± 2.0%, $P < 0.05$; Fig. 3a).

Neither CNH nor infliximab significantly affected the total number of ischaemic arrhythmias (Fig. 3b). At the start of reperfusion, infliximab almost doubled the number of arrhythmias in normoxic rats from 72 ± 22 in untreated group to 134 ± 24, but this effect was not statistically significant due to high variability within the groups. CNH markedly reduced the total number of reperfusion arrhythmias in both untreated (23 ± 6; $P = 0.083$) and infliximab-treated (36 ± 7; $P < 0.05$) groups when compared to the corresponding normoxic animals (Fig. 3c).

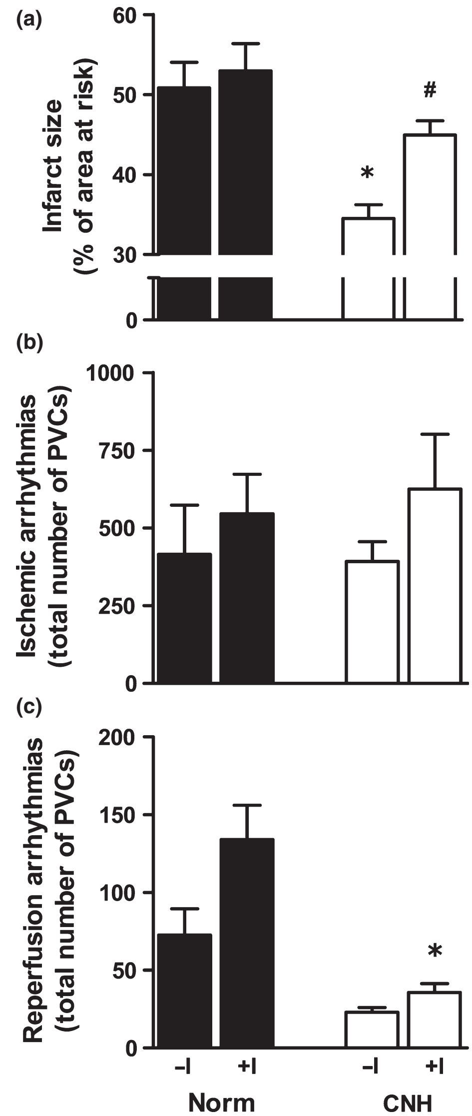

Figure 3 The effect of infliximab (I) on (a) myocardial infarct size, (b) the total number of premature ventricular complexes (PVCs) during 20 min of ischaemia and (c) the number of PVCs during the first 3 min of reperfusion in rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (Norm). Values are means ± SEM from 8 to 11 hearts in each group; *$P < 0.05$ vs. corresponding normoxic group; $^{\#}P < 0.05$ vs. corresponding untreated group.

Effect of infliximab on the expression of TNF-α receptors

Adaptation to CNH did not change the expression of TNFR1 in LV myocardium but increased the protein level of TNFR2 by 135% ($P < 0.05$) that was completely inhibited by infliximab treatment (Fig. 4a,b). Infliximab had no effect on protein expression of TNF-α receptors in LV of normoxic rats.

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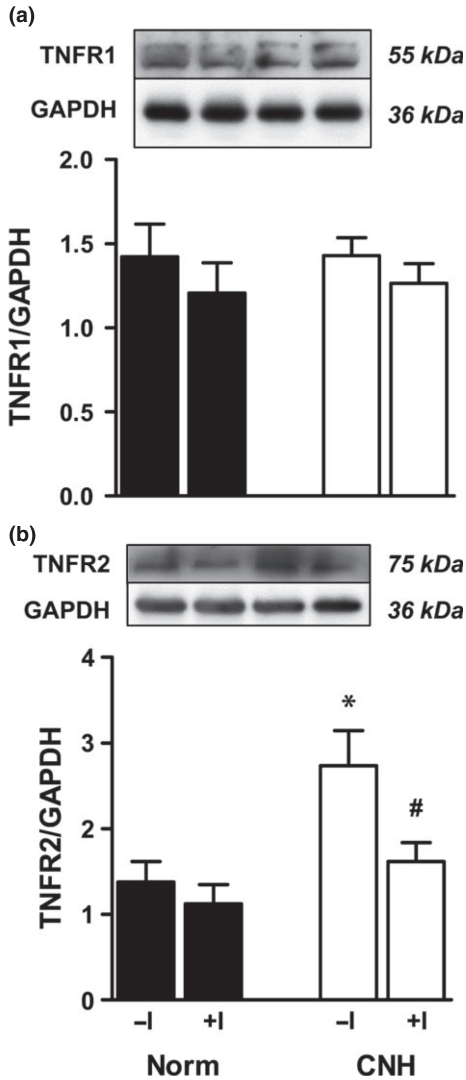

Figure 4 The effect of infliximab (I) on myocardial levels of TNF-α (a) receptor 1 (TNFR1) and (b) receptor 2 (TNFR2) in left ventricle of rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (Norm). Representative Western blots of TNFR1 and TNFR2 are shown; GAPDH was used as a loading control. Values are means ± SEM from 6 to 8 hearts in each group; *$P < 0.05$ vs. corresponding normoxic group; $^{\#}P < 0.05$ vs. corresponding untreated group.

Expression of NF-κB and cardioprotective signalling molecules

In LV of rat hearts adapted to CNH, increased TNF-α level and the expression of TNFR2 was accompanied by elevated expression of NF-κB (by 53%; $P < 0.05$), which was also abolished by infliximab; no effect of treatment was observed in normoxic hearts (Fig. 5a). CNH increased expression of iNOS and COX-2 (by 162 and 46%, respectively; $P < 0.05$; Fig. 5c,d). Chronic infliximab treatment had no significant effect on iNOS and COX-2 in both normoxic and CNH hearts. Nevertheless, the trend of decreasing iNOS expression in CNH hearts treated by infliximab was apparent ($P = 0.097$; Fig. 5c). Neither CNH nor infliximab significantly affected myocardial concentration of MCP-1 and expression of ALDH-2 and HO-1 (Fig. 5b,e,f).

Expression of MnSOD and oxidative stress markers

CNH increased the myocardial expression of mitochondrial MnSOD and the concentrations of oxidative stress markers, MDA and 3-nitrotyrosine by 64–72% compared to the normoxic values ($P < 0.05$). Chronic infliximab treatment completely eliminated these effects of CNH without affecting MnSOD and oxidative stress markers in normoxic controls (Fig. 6a–c).

Discussion

The main finding of the present study is that adaptation to CNH improved cardiac ischaemic tolerance in rats that was accompanied by increased myocardial concentration of proinflammatory cytokine TNF-α and its receptor TNFR2. Chronic treatment with TNF-α inhibitor infliximab during adaptation attenuated the infarct size-limiting effect of CNH. CNH increased myocardial oxidative stress and induced overexpression of transcription factor NF-κB and MnSOD that were abolished by infliximab treatment.

Adaptation to chronic hypoxia represents the protective phenomenon that improves cardiac ischaemic tolerance with a similar efficiency as different forms of acute conditioning (pre-, per- and post-conditioning). However, as compared to the fast activation of protection by conditioning, the development of ischaemia-tolerant phenotype of chronically hypoxic hearts needs more time – from several days to weeks (Asemu *et al.* 2000, Zhang *et al.* 2000, Neckář *et al.* 2013). Moreover, the cardioprotection afforded by chronic hypoxia persists for weeks (Neckář *et al.* 2004, Fitzpatrick *et al.* 2005), that is much longer than short-lived effects of conditioning. Therefore, the improved ischaemic tolerance of chronically hypoxic hearts can be considered as a form of sustained cardioprotection (Peart & Headrick 2008). However, its underlying mechanism has not been fully elucidated (Ošťádal & Kolář 2007). In the present study, we indicate for the first time a role of TNF-α signalling in the cardioprotective mechanism of chronic hypoxia.

We demonstrated that CNH markedly increased TNF-α level in both RV and LV. This finding is in agreement with the increased expression of TNF-α and pro-inflammatory genes in hearts of chronically hypoxic adult rats or foetal guinea-pigs (Chen *et al.*

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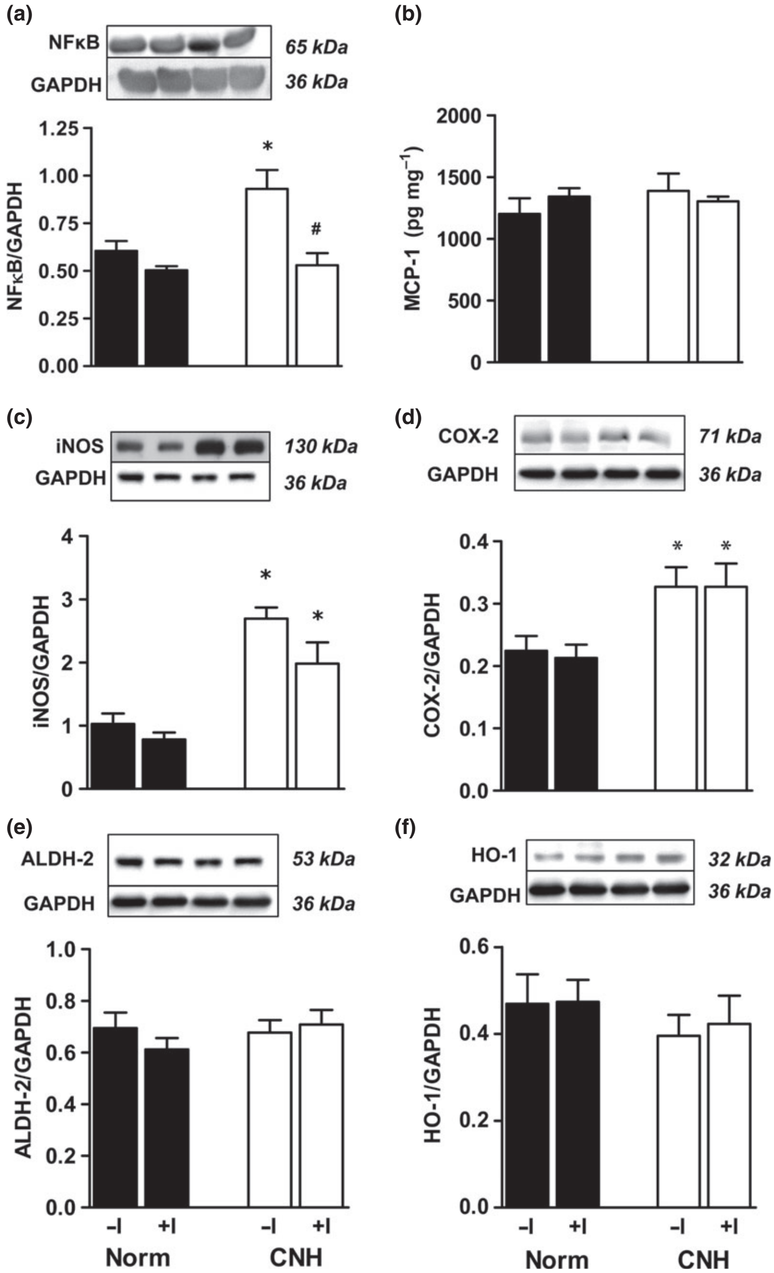

Figure 5 The effect of infliximab (I) on myocardial level of (a) nuclear factor κB (NF-κB), concentration of (b) monocyte chemoattractant protein-1 (MCP-1), and levels of (c) inducible nitric oxide synthase (iNOS), (d) cyclooxygenase 2 (COX-2), (e) aldehyde dehydrogenase 2 (ALDH-2) and (f) haeme oxygenase 1 (HO-1) in left ventricle of rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (Norm). Representative Western blots of the analysed proteins are shown; GAPDH was used as a loading control. Values are means ± SEM from 6 to 8 hearts in each group; $*P < 0.05$ vs. corresponding normoxic group; $^{\#}P < 0.05$ vs. corresponding untreated group.

2007, Oh *et al.* 2008, Klusoňová *et al.* 2009). Interestingly, Smith *et al.* (2001) showed that knockout TNF-$\alpha^{-/-}$ mice exhibited lower pulmonary hypertension and RV hypertrophy upon adaptation to chronic hypoxia than wild-type animals. Although we did not detect a significant effect of TNF-α inhibition on RV hypertrophy, the erythropoietic response to CNH was attenuated in infliximab-treated rats, as indicated by a smaller increase in haematocrit. Altogether, these results suggest that TNF-α plays a role not only in the induction of the improved cardiac ischaemic tolerance but also in other adaptive responses of the organism to chronic hypoxia.

TNF-α is generated as a precursor called transmembrane TNF-α, a 26-kDa protein. This form is subsequently cleaved by TNF-α-converting enzyme to the secreted (soluble) and active form of TNF-α (17 kDa) that mediates its biological action through types 1 and 2 TNF-α receptor (TNFR1 and TNFR2). The activation of TNF-α receptor-specific response was shown as an important event in cardiac ischaemic tolerance. While an excessive TNF-α expression and subsequent TNFR1 activation are deleterious, a lower TNF-α concentration and TNFR2 activation are protective (Flaherty *et al.* 2008, Lacerda *et al.* 2009, Schulz & Heusch 2009, Katare *et al.* 2010). Previously, Ramirez

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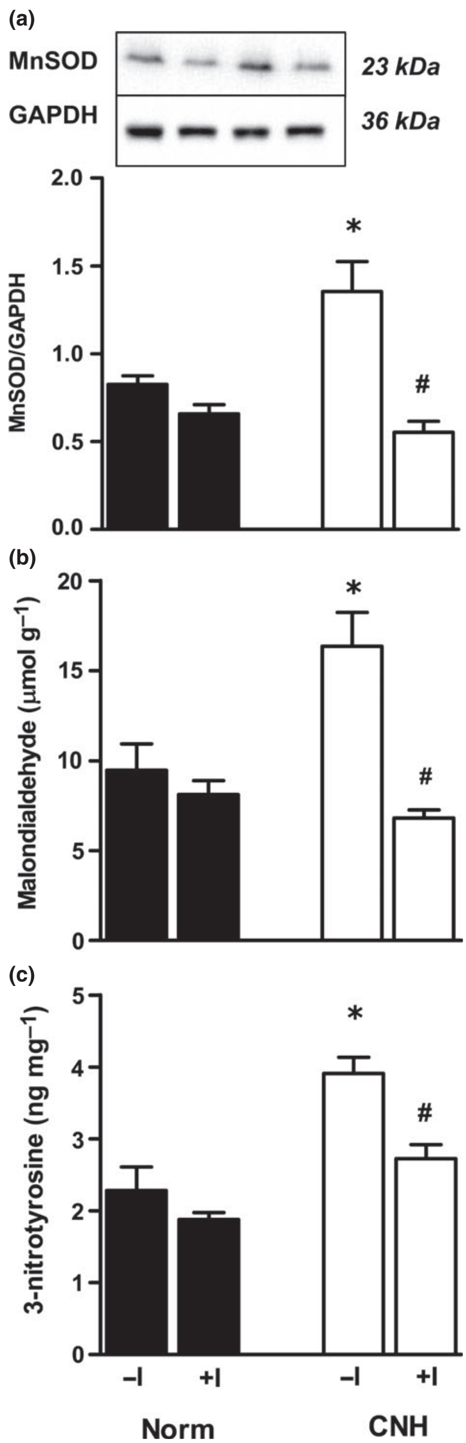

Figure 6 The effect of infliximab (I) on myocardial level of (a) mitochondrial manganese superoxide dismutase (MnSOD) and concentrations of (b) malondialdehyde and (c) 3-nitrotyrosine in left ventricle of rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (Norm). Representative Western blot of MnSOD is shown; GAPDH was used as a loading control. Values are means ± SEM from 6 to 8 hearts in each group; $^*P < 0.05$ vs. corresponding normoxic group; $^\#P < 0.05$ vs. corresponding untreated group.

et al. (2012) observed decreased gene expression of TNFR1 in chronically hypoxic rat hearts. Our results showed increased expression of TNFR2 but not TNFR1 in LV of rats adapted to CNH. Moreover, chronic treatment by TNF-α inhibitor infliximab abolished the increased TNFR2 level and blunted infarct size-limiting effect of CNH. These data suggest that adaptation to CNH improved cardiac ischaemic tolerance in rat hearts by activation of protective TNFR2 signalling but had no effect on detrimental signalling mediated by TNFR1.

Not only secreted TNF-α but also its transmembrane form exerts various biological actions that modulate the local inflammation and contribute to physiological as well as pathophysiological responses (Horiuchi *et al.* 2010). Transmembrane TNF-α mediates its biological activities mainly through TNFR2 (Grell *et al.* 1995) which is the key receptor for the beneficial role of TNF-α in cardiac I/R injury. With this background, we analysed the expression of TNF-α in both cytosolic and particulate (membrane) fractions of LV collected from normoxic and chronically hypoxic rats. CNH increased the TNF-α level equally in both subcellular fractions, and infliximab treatment abolished these effects. Therefore, our results do not allow to suggest whether the membrane-bound TNF-α precursor or the secreted form of TNF-α primarily contributes to the cardioprotective phenotype of CNH rats. However, we cannot exclude their specific role in the progression of myocardial remodelling due to chronic hypoxia as was suggested earlier based on the responses of transgenic mice overexpressing a mutated non-cleavable transmembrane TNF-α or secreted form of TNF-α (Diwan *et al.* 2004).

Chronic hypoxia induces expression of more than 20 transcription factors. NF-κB is one of the most important transcription factors that play the pivotal role in regulating both beneficial and detrimental processes (Cummins & Taylor 2005). NF-κB signalling constitutes the complex of anti-inflammatory and pro-inflammatory signals, including cytokines (Diwan *et al.* 2004, Taylor & Cummins 2009). As was shown earlier in cell lines, unlike TNFR1, TNFR2 stimulation *via* transmembrane TNF-α can induce long-lasting activation of NF-κB and NF-κB-associated signalling (reviewed in Naudé *et al.* 2011). The activation of NF-κB has been demonstrated in hearts subjected to a delayed preconditioning (Morgan *et al.* 1999, Xuan *et al.* 1999, Qiao *et al.* 2013). Similarly, in the present study, the expression of NF-κB was markedly elevated in ischaemia-tolerant CNH hearts and infliximab treatment abolished both NF-κB overexpression and cardioprotection. These findings suggest a close relationship between TNF-α, TNRF2, NF-κB and the cardioprotective phenotype afforded by CNH.

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In the present study, CNH had no effect on myocardial level of MCP-1 and the expression of ALDH-2 and HO-1. Although these molecules were earlier described as protective against myocardial I/R injury (Hangaishi *et al.* 2000, Martire *et al.* 2003, Chen *et al.* 2008), it seems unlikely that they play a major role in cardiac ischaemic tolerance afforded by CNH. As compared with the above-mentioned molecules, CNH increased expression of iNOS and COX-2. However, chronic infliximab treatment had no effect on COX-2 level and only slightly reduced iNOS expression in CNH rat hearts. Similarly, the blockade of TNF-α signalling by other TNF-α inhibitor etanercept did not prevent the increased expression of iNOS in RV of chronically hypoxic juvenile rats (Dunlop *et al.* 2014). These findings suggest that CNH-induced cardiac overexpression of iNOS and COX-2, respectively, is related to TNF-α-mediated cell signalling. As shown previously, iNOS and COX-2 were revealed as important protective mediators/effectors of the late phase of preconditioning (Bolli 2000). Therefore, we cannot exclude that increased LV expression of these molecules contributes to protective cardiac phenotype conferred by chronic hypoxia.

In the present study, CNH led to lipid peroxidation and protein nitrosylation as indicated by increased myocardial levels of MDA and nitrotyrosine respectively. This is in line with our previous observation of a homogeneously increased immunofluorescent staining of nitrosylated proteins in LV myocardium of chronically hypoxic rats (Hlaváčková *et al.* 2010). The surge of both markers induced by CNH appears to be linked to TNF-α as it was completely abolished by infliximab.

It has been suggested that ROS play an important role in the cell survival and death triggered by TNF-α signalling. The main sources of TNF-α-induced ROS generation are mitochondria (Kim *et al.* 2010) where MnSOD is the dominant antioxidative enzyme. Previous reports showed that TNF-α increased MnSOD expression and activity in a delayed form of preconditioning and TNF-α antibodies blocked cardioprotection (Nelson *et al.* 1995, Yamashita *et al.* 1999, 2000). Similarly, in the present study, chronic infliximab treatment abolished the CNH-induced increase of myocardial expression of MnSOD. As shown previously, the improved cardiac ischaemic tolerance conferred by adaptation to chronic hypoxia was associated with the increased expression of MnSOD but not cytosolic Cu,ZnSOD (Guo *et al.* 2009, Neckář *et al.* 2013). Furthermore, Balková *et al.* (2011) demonstrated that MnSOD expression and activity in myocardial mitochondria negatively correlated with infarct size in rats adapted to a cardioprotective regimen of chronic intermittent hypoxia. Therefore, the increase of MnSOD expression likely represents the key cardioprotective action during adaptation to CNH that is dependent on TNF-α-induced ROS generation.

In conclusion, the present study demonstrates that TNF-α is involved in the cardioprotective mechanism afforded by CNH. TNF-α contributes to the improved cardiac ischaemic tolerance of CNH rats possibly *via* its receptor TNFR2 and the NF-κB-dependent activation of protective redox signalling with increased antioxidant defence.

Conflict of interest

The authors declare no conflict of interest.

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Myocardial ischemic tolerance in rats subjected to endurance exercise training during adaptation to chronic hypoxia

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Running head: Cardioprotection by chronic hypoxia and exercise

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ABSTRACT

Chronic hypoxia and exercise are natural stimuli that confer sustainable cardioprotection against ischemia/reperfusion (I/R) injury but it is unknown whether they can act in synergy to enhance ischemic resistance. Inflammatory response mediated by tumor necrosis factor-α (TNF-α) plays a role in the infarct size-limitation by continuous normobaric hypoxia (CNH) whereas exercise is associated with anti-inflammatory effects. This study was conducted to determine whether exercise training performed under conditions of CNH (12% O_2) affects myocardial ischemic resistance with respect to inflammatory and redox status. Adult male Wistar rats were assigned to one of following groups: normoxic sedentary, normoxic trained, hypoxic sedentary, and hypoxic trained. ELISA and Western blot, respectively, were used to quantify myocardial cytokines and the expression of TNF-α receptors, nuclear factor-κB (NF-κB) and selected components of related signaling pathways. Infarct size and arrhythmias were assessed in open-chest rats subjected to I/R. CNH increased TNF-α and interleukin-6 levels and the expression of TNF-α type 2 receptor, NF-κB, inducible nitric oxide synthase (iNOS), cytosolic phospholipase $A_2\alpha$, cyclooxygenase-2, manganese superoxide dismutase (MnSOD) and catalase. None of these effects occurred in normoxic trained group, whereas exercise in hypoxia abolished or significantly attenuated CNH-induced responses, except for iNOS and MnSOD. Both CNH and exercise reduced infarct size but their combination provided the same degree of protection as CNH alone. In conclusion, exercise training does not amplify the cardioprotection conferred by CNH. High ischemic tolerance of the CNH hearts persists after exercise, possibly by maintaining the increased antioxidant capacity despite attenuating TNF-α-dependent protective signaling.

Key words: chronic hypoxia, exercise training, cardioprotection, cytokines, antioxidants

NEW & NOTEWORHY

Chronic hypoxia and regular exercise are natural stimuli that confer sustainable myocardial protection against acute ischemia/reperfusion injury. Signaling mediated by TNF-α *via* its type 2 receptor plays a role in the cardioprotective mechanism of chronic hypoxia. In the present study, we found that exercise training of rats during adaptation to hypoxia does not amplify the infarct size-limiting effect. Ischemia-resistant phenotype is maintained in the combined hypoxia/exercise setting, despite exercise-induced attenuation of TNF-α-dependent protective signaling.

INTRODUCTION

Given the worldwide epidemic prevalence of ischemic heart disease representing the leading cause of mortality, the search for effective approaches to improve myocardial ischemic tolerance and delay the onset of cell death became crucially important. It is well recognized that the heart has the capability to protect itself from lethal ischemia/reperfusion (I/R) injury if subjected to appropriate stimuli. Among them, chronic hypoxia and exercise training have received an increasing attention as natural and clinically relevant stimuli that can induce considerably prolonged or sustainable cardioprotective states.

In line with the results of human epidemiological surveys (2, 15, 26), the vast majority of animal studies demonstrated that chronic hypoxia confers the protective cardiac phenotype against major endpoints of acute I/R injury (19). Importantly, the significant infarct size-limiting effect of chronic hypoxia lasts at least for five weeks after the cessation of hypoxia (31). Moreover, as demonstrated recently, rats exposed to chronic hypoxia several days after the induction of myocardial infarction exhibit better heart function and less progressive remodeling than infracted normoxic animals (43).

The benefits of regular exercise for healthy heart have been well recognized and a strong correlation exists between physical activity and the rate of survival after myocardial infarction (25, 35). The lack of physical activity is now considered as a major risk factor for cardiovascular diseases. Similarly as chronic hypoxia, exercise has been shown to mitigate myocardial injury caused by acute I/R insult in various experimental settings (6, 13, 36). Although the protective effects depend on the type and intensity of exercise (10), only a few daily sessions of exercise are sufficient to achieve the maximum level of protection in rats, which is then sustainable for months of regular exercise training (9, 22) and still persists after four weeks of detraining (10). Thus, in analogy to chronic hypoxia, regular exercise training induces the protective cardiac phenotype without tachyphylaxis that may last long after the initial stimulus withdrawal. Detailed understanding the underlying molecular mechanism(s) may offer greater potential for therapeutic exploitation than various short-lived forms of cardioprotection. Although chronic hypoxia and exercise obviously share several important signaling pathways, limited evidence exists suggesting that there are some differences in detailed protective mechanism(s). It is unknown, whether these two protective measures can act in synergy in improving myocardial survival upon ischemic insult.

It is now well established that reactive oxygen species (ROS) play a dual role in myocardial I/R injury: while excess ROS can trigger oxidative damage of biological structures, they also serve as important elements in protective signaling at physiologically relevant levels (4). We have shown recently that chronic hypoxia is associated with the increased formation of ROS which plays an important role in the induction of the protective cardiac phenotype as various antioxidant treatments applied during the adaptation period eliminated its infarct size-limiting effect (20, 33). Similarly, the exercise-induced cardioprotection was abolished by an antioxidant given during exercise sessions (1).

ROS stimulate myocardial inflammatory reaction by promoting tumor necrosis factor-α (TNF-α) activity leading to pro-inflammatory cytokine cascade (12, 17). This is a self-amplifying process as TNF-α is involved in further production of ROS (27). Similarly as ROS, TNF-α can contribute to both myocardial I/R injury (12, 44) and protective signaling [23,24]. In our recent study, we observed the increased myocardial levels of TNF-α and its type 2 receptor (TNFR2) together with the increased antioxidant capacity in chronically hypoxic rats. Moreover, the treatment of animals during adaptation with an antibody against TNF-α suppressed not only these responses but also the infarct-sparing effect (8). In contrast, exercise can result in an anti-inflammatory phenotype as indicated by the repression of related myocardial transcripts (7). Interestingly, regular exercise completely abolished the myocardial

TNF-α increase induced by the chronic stimulation of β-adrenoceptors (40). This observation led us to hypothesize that exercise can also suppress TNF-α and the pro-inflammatory myocardial phenotype of chronically hypoxic rats. In this study, we therefore attempted to determine how regular exercise training performed under conditions of continuous chronic hypoxia affects myocardial ischemic tolerance with respect to the inflammatory reaction and redox status.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (initial body wt 250–280 g, Charles River, Germany) were housed in a controlled environment (23 °C; 12-h light-dark cycle; light from 5:00 AM) with free access to water and standard chow diet. Animals were randomly assigned to one of the following experimental groups: normoxic sedentary, normoxic trained, hypoxic sedentary, and hypoxic trained. The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Academy of Science, National Academy Press, Washington, D.C.). The experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology of the Czech Academy of Sciences.

Chronic hypoxia and exercise training

Rats were exposed to moderate continuous normobaric hypoxia (CNH; inspired O_2 fraction 0.12) for 3 weeks in a normobaric chamber (6 m^3) equipped with hypoxic generators

(Everest Summit, Hypoxico Inc., NY). Additional subgroup of animals (n=6) was exposed to CNH for only one week. No reoxygenation occurred during this period. The control normoxic rats were kept for the same period of time at room air.

Rats assigned to exercise groups were habituated to forced treadmill running by increasing the speed (from 25 to 30 $m.min^{-1}$) and duration (from 10 min to 50 min) of daily exercise session stepwise for 5 consecutive days. After 2 days of rest, the exercise protocol involved 5 days of running at 30 $m.min^{-1}$ for 60 min with a 0° inclination. Normoxic and hypoxic animals were trained either at room air or in the hypoxic chamber, respectively, during the light period. Habituation to running started after the first week of hypoxic exposure. Corresponding sedentary and trained rats were housed in the same room. Animals were used immediately after the cessation of hypoxic exposure and/or the next day after the last exercise session.

The compliance of each rat with exercise training was evaluated during each session by a 5-point score: a score of 1 was given to well-compliant rats while a score of 5 was given to totally non-compliant ones. Mean exercise compliance score during the whole training protocol was calculated. Two insufficiently compliant rats out of 15 in the combined CNH/exercise group were excluded from evaluations.

Myocardial ischemia/reperfusion

Acute I/R insult was performed as described previously (32). Anesthetized (sodium pentobarbital, 60 $mg.kg^{-1}$ i.p.) rats were ventilated (Ugo Basile, Italy) with room air. Electrocardiogram and blood pressure in the carotid artery were continuously recorded (Gould P23Gb) and subsequently analyzed by a custom-designed software. The rectal temperature was maintained between 36.5 and 37.5 °C throughout the experiment. Left thoracotomy was performed and, after 10-min stabilization, ischemia was induced by the tightening of a suture placed around the left anterior descending (LAD) coronary artery about 1–2 mm distal to its origin. After a 20-min occlusion period, the ligature was released, chest was closed and myocardial reperfusion continued for 3 h in spontaneously breathing animals maintained in deep anesthesia.

Infarct size determination

Hearts were excised and washed with saline *via* aorta. The area at risk was delineated by perfusion with 5% potassium permanganate after the LAD reocclusion (32). Frozen hearts were cut into slices 1 mm thick, stained with 1% 2,3,5-triphenyltetrazolium chloride (pH 7.4; 37 °C) for 30 min and fixed in formaldehyde solution. The infarct size (IS), the size of the area at risk (AR) and the size of the left ventricle (LV) were determined by computerized planimetric using the software Ellipse (ViDiTo, Slovakia). The size of AR was normalized to LV (AR/LV) and the IS was normalized to the AR (IS/AR). The incidence and severity of ventricular arrhythmias during the 20-min ischemic insult and during the first 3 min of reperfusion were assessed as previously described (3).

Tissue processing

The separate groups of animals (not subjected to myocardial I/R) assigned to biochemical analyses were euthanized by cervical dislocation, hearts were rapidly excised, washed in cold (0 °C) saline, dissected into the right ventricle (RV), free wall of LV, and the septum and weighed; LV was frozen in liquid nitrogen and stored at –80 °C until use.

Immunoblotting

Frozen LV were pulverized to fine powder with liquid nitrogen and subsequently homogenized in eight volumes of ice-cold homogenization buffer containing: 12.5 mM Tris (pH 7.4), 250 mM sucrose, 2.5 mM EGTA, 1 mM EDTA, 100 mM NaF, 0.3 mM phenylmethylsulfonyl fluoride, 6 mM β-mercaptoethanol, 10 mM glycerol-2-phosphate, 0.2 mM leupeptin, 0.02 mM aprotinin and 0.1 mM activated sodium orthovanadate. All steps were performed at 4 °C. The homogenate aliquots were stored at –80 °C until use.

Proteins were separated by SDS-PAGE electrophoresis (10% or 15% gels) and transferred to PVDF membranes (BioRad, Prague, Czech Republic). After blocking with 5% dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 1 h at room temperature, membranes were washed and probed at 4 °C with the following primary antibodies against: catalase (CAT) (Abcam, Cambridge, MA; ab16731, 1:2000), citrate synthase (CS) (Abcam; ab-96600, 1:2000), cyclooxygenase-1 (COX-1) (Santa Cruz Biotechnology, Dallas, TX; sc-1752, 1:1000), cyclooxygenase-2 (COX-2) (Santa Cruz Biotechnology; sc-1747, 1:1000), cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) (Cell Signaling, Danvers, MA; 2832S, 1:2000), anti-p-$cPLA_2\alpha$ (Cell Signaling; 2831S, 1:2000), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology; sc-25778, 1:500), inducible nitric oxide synthase (iNOS) (BD Biosciences, San Jose, CA; 610432, 1:500), manganese superoxide dismutase (MnSOD) (Sigma Aldrich, Prague, Czech Republic; S5069, 1:1000), nuclear factor- κB (NF-κB) p65 (Santa Cruz Biotechnology; sc-372, 1:500), TNF-α type 1 receptor (TNFR1) (Santa Cruz Biotechnology; sc-1070, 1:1000) and TNF-α type 2 receptor (TNFR2) (Santa Cruz Biotechnology; sc-7862, 1:1000). Following overnight incubation, the membranes were washed and incubated for 1 h at room temperature with anti-rabbit (Bio-Rad; 170-6515), anti-mouse (Thermo Fisher Scientific, Prague, Czech Republic; 31432) and anti-goat (Sigma

Aldrich; A8919), respectively, horseradish peroxidase-labelled secondary antibodies. Bands were visualized by enhanced chemiluminescence on the LAS system or on the medical X-ray films (Agfa, Berlin, Germany). ImageJ (Java Technology, Cupertino, CA) software was used for the quantification of the relative abundance of proteins. To ensure the specificity of immunoreactive proteins, prestained molecular weight protein standards (BioRad) were used. The samples from each experimental group were run on the same gel and quantified on the same membrane. Neither CNH nor exercise affected the expression of GAPDH, which was used as a loading control.

Inflammatory cytokines assay

For the measurement of TNF-α and interleukin-6 (IL-6), the ELISA kits (eBioscience, Vienna, Austria) were used. These assays were performed on the homogenized samples of LV myocardium from different experimental groups according to the standards described by the manufacturers. The results are expressed per mg of total protein.

Malondialdehyde (MDA) assay

Myocardial samples for determination of lipid peroxidation marker MDA were processed as described earlier (8) and analyzed by an HPLC system (Shimadzu, Japan; column EC Nucleosil 100-5 C18; 4.6 mm × 125 mm; flow 1.0 $ml.min^{-1}$; sampling volume 30–100 μl) with the UV detection set on 310 nm. MDA concentration was normalized to total protein content.

Statistical analyses

Normally distributed variables are expressed as mean ± SE. One-way ANOVA and subsequent Tukey's multiple comparison tests were used to examine differences between the groups. Not normally distributed data (arrhythmias) are expressed as median ± interquartil range. Differences in the number of premature ventricular complexes between the groups were compared by the Kruskal–Wallis non-parametric test. The incidence of ventricular tachycardia and fibrillation was examined by Fisher's exact test. Differences were assumed statistically significant when $P < 0.05$. Statistical analyses were performed using GraphPad Prism 6.01 (Graphpad Software Inc., CA).

RESULTS

Body and heart wt and hematocrit

Adaptation of rats to moderate continuous hypoxia did not significantly affect body wt, while exercise training caused growth retardation, which was more pronounced in animals trained under hypoxic conditions. No significant differences in LV wt were observed among the groups, except for the hypoxic exercised rats, which showed increased LV wt normalized to body wt. CNH led to RV hypertrophy and increased hematocrit. These variables were not affected by exercise training (Table 1).

Myocardial infarct size and arrhythmias

Baseline values of mean arterial pressure were slightly but significantly higher in both hypoxic groups compared to their normoxic counterparts. This difference persisted throughout ischemia and reperfusion in sedentary rats only. Neither CNH nor exercise training affected heart rate before ischemia. Both hypoxic groups exhibited higher heart rate at the end of reperfusion than at baseline and the rats trained at normoxia had lower heart rate than their sedentary controls (Table 2).

The mean normalized AR (AR/LV) was 39–43% and did not differ among the groups. The IS reached 54.1 ± 4.0% of the AR in the normoxic group and exercise training decreased it to 44.3 ± 2.7%. CNH reduced IS to 36.7 ± 3.3% but exercise at hypoxia did not provide any additive protection (37.4 ± 3.7%) (Fig. 1).

Neither CNH nor exercise training significantly affected the total number of ischemic ventricular arrhythmias (Fig. 2A) and the total duration of tachyarrythmias (tachycardia and reversible fibrillation; Fig. 2B). However, animals trained at hypoxia were significantly more susceptible to ischemic arrhythmias than their normoxic counterparts. Sustained fibrillation (> 2-min duration) occurred in 18–32% of rats, except for the sedentary hypoxic group which exhibited only reversible fibrillation; the differences among groups did not reach statistical significance (Fig. 2C). CNH reduced the total number of arrhythmias occurring at the beginning of reperfusion but exercise abolished this effect (Fig. 2D).

The mean exercise compliance score of normoxic and chronically hypoxic animals was 1.29 and 2.06, respectively. To verify that the somewhat worse compliance of rats exercising at hypoxia compared to those trained at room air did not affect myocardial ischemic tolerance, we selected well-compliant animals (score of 1.0–1.5) from both groups. The mean score was 1.23 and 1.24 in selected normoxic and chronically hypoxic subgroups, respectively. Fig. 1 and Fig. 2 show that this selection had no significant effect on infarct size and the susceptibility to arrhythmias.

IL-6, TNF-α and its receptors

Adaptation to CNH for 3 weeks increased myocardial levels of TNF-α and IL-6 by 53% and 88%, respectively, compared to the normoxic sedentary group. No increase was absent when TNF-α was measured after the first week of the hypoxic exposure (93% of normoxic level). Exercise training had no effect on TNF-α and IL-6 in the hearts of normoxic rats but it significantly attenuated their increase induced by CNH (Fig. 3A,B). CNH had no effect on the myocardial protein level of TNFR1 while significantly increasing TNFR2 level (by 102%). Exercise training of normoxic rats affected neither TNFR1 nor TNFR2 but it prevented the increase in TNFR2 level in the group adapted to CNH (Fig. 3C,D).

NF-κB and related signaling

The expression of transcription factor NF-κB was increased by CNH by 71%. This increase was reduced by exercise training which had no effect in normoxic rats. Nevertheless, NF-κB level still remained significantly higher in rats exercising at hypoxia compared to their normoxic counterparts (Fig. 4A). CNH increased the expression of iNOS (by 63%) which was not significantly affected by exercise (Fig. 4B). Both $cPLA_2α$ and its phosphorylated form were upregulated by CNH by 13% and 26%, respectively. These increases were abolished by exercise training which had no effect in the normoxic group (Fig. 4C,D). Neither CNH nor exercise affected COX-1 level, while COX-2 level was increased by 43% in the CNH group, the effect being attenuated by exercise (Fig. 4E,F).

MDA and antioxidant enzymes

Myocardial MDA concentration increased by 76% and the expression of MnSOD and catalase rose by 75% and 24%, respectively, in the hearts of rats adapted to CNH for 3 weeks. MnSOD measured after the first week of the hypoxic exposure remained unaffected, reaching 101% of normoxic value. Exercise training had no effect in the normoxic animals and it only tended to attenuate the CNH-induced increases of MDA, MnSOD and catalase without reaching statistical significance (Fig. 5A,B,C). Neither CNH nor exercise affected the expression of CS which is commonly used as a marker of mitochondrial mass (Fig. 5D).

DISCUSSION

The present study was designed to determine whether a combination of two well-established forms of sustainable cardioprotection induced by chronic hypoxia and exercise training can result in the amplification of ischemia-resistant cardiac phenotype. Our data are in line with a number of earlier reports showing that these adaptive interventions acting separately to reduce myocardial infarct size induced by acute I/R insult. The novel finding is that rats subjected to regular exercise during continuous exposure to hypoxic atmosphere exhibited the same infarct-sparing effect as their sedentary counterparts. CNH led to pro-inflammatory response, increased myocardial expression of several related potentially protective mediators and antioxidant enzymes while none of these effects were observed in the rats exercising at room air. On the other hand, exercise in hypoxia abolished or significantly attenuated most of the CNH-induced responses related to inflammation, including the increased TNF-α and IL-6 levels and the overexpression of TNFR2, NF-κB, $cPLA_2α$ and COX-2, without significantly affecting the upregulation of iNOS and antioxidant enzyme MnSOD.

We reported recently that the treatment of rats with antibodies against TNF-α during adaptation to CNH suppressed the infarct size-limiting effect and eliminated the CNH-induced increases in myocardial levels of TNF-α, its receptor TNFR2, NF-κB and MnSOD. These results led us to conclude that TNF-α is involved in the protective mechanism of CNH, its effect being possibly mediated by TNFR2 and the NF-κB-dependent activation of redox signaling with increased antioxidant defense (8). TNF-α is a key cytokine which plays an essential role in the initiation of inflammatory response. While excessive levels of TNF-α have detrimental actions on the heart mediated by TNFR1 (which was not affected in our study), the activation of TNFR2 by low levels of this cytokine is protective (39). Several studies have demonstrated that TNF-α can also induce various forms of conditioning (21, 34).

Regarding the involvement of cytokines in exercise-induced cardioprotection, the available data are scarce and conflicting. Serra et al. (40) did not observed any effect of regular exercise training itself on the myocardial levels of TNF-α and IL-6 in rats. On the other hand, TNF-α neutralization blunted the protection induced by a single exercise session, likely *via* the prevention of antioxidant response (45). Regarding IL-6, a recent report indicated that this myokine released from skeletal muscles mediated cardioprotective effects of exercise in mice. Exercise did not affect myocardial IL-6 level but it upregulated its receptor and activated IL-6 signaling pathways (24). Thus, the absence of any effect of exercise alone on myocardial cytokines in our study does not necessarily mean that they are not involved in the induction of protected cardiac phenotype.

Exercise has been shown to reduce sympathetic activation and stimulation of myocardial adrenoceptors associated with the adaptation to chronic hypoxia (11) which plays an important role in the cardioprotection conferred by hypoxic conditioning (23). Interestingly, exercise training completely abolished the increase of myocardial TNF-α and IL-6 levels caused by the sustained pharmacological stimulation of β-adrenoceptors (40).

Given our finding that TNF-α plays a role in the induction of the ischemia-resistant phenotype of CNH hearts (8), its blunted response to hypoxia in exercised rats may be expected to attenuate the protective effect. However, here we show that exercise training abolished only the CNH-induced suppression of early reperfusion arrhythmias, whereas the infarct-sparing effect remained unaffected. This can be possibly explained by another protective mechanism activated by exercise that just compensated for the blunted TNF-α signaling. The absence of any influence of exercise training alone on the potentially protective molecules detected in our study seems to support this view. Nevertheless, it should be noted that NF-κB and iNOS upregulated by CNH remained significantly higher in exercised hearts compared to their normoxic counterparts, and COX-2 also exhibited similar expression pattern. Both iNOS and COX-2 have been shown to play a role in delayed forms of cardioprotection (5). Thus, their levels might be still sufficient to maintain ischemia-resistant cardiac phenotype in the present combined CNH/exercise setting.

It can be assumed that TNF-α increase occurred already during the first week of CNH exposure when the animals did not exercise and this initial response was able to induce the persisting cardioprotected state. Indeed, it has been shown that TNF-α can result in the long-lasting activation of NF-κB and its downstream targets (29). However, our observation of unchanged levels of TNF-α and MnSOD after one week of hypoxia seems to rule out this possibility. Accordingly, previously we did not detect any reduction of infarct size during the first week of exposure to CNH (42).

Inflammation and oxidative stress are mutually related. Specifically, TNF-α stimulates ROS production while ROS can promote the TNF-α-induced inflammatory cascade (17, 27, 38). It has been shown that mitochondria are the principal source of ROS formation in the TNF-α pathway (42). Signaling *via* ROS-dependent pathways appears to play a key role in cardioprotection against I/R insult conferred by various stimuli including chronic hypoxia

(20) and exercise training (1) as indicated by the elimination of their infarct-sparing effects by antioxidant treatments during hypoxic exposure and training sessions, respectively. Numerous but not all studies demonstrated the increased myocardial capacity of antioxidant defense systems induced by chronic hypoxia or exercise as a prerequisite for their salutary effects against I/R injury. The enhanced expression of MnSOD and catalase in hearts of CNH rats in the present study is in line with these results. Excess formation of ROS without adequate activation of cellular antioxidants caused by a brief periodic interruption of hypoxic exposure may result in a disturbed redox balance and a loss of protection (16, 30). Regarding exercise training, we did not detect any significant effect on MDA and antioxidant enzymes in ventricular homogenate in agreement with a number of reports summarized in a recent review (37). Nevertheless, it has been suggested that cardioprotection induced by a longer duration of exercise is mediated, at least in part, by MnSOD (13, 37), the primary mitochondrial antioxidant enzyme. Indeed, some studies detected the increased level and activity of this enzyme following exercise in myocardial mitochondrial fraction (18, 41). Although we cannot exclude that exercise led to the upregulation of MnSOD in mitochondria also in our present study, the unchanged level of CS reflecting mitochondrial mass makes this an unlikely possibility.

It is concluded that regular exercise training of rats during their adaptation to CNH conferred the same infarct size-limiting effect as CNH alone, despite attenuating the CNH-induced increase in myocardial inflammatory response and related cardioprotective signaling. The maintenance of ischemia-resistant cardiac phenotype in CNH combined with exercise can be possibly attributed to the persisting activity of NF-κB/iNOS pathway and increased myocardial antioxidant defense capacity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

F.K. developed the study concept and drafted the manuscript. All authors were involved in performing the experiments, data collection, analysis and interpretation, contributed to the intellectual content and editing of the manuscript, and approved its final version.

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Table 1

Body and heart weight parameters and hematocrit in chronically hypoxic and normoxic sedentary and exercise-trained rats.

Group	n	BW (g)	LVW (mg)	LVW/BW (mg/g)	RVW (mg)	RVW/BW (mg/g)	Hematocrit (%)
Normoxic sedentary	8	422 ± 9	538 ± 20	1.275 ± 0.038	229 ± 6	0.542 ± 0.008	45.7 ± 0.8
Normoxic trained	9	$380 \pm 7^{\dagger}$	528 ± 20	1.391 ± 0.044	205 ± 5	0.541 ± 0.014	45.2 ± 0.8
Hypoxic sedentary	8	397 ± 6	506 ± 22	1.271 ± 0.039	341 ± 15*	0.857 ± 0.033*	53.1 ± 1.3*
Hypoxic trained	8	$330 \pm 3^{*\dagger}$	474 ± 12	1.436 ± 0.031*	$288 \pm 7^{*\dagger}$	0.875 ± 0.027*	55.9 ± 0.8*

BW, body weight; LVW, left ventricle weight; LVW/BW, relative left ventricle weight; RVW, right ventricle weight; RVW/BW, relative right ventricle weight; n, number of rats. Values are means ± SE; $^*P < 0.05$ vs. corresponding normoxic group; $^{\dagger}P < 0.05$ vs. corresponding sedentary group.

Table 2

Heart rate and mean arterial blood pressure after stabilization (Baseline), at the end of 20-min coronary artery occlusion (Ischemia) and at the end of 3-h reperfusion in chronically hypoxic and normoxic sedentary and exercise-trained rats.

	n	Baseline	Ischemia	Reperfusion
Heart rate (beats.min^{-1})				
Normoxic sedentary	8	392 ± 8	404 ± 11	416 ± 11
Normoxic trained	15	372 ± 8	379 ± 12	378 ± 9†
Hypoxic sedentary	13	371 ± 9	407 ± 5‡	393 ± 8‡
Hypoxic trained	13	368 ± 8	374 ± 10	396 ± 8‡
Blood pressure (mmHg)				
Normoxic sedentary		101 ± 4	87 ± 7	84 ± 4
Normoxic trained		107 ± 4	103 ± 5	95 ± 5
Hypoxic sedentary		122 ± 5*	121 ± 6*	105 ± 6*
Hypoxic trained		122 ± 4*	114 ± 6	108 ± 7

n, number of rats. Values are means ± SE; *$P < 0.05$ vs. corresponding normoxic group; $^{\dagger}P < 0.05$ vs. corresponding sedentary group; $^{\ddagger}P < 0.05$ vs. Baseline.

FIGURE CAPTIONS

Figure 1. Myocardial area at risk (A) and infarct size (B) induced by coronary artery occlusion and reperfusion in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Tc denotes the subgroups well-compliant to exercise training (see the Methods section for details). Values are means ± SE from 7–15 hearts in each group; **P* < 0.05 vs. corresponding normoxic group; †*P* < 0.05 vs. corresponding sedentary group.

Figure 2. Total number of premature ventricular complexes (PVCs)(A), total duration of tachyarrhythmias (B) and the incidence of reversible/sustained ventricular fibrillation (C) during coronary artery occlusion, and total number of PVCs during the first 3 min of reperfusion (D) in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Tc denotes the subgroups well-compliant to exercise training (see the Methods section for details). Values (graphs A, B and D) are shown as median with interquartil range from 7–15 hearts in each group; **P* < 0.05 vs. corresponding normoxic group; †*P* < 0.05 vs. corresponding sedentary group.

Figure 3. Myocardial levels of tumor necrosis factor-α (TNF-α)(A), interleukin-6 (IL-6)(B), TNF-α type 1 receptor (TNFR1)(C) and TNF-α type 2 receptor (TNFR2)(D) in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Representative Western blots are shown (E); GAPDH was used as a loading control. Values are means ± SE from 8 hearts in each group; **P* < 0.05 vs. corresponding normoxic group; †*P* < 0.05 vs. corresponding sedentary group.

Figure 4. Myocardial levels of nuclear factor-κB (NF-κB)(A), inducible nitric oxide synthase (iNOS)(B), cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$)(C), phosphorylated form of $cPLA_2\alpha$ (p-$cPLA_2\alpha$)(D), cyclooxygenase-1 (COX-1)(E) and cyclooxygenase-2 (COX-2)(F) in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Representative Western blots are shown (D); GAPDH was used as a loading control. Values are means ± SE from 8 hearts in each group; $^*P < 0.05$ vs. corresponding normoxic group; $^\dagger P < 0.05$ vs. corresponding sedentary group.

Figure 5. Concentration of malondialdehyde (MDA)(A) and myocardial levels of manganese superoxide dismutase (MnSOD)(B), catalase (CAT)(C) and citrate synthase (CS)(D) in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Representative Western blots are shown (E); GAPDH was used as a loading control. Values are means ± SE from 8 hearts in each group; $^*P < 0.05$ vs corresponding normoxic group.

Figure 1:

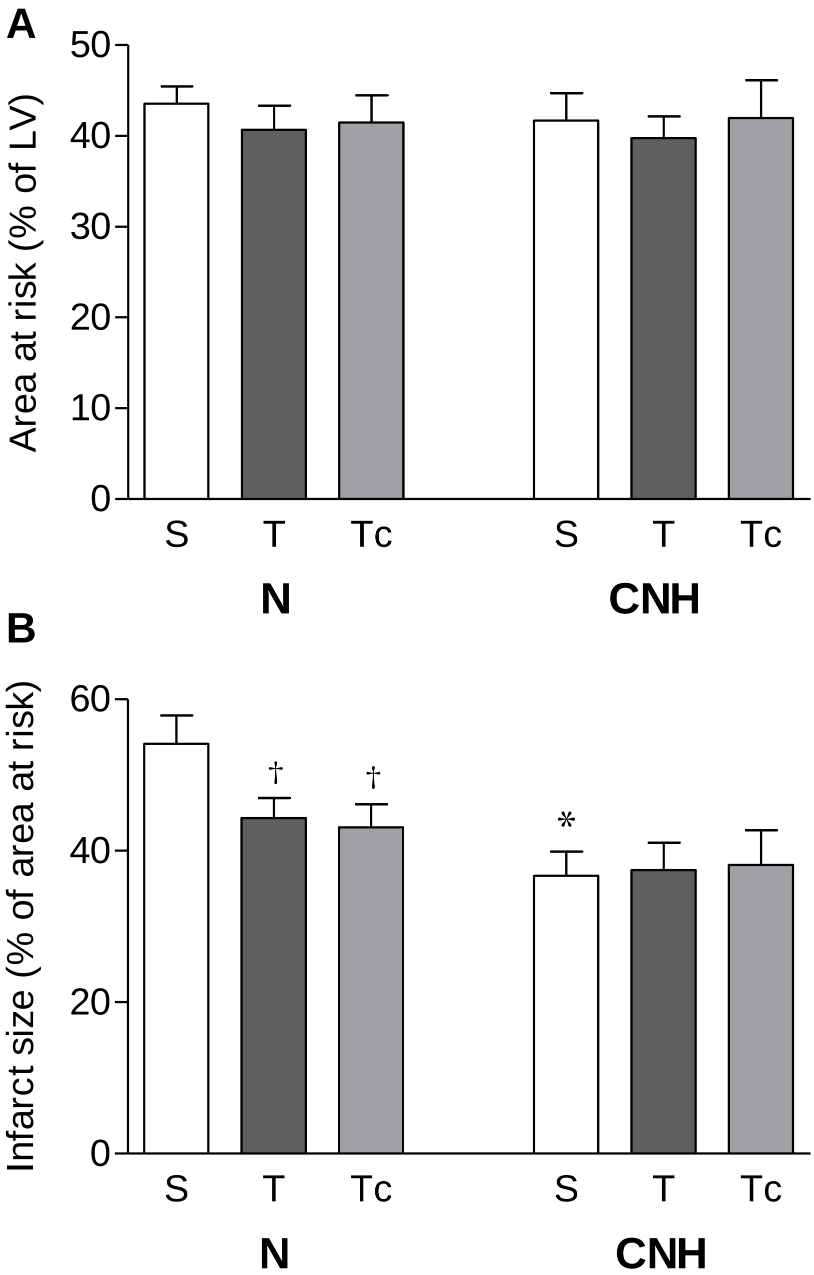

A
50
40
30
20
10
0
Area at risk (% of LV)
S
T
Tc
S
T
Tc
N
CNH
B
60
40
20
0
Infarct size (% of area at risk)
†
†
*
S
T
Tc
S
T
Tc
N
CNH

Figure 2:

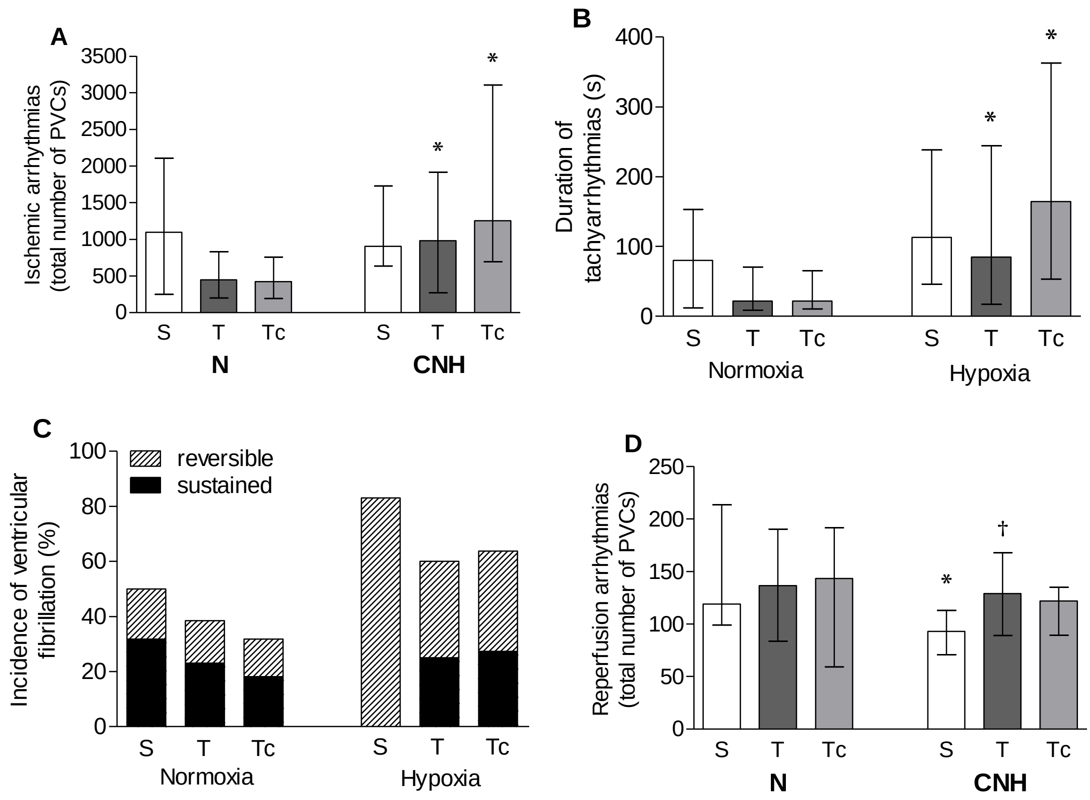
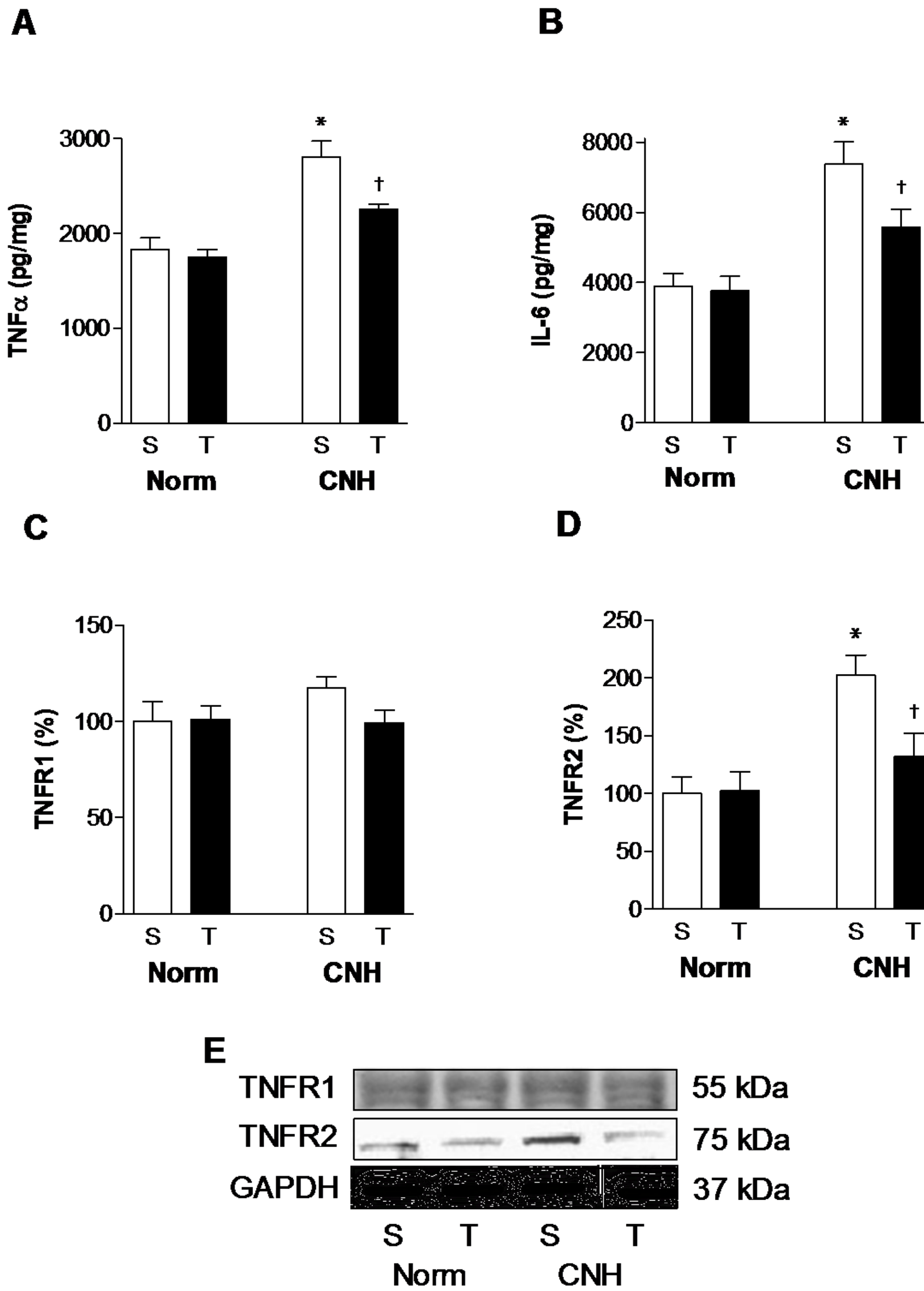

A
Ischemic arrhythmias (total number of PVCs)
3500
3000
2500
2000
1500
1000
500
0
S
T
Tc
N
CNH
*
B
Duration of tachyarrhythmias (s)
400
300
200
100
0
Normoxia
Hypoxia
C
Incidence of ventricular fibrillation (%)
100
80
60
40
20
reversible
sustained
D
Reperfusion arrhythmias (total number of PVCs)
250
200
150
100
50
†

Figure 3:



A
TNFα (pg/mg)
3000
2000
1000
0
*
†
S
T
S
T
Norm
CNH
B
IL-6 (pg/mg)
8000
6000
4000
2000
0
*
†
S
T
S
T
Norm
CNH
C
TNFR1 (%)
150
100
50
0
S
T
S
T
Norm
CNH
D
TNFR2 (%)
250
200
150
100
50
0
*
†
S
T
S
T
Norm
CNH
E
TNFR1
55 kDa
TNFR2
75 kDa
GAPDH
37 kDa
S
T
S
T
Norm
CNH

Figure 4:

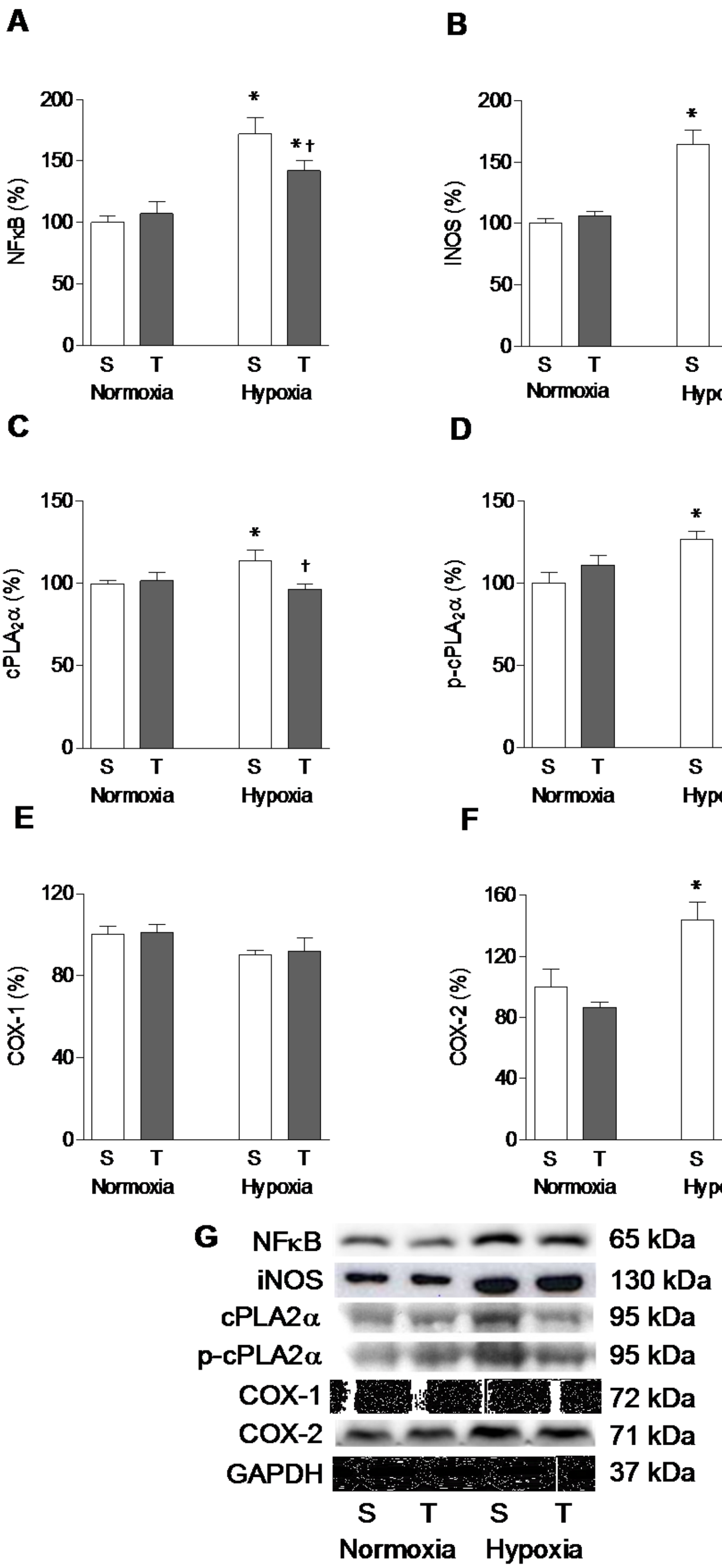
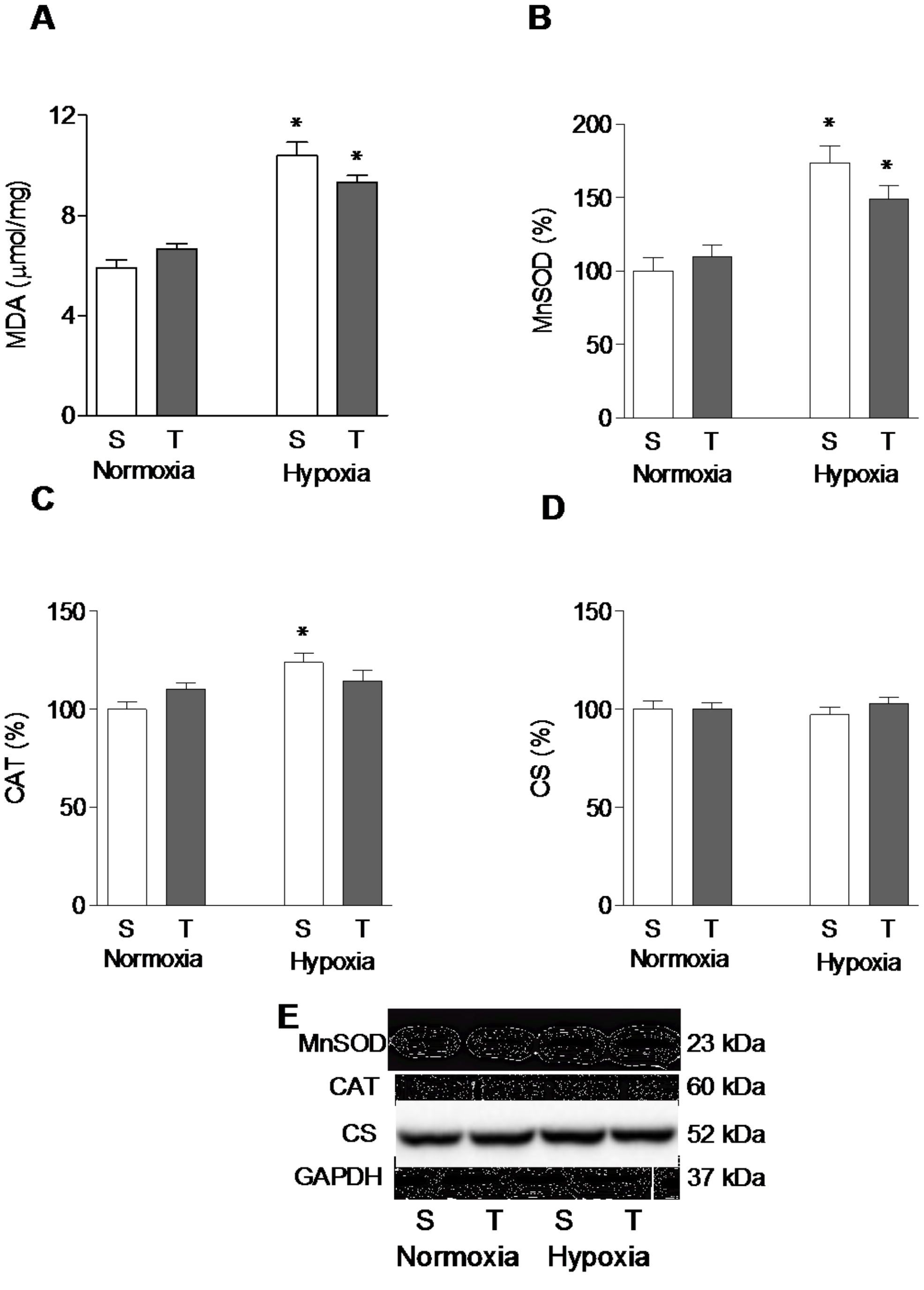

A
200
150
100
50
0
NFκB (%)
*
* †
S T S T
Normoxia Hypoxia
B
200
150
100
50
0
iNOS (%)
*
*
S T S T
Normoxia Hypoxia
C
150
100
50
0
cPLA2α (%)
*
†
S T S T
Normoxia Hypoxia
D
150
100
50
0
p-cPLA2α (%)
*
†
S T S T
Normoxia Hypoxia
E
120
80
40
0
COX-1 (%)
S T S T
Normoxia Hypoxia
F
160
120
80
40
0
COX-2 (%)
*
* †
S T S T
Normoxia Hypoxia
G
NFκB 65 kDa
iNOS 130 kDa
cPLA2α 95 kDa
p-cPLA2α 95 kDa
COX-1 72 kDa
COX-2 71 kDa
GAPDH 37 kDa
S T S T
Normoxia Hypoxia

Figure 5:


A
MDA (μmol/mg)
12
8
4
0
S
T
S
T
Normoxia
Hypoxia
B
MnSOD (%)
200
150
100
50
0
S
T
S
T
Normoxia
Hypoxia
C
CAT (%)
150
100
50
0
S
T
S
T
Normoxia
Hypoxia
D
CS (%)
150
100
50
0
S
T
S
T
Normoxia
Hypoxia
E
MnSOD
23 kDa
CAT
60 kDa
CS
52 kDa
GAPDH
37 kDa
S
T
S
T
Normoxia
Hypoxia