ACTA PHYSIOLOGICA

Acta Physiol 2015, **214**, 97–108

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

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Received 10 April 2014,
revision requested 6 June 2014,
revision received 12 February 2015,
accepted 9 March 2015

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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 ± 4.3% of the area at risk in normoxic animals to 35.5 ± 2.4%. Infliximab abolished the protective effect of CNH (44.9 ± 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

 

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 (Ošťádal & 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.

  doi: 10.1111/apha.12489

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

 

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.

  doi: 10.1111/apha.12489

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).

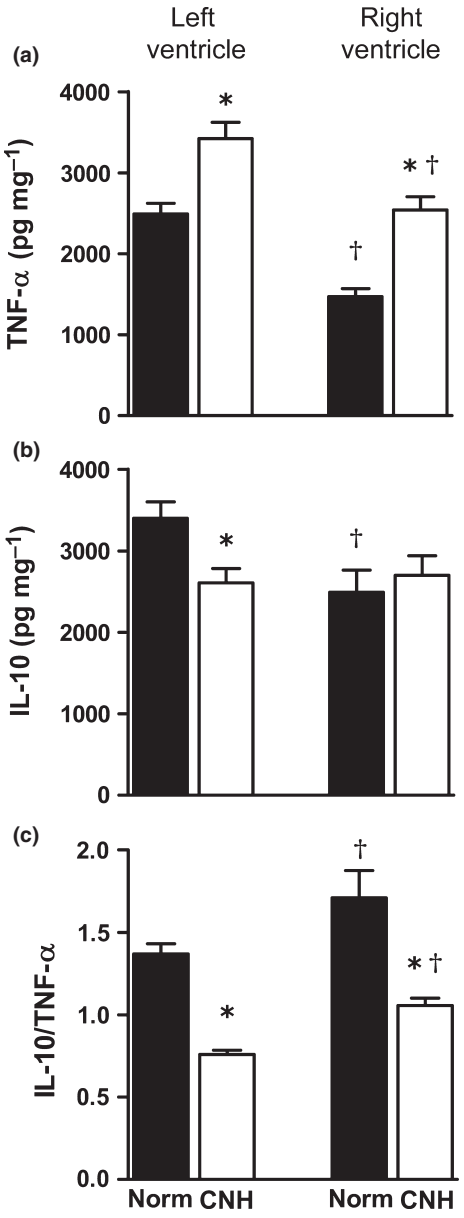


**Figure 1** Myocardial levels of (a) TNF-α, (b) IL-10 and (c) IL-10/TNF-α ratio from homogenates of left and right ventricles of rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (Norm). Values are means ± SEM from 6 to 8 hearts in each group; *$P < 0.05$ vs. corresponding normoxic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

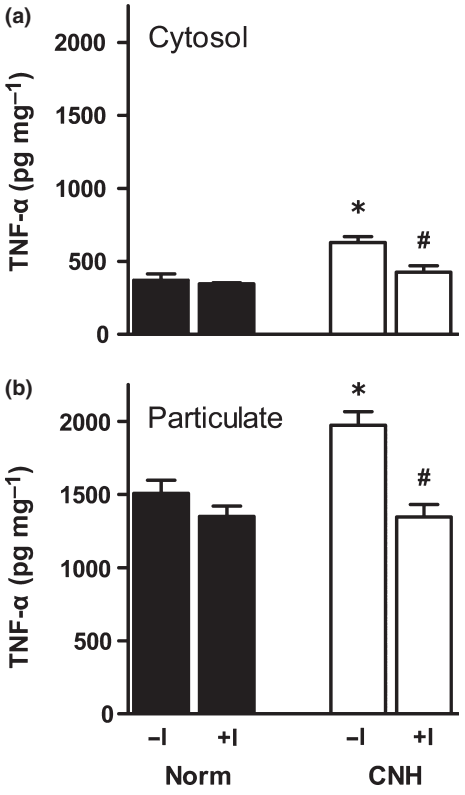


**Figure 2** The effect of infliximab (I) on myocardial levels of TNF-α in (a) cytosolic and (b) particulate fractions of left ventricle from rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (Norm). Values are means ± SEM from 6 to 8 hearts in each group; *$P < 0.05$ vs. corresponding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

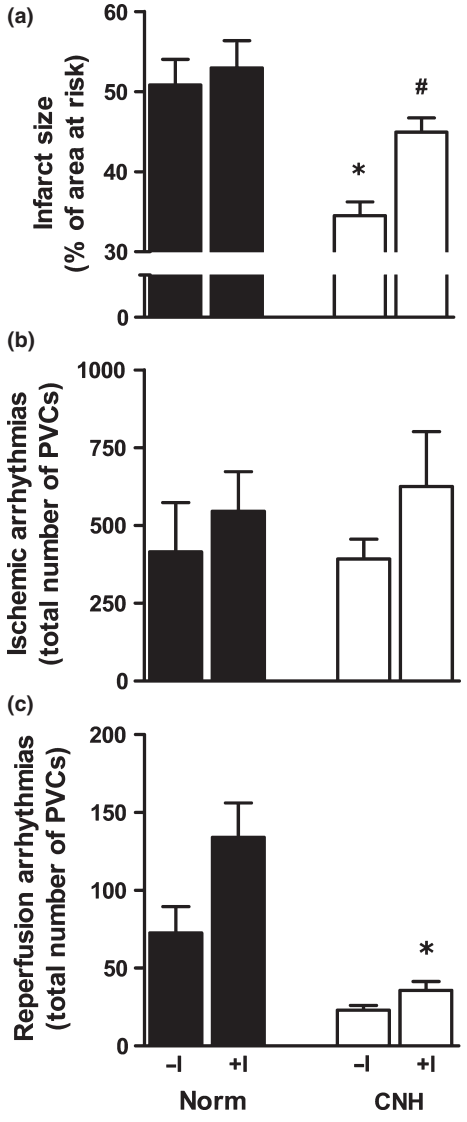


**Figure 3** The effect of infliximab (I) on (a) myocardial infarct size, (b) the total number of premature ventricular complexes (PVCs) during 20 min of ischaemia and (c) the number of PVCs during the first 3 min of reperfusion in rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (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.

 

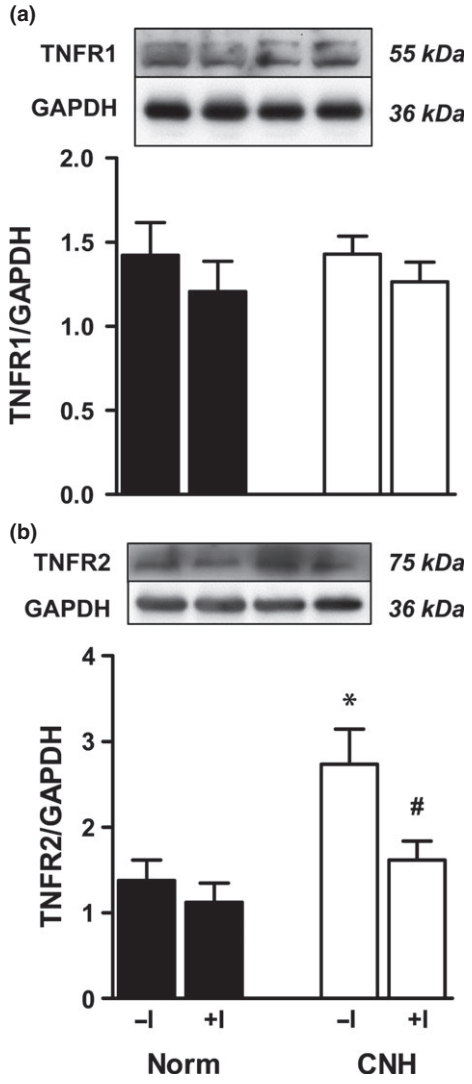


**Figure 4** The effect of infliximab (I) on myocardial levels of TNF-α (a) receptor 1 (TNFR1) and (b) receptor 2 (TNFR2) in left ventricle of rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (Norm). Representative Western blots of TNFR1 and TNFR2 are shown; GAPDH was used as 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.*

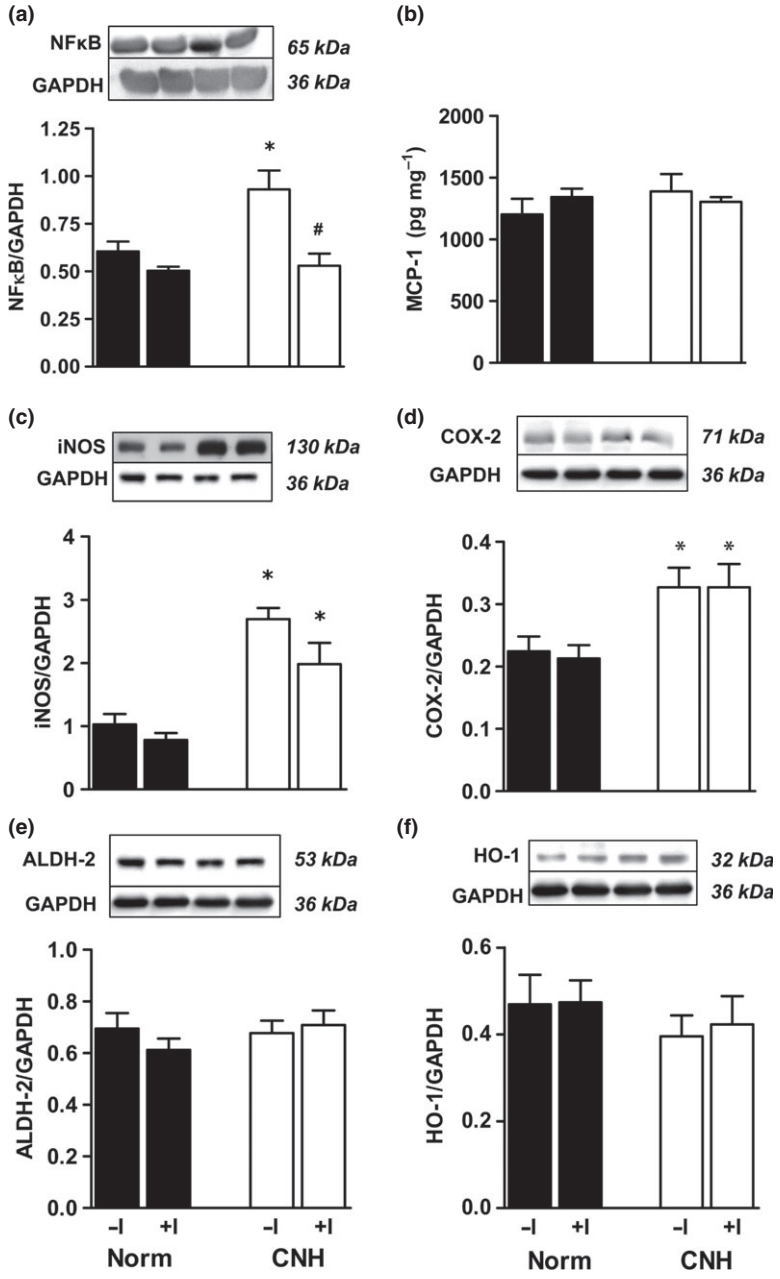


**Figure 5** The effect of infliximab (I) on myocardial level of (a) nuclear factor κB (NF-κB), concentration of (b) monocyte chemoattractant protein-1 (MCP-1), and levels of (c) inducible nitric oxide synthase (iNOS), (d) cyclooxygenase 2 (COX-2), (e) aldehyde dehydrogenase 2 (ALDH-2) and (f) haeme 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

 

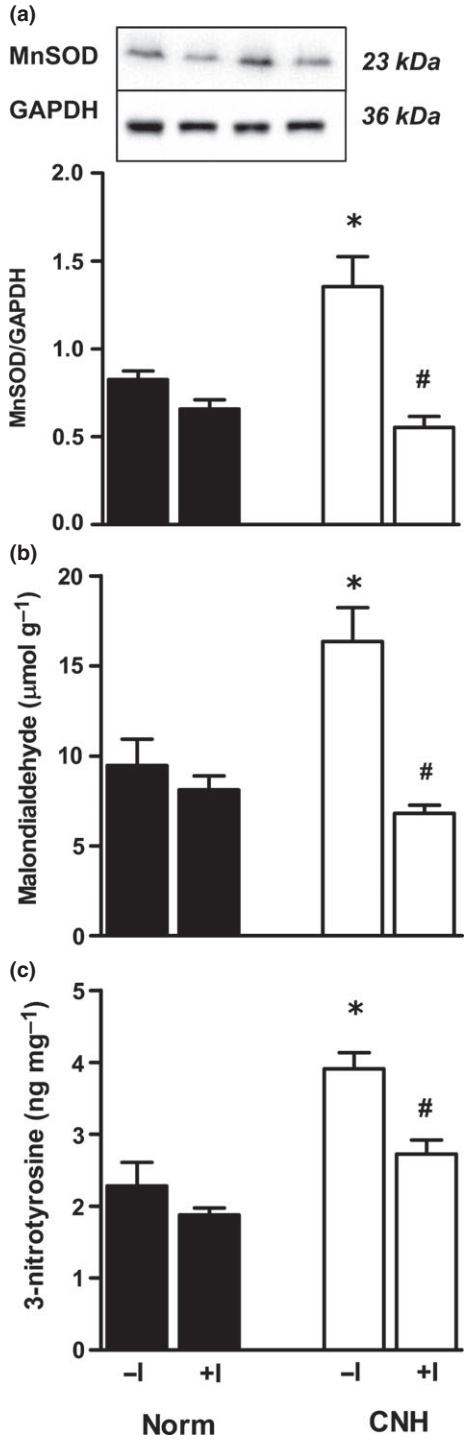


**Figure 6** The effect of infliximab (I) on myocardial level of (a) mitochondrial manganese superoxide dismutase (MnSOD) and concentrations of (b) malondialdehyde and (c) 3-nitrotyrosine in left ventricle of rats adapted to continuous normobaric hypoxia (CNH) and normoxic controls (Norm). Representative 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.

 

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.

This work was supported by the Czech Science Foundation (13-10267S to J.N. and 303/12/1162 to F.K.), the Grant Agency of the Charles University in Prague (1592614 to A.C.) and the institutional research projects 67985823 (Institute of Physiology, CAS) and 00023001 (IKEM). The Center for Experimental Medicine (IKEM) received financial support from the European Commission within the Operational Program Prague–Competitiveness; project 'CEVKOON' (#CZ.2.16/3.1.00/22126). The authors thank J. Vašinová for excellent technical assistance.

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  doi: 10.1111/apha.12489

# 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 $A_2$α, 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 $cPLA_2\alpha$ 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. 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, $cPLA_2\alpha$ 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.

**GRANTS**

Funding support was provided by the Czech Science Foundation grants 302/12/1162 (F.K.) and 13-10267 (J.N.), and the Grant Agency of the Charles University in Prague 798813 (J.H.).

## 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 ± 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 ± 3*$^{\dagger}$ | 474 ± 12 | 1.436 ± 0.031* | 288 ± 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$^{\dagger}$ |
| Hypoxic sedentary | 13 | 371 ± 9 | 407 ± 5$^{\ddagger}$ | 393 ± 8$^{\ddagger}$ |
| Hypoxic trained | 13 | 368 ± 8 | 374 ± 10 | 396 ± 8$^{\ddagger}$ |
| 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; $^{\dagger}P$ < 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; $^{\dagger}P$ < 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; $^{\dagger}P$ < 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:

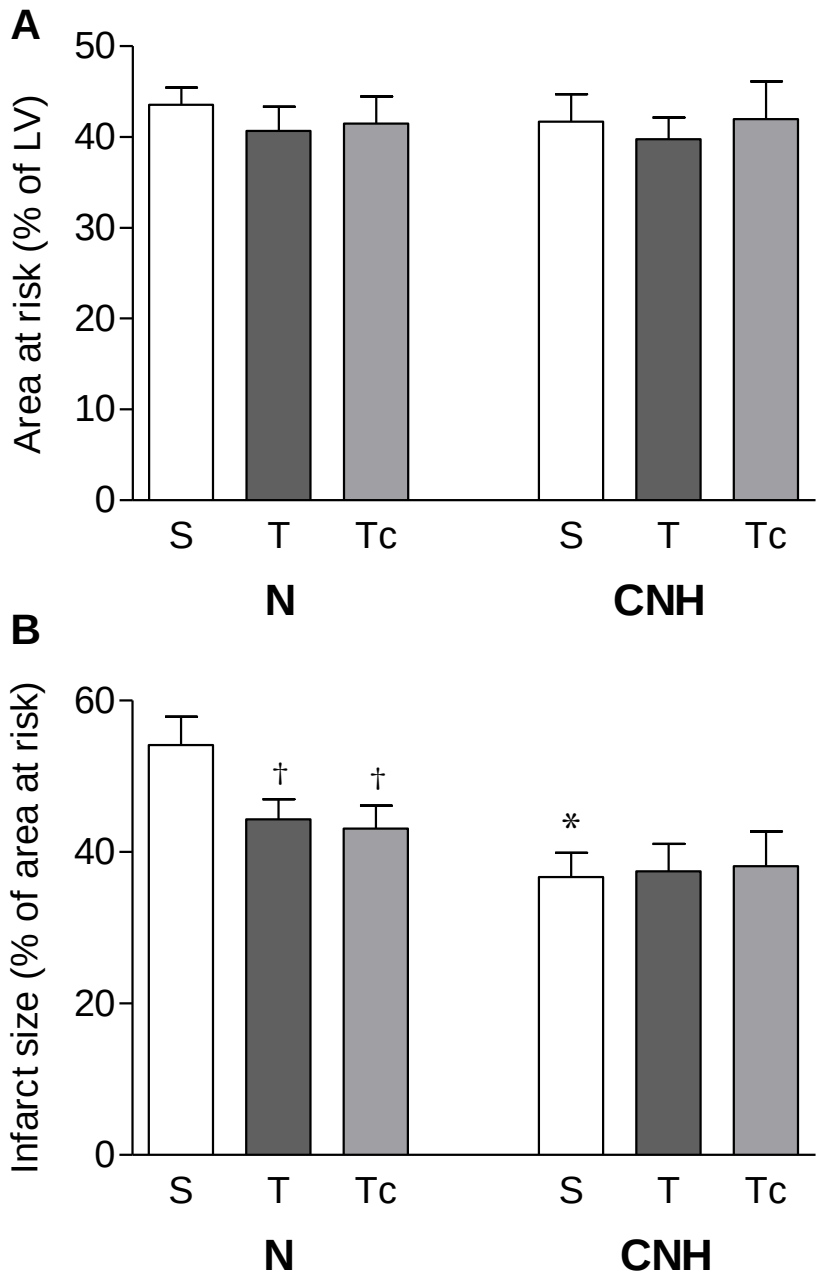

A
Area at risk (% of LV)
50
40
30
20
10
0
S
T
Tc
S
T
Tc
N
CNH
B
Infarct size (% of area at risk)
60
40
20
0
†
†
*
S
T
Tc
S
T
Tc
N
CNH

Figure 2:

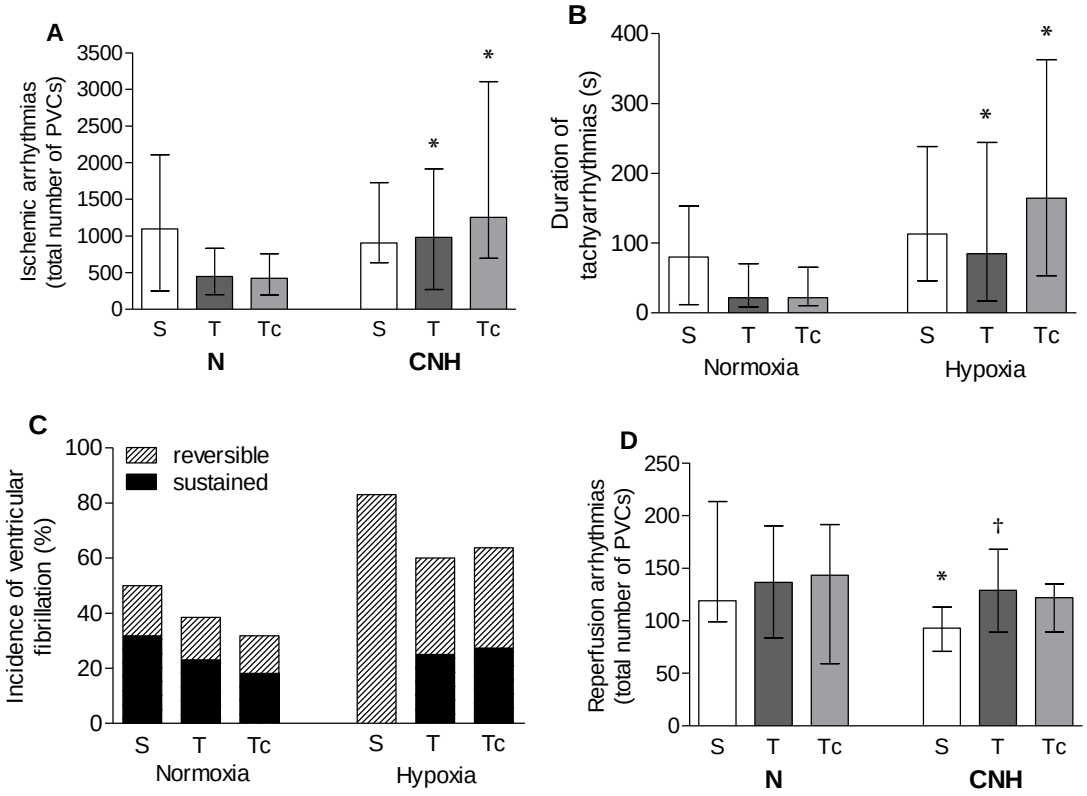

A
Ischemic arrhythmias (total number of PVCs)
3500
3000
2500
2000
1500
1000
500
0
S T Tc S T Tc
N CNH
B
Duration of tachyarrhythmias (s)
400
300
200
100
0
S T Tc S T Tc
Normoxia Hypoxia
C
Incidence of ventricular fibrillation (%)
reversible
sustained
100
80
60
40
20
0
S T Tc S T Tc
Normoxia Hypoxia
D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

Figure 3:

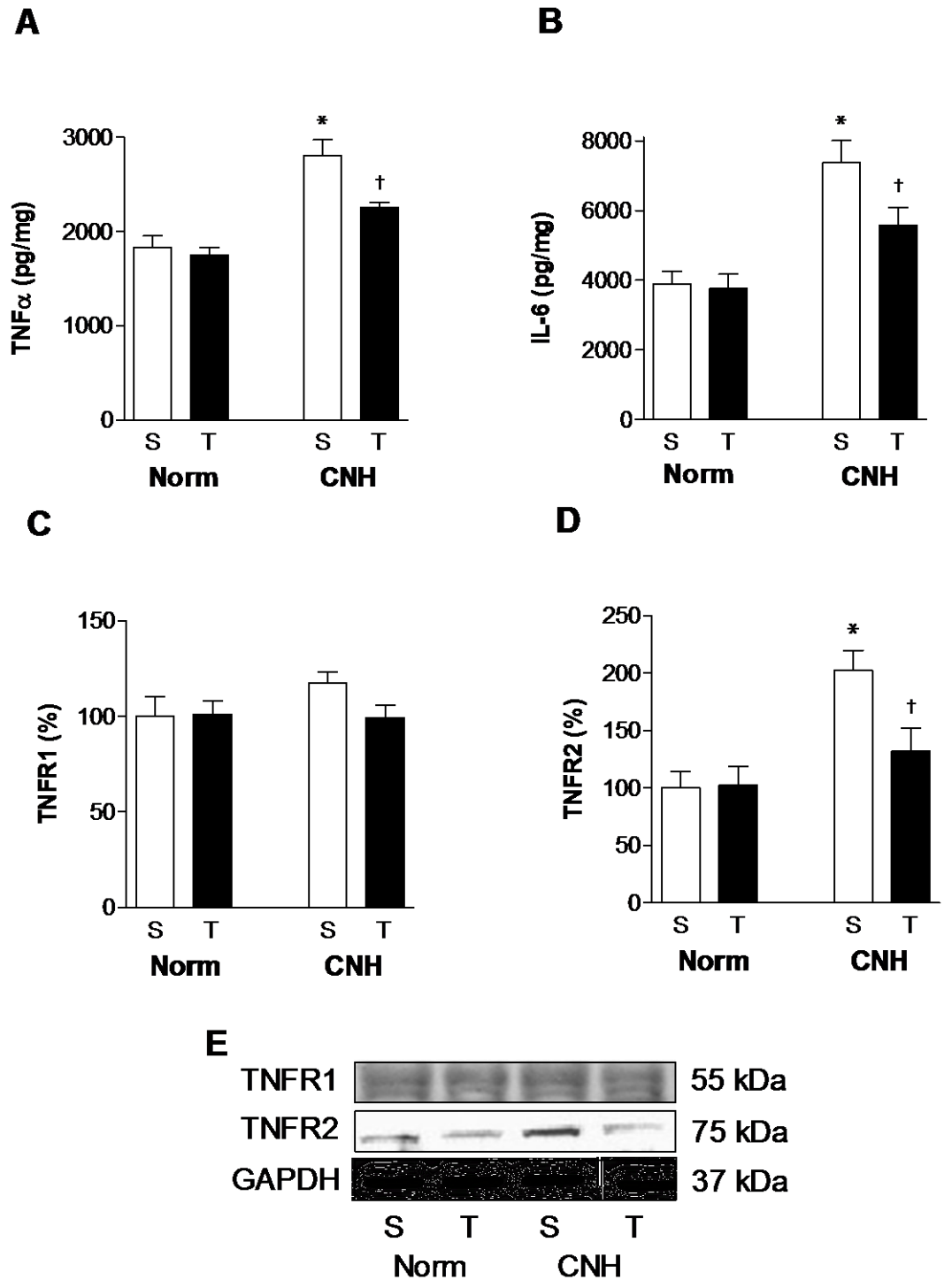

A
TNFα (pg/mg)
3000
2000
1000
0
*
†
S T S T
Norm CNH
B
IL-6 (pg/mg)
8000
6000
4000
2000
0
*
†
S T S T
Norm CNH
C
TNFR1 (%)
150
100
50
0
S T S T
Norm CNH
D
TNFR2 (%)
250
200
150
100
50
0
*
†
S T S T
Norm CNH
E
TNFR1
TNFR2
GAPDH
55 kDa
75 kDa
37 kDa
S T S T
Norm CNH

Figure 4:

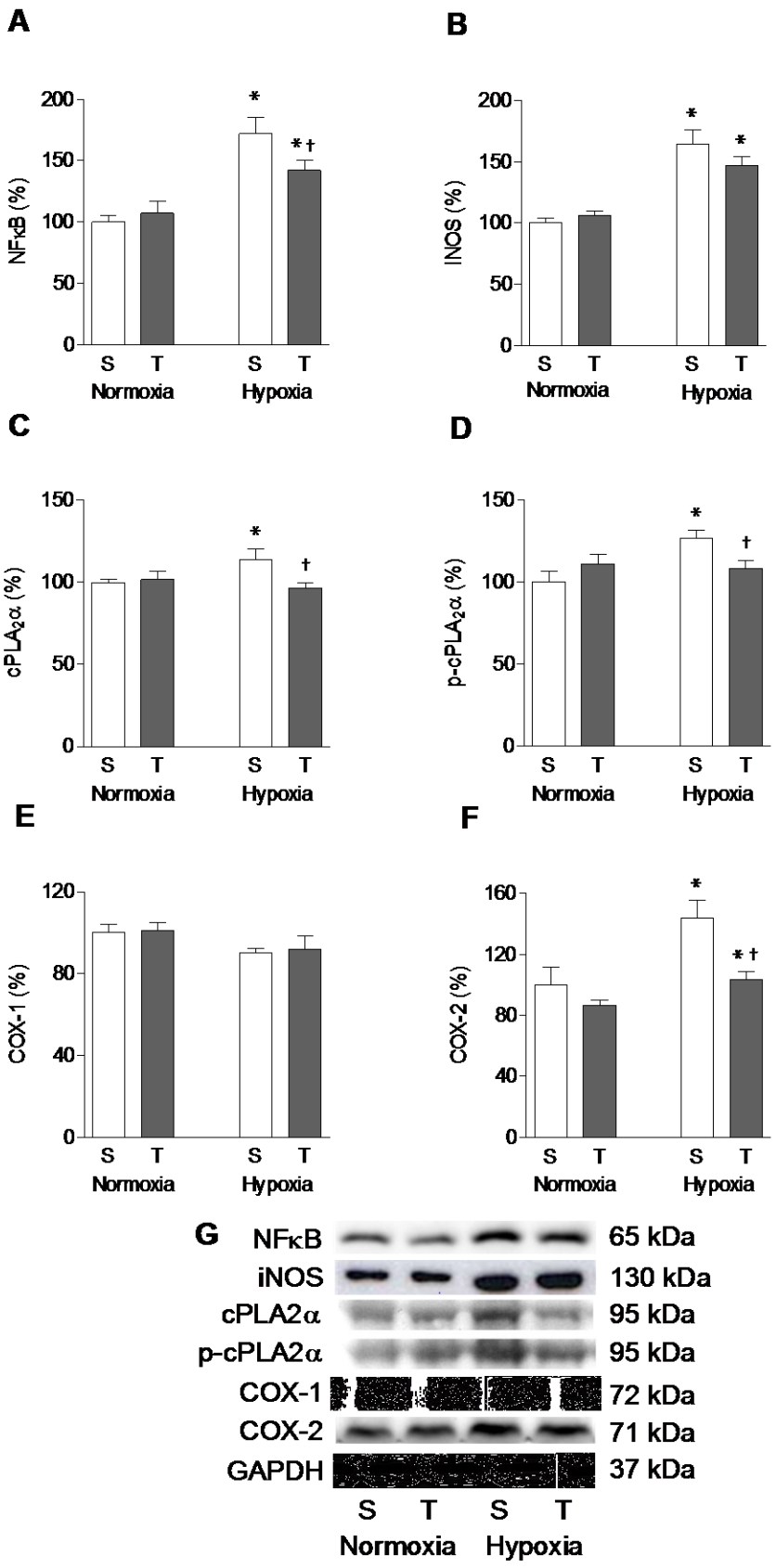
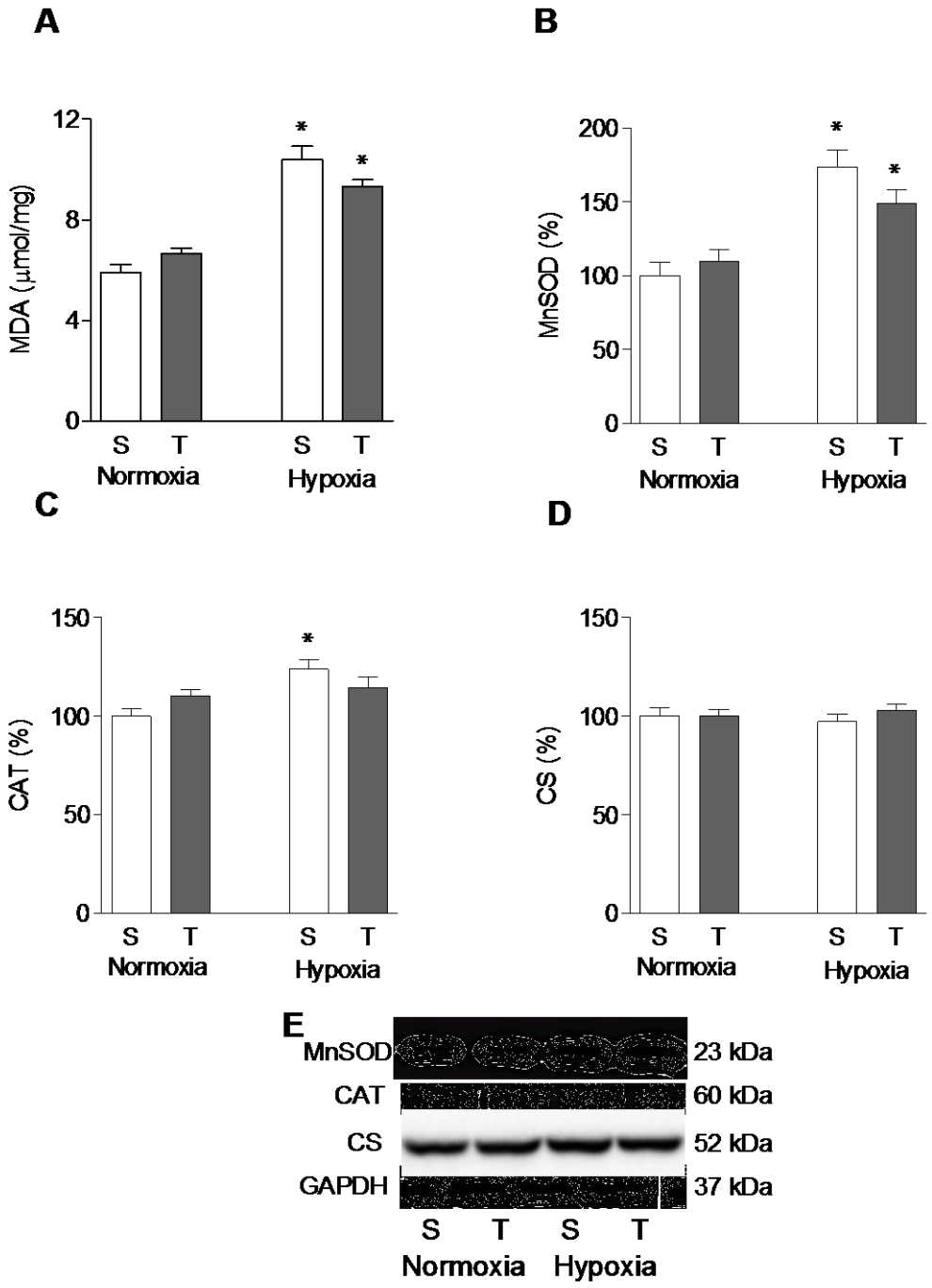

A
NFκB (%)
200
150
100
50
0
*
*†
S T S T
Normoxia Hypoxia
B
iNOS (%)
200
150
100
50
0
*
*
S T S T
Normoxia Hypoxia
C
cPLA2α (%)
150
100
50
0
*
†
S T S T
Normoxia Hypoxia
D
p-cPLA2α (%)
150
100
50
0
*
†
S T S T
Normoxia Hypoxia
E
COX-1 (%)
120
80
40
0
S T S T
Normoxia Hypoxia
F
COX-2 (%)
160
120
80
40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