High-intensity training reduces intermittent hypoxia-induced ER stress and myocardial infarct size

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Abstract

Background  Chronic intermittent hypoxia (IH) is described as the major detrimental factor leading to cardiovascular morbi-mortality in obstructive sleep apnea (OSA) patients. OSA patients exhibit an increased infarct size following a myocardial event and previous animal studies have shown that chronic IH could be the main mechanism. Endoplasmic reticulum (ER) stress plays a major role in the pathophysiology of cardiovascular disease. High-intensity training (HIT) exerts beneficial effects on the cardiovascular system. Thus, we hypothesized that HIT could prevent the IH-induced ER stress and increase in infarct size.

Methods  Wistar male rats were exposed to 21 days of IH (21–5% FiO₂, 60s cycle, 8 h/day) or normoxia (N). After one week of IH alone, rats were submitted daily to both IH and HIT (2*24min, 15 to 30m/min). Rat hearts were either rapidly frozen to evaluate ER stress by Western Blot or submitted to an ischemia-reperfusion protocol ex-vivo (30 min-global ischemia/120 min-reperfusion).

Results  IH induced a cardiac pro-apoptotic ER stress, characterized by increased expression of GRP78, pPERK, ATF4 and CHOP. IH-induced myocardial apoptosis was confirmed by the increased expression of cleaved caspase-3. These IH-associated pro-apoptotic alterations were associated with a significant increase in infarct size (35.4±3.2% vs 22.7±1.7% of ventricles, in IH/Sed and N/Sed respectively, p<0.05). HIT prevented both the IH-induced pro-apoptotic ER stress and the increased myocardial infarct size (28.8±3.9% and 21.0±5.1%, in IH/HIT and N/HIT groups respectively, p=0.28).

Conclusion  These findings suggest that HIT could represent a preventive strategy to limit IH-induced myocardial ischemia-reperfusion damages in OSA patients.

Keywords  Obstructive sleep apnea, intermittent hypoxia, ischemia-reperfusion, high-intensity aerobic training, endoplasmic reticulum stress
New & Noteworthy

We demonstrated that intermittent hypoxia induced a cardiac pro-apoptotic ER stress and an increased infarct size, which were prevented by high-intensity aerobic training. These results strengthen the need for early identification of patients with sleep apnea at risk for cardiovascular complications and suggest that exercise can be used as a new preventive strategy for these patients.
**Introduction**

Obstructive sleep apnea (OSA) syndrome is a common disease characterized by recurrent episodes of pharyngeal collapses occurring during sleep, leading to intermittent hypoxia (IH), sleep fragmentation and variations of intra-thoracic pressure. OSA is recognized as an independent risk factor for the development of cardiovascular disease, as it is independently associated with hypertension, atherosclerosis, cardiac rhythm disorders and coronary artery disease (3). Clinical and experimental studies suggest that chronic IH is the major stimulus leading to cardiovascular morbi-mortality in OSA patients (45). Recently, Buchner et al. demonstrated that OSA patients exhibit an increase in infarct size following an ischemic event compared to patients without OSA (9). Several animal studies have confirmed these clinical observations and demonstrated that IH induces elevation of mean arterial blood pressure and increases the myocardial infarct size following ischemia-reperfusion protocols (6, 35, 54, 57, 62).

The first-line therapy for OSA remains the application of continuous positive airway pressure (CPAP), which substantially decreases the number and severity of respiratory events in patients (3). However, as the effects of CPAP on blood pressure are limited (7, 55) and depend on the duration of CPAP use during the night (4), further large randomized clinical trials are needed to clarify the real impact of CPAP treatment to reduce cardiovascular risk. A better understanding of the pathophysiological mechanisms involved in the deleterious effects of IH is a major research priority and could help to provide new alternative or complementary treatment to CPAP in the management of OSA-associated cardiovascular risk.

The involvement of endoplasmic reticulum (ER) stress in the pathophysiology of cardiovascular diseases is well documented (47). ER is a multifunctional organelle that controls the synthesis, folding, assembly and transport of proteins. Oxidative stress, calcium dysregulation or ischemia can interfere with ER function, leading to accumulation of unfolded proteins to exceed its processing capacities. The resulting ER stress triggers the unfolded protein response (UPR) that induces signal transduction events to restore ER homeostasis (47). The glucose regulated protein kinase 78 (Grp78), an ER chaperone, dissociates from three distinct UPR sensors and activates specific pathways: (i) double-stranded RNA-activated protein kinase-like ER kinase (PERK) phosphorylation leads to translational inhibition through the transient phosphorylation of eukaryotic translation initiation factor-2α
(eIF2α); (ii) Inositol-requiring protein-1 (IRE1α), through the splicing of the mRNA encoding the transcriptional factor X-box binding protein 1 (XBP1) mRNA, induces expression of UPR-related genes that are known to fixe or degrade proteins; (iii) activating transcription factor-6 (ATF6) serves also as a preferential transcription activator (47). The three arms of the UPR coordinately regulate the transcription of UPR-related genes encoding ER chaperones and protein folding enzymes, to reduce the accumulation of unfolded proteins (31). However, during long term activation of the ER stress response, UPR fails to control the level of unfolded and misfolded proteins and ER-initiated pro-apoptotic signaling is induced (47). Indeed, PERK, through activating transcription factor-4 (ATF4) contributes to apoptotic cell death via activation of C/EBP HOmologous Protein (CHOP)(25) and caspase pathway (i.e. caspase-3) activation (51); and ATF6 branch is also involved in apoptotic cell death (50). Few studies have investigated the role of ER stress in the context of IH. ER stress has been shown to underlie neural injuries in animal model of IH (11) and two recent studies described a beneficial effect of both adiponectin (18) and metallothionein (70) on IH-induced myocardial ER stress and cell death. Therefore, we first hypothesized that IH induces a pro-apoptotic ER stress that could underlie cardiac damages following myocardial infarction.

Beneficial effects of aerobic exercise on cardiovascular health are well established (32). In animals, the most commonly used exercise model is running at a moderate intensity on a treadmill (i.e. 30-60 min of running at treadmill speeds of 15-20m/min, for at least 5 to 12 weeks) and this kind of exercise has been shown to reduce myocardial infarct size following ischemia-reperfusion (19, 20, 29). Shorter low intensity exercise protocols also attenuate post-ischemic myocardial injuries, as 1-3 consecutive days of exercise result in both enhanced post-ischemic recovery and reduced infarct size (17, 23, 24, 68). In addition to protocol duration, it seems that training intensity may also represent a crucial factor in exercise-induced cardioprotection (1, 44, 60) and a growing body of evidence from both human and animal studies suggest a greater effect of high-intensity aerobic training (HIT) compared to moderate intensity training on cardiovascular, muscle and metabolic adaptations (38, 49, 61, 64, 66). Among putative mechanisms that have been proposed to explain exercise-induced cardioprotection, several studies focused on the exercise-associated increase in myocardial antioxidant capacity (29, 56) and improvement of calcium homeostasis (20, 23, 24, 34, 65). More recently, it has been shown that exercise could also exert beneficial effects through a reduction of ER stress in several contexts such as Alzheimer’s disease (36), insulin resistance (14) and muscle apoptosis (39). To our knowledge, there is only one study reporting a
protective effect of exercise in the context of IH. The authors reported a beneficial effect of exercise on IH-induced myocardial oxidative stress and apoptosis (10). In the present study, we first tested whether HIT would exert greater effects on performance and mitochondrial adaptations than moderate-intensity training (MIT) and then we hypothesized that HIT would reduce IH-induced ER stress and associated post-ischemic myocardial damages.

As previously mentioned, the first line treatment of OSA is CPAP which appears to be insufficient to reduce cardiovascular risk in many patients. Therefore a combination of CPAP with other therapeutic strategies is a growing field of research. If our hypotheses are verified, targeting IH-induced ER stress by HIT could represent a new promising preventive strategy to limit IH-induced post-ischemic myocardial damages in OSA patients.
Methods

Animals
This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

Adult male Wistar rats (2 months old, 300-350g) were used for this study. They were obtained from Janvier labs (Le Genest-Saint-Isle, France). The study was approved by the University Grenoble Alpes Animal Research Ethic Committee (ComEth) (authorization number: 184_UHTA_U1042_CA_03). Rats were housed (n=4 per cage) at the animal care facility of the HP2 laboratory (approval: A38 516 10006), under a 12-hour light-dark cycle with 20-22°C and allowed free access to standard food and water.

Experimental design
A first set of experiments was achieved in order to validate the type of aerobic training. Rats were randomized to sedentary conditions (Sed), moderate-intensity (MIT) or high-intensity (HIT) aerobic training conditions. Exercise effects on muscle citrate synthase activity, maximal aerobic speed and endurance were then evaluated after 2 weeks of training (n=4-8 per condition).

Another group of rats were randomly assigned to normoxia (N) or intermittent hypoxia (IH) for a 21 day exposure. In both conditions, rats were assigned to sedentary (Sed) or HIT conditions, to constitute 4 groups: N/Sed, IH/Sed, N/HIT and IH/HIT. Training began after the first week of N or IH and lasted until the end of exposure. In each group, two sets of experiments were performed: the first set of rats was used to investigate the effect of IH and HIT on ER stress (n=6 per condition), and the second set was used to evaluate the effects of IH and HIT on myocardial infarct size (n=11-15 per condition). Arterial pressure was recorded in all animals (Figure 1).

Aerobic trainings
Training was carried out on a motorized treadmill (0% grade; Bioseb, Vitrolles, France). Rats were first accustomed to the treadmill running for 4 days during the first week of exposure (no more than 15 min per session). Then, MIT or HIT was performed for 10 days during the last 2 weeks of N or IH exposure (5 days per week).
Moderate-intensity aerobic training protocol
MIT was performed at a constant speed of 15m/min corresponding to 60% maximal aerobic speed (MAS) during 60 min. Twenty four hours after the last training session, rats were anesthetized with intraperitoneal (ip) injection of sodium pentobarbital (60mg/kg) and muscle samples were taken.

High-intensity aerobic training protocol
HIT consisted in 2 bouts of 24 min exercise, interspaced by 30 min recovery. The treadmill speed was set at 15m/min for the first step and corresponds to 6 min warm-up (50% MAS), followed by six steps of 3 min with increased intensity to reach 30m/min (corresponding to 65, 70, 75, 80, 85, 90% MAS). The highest intensity corresponded to approximately 90% MAS. Twenty four hours after the last training session, rats were anesthetized with intraperitoneal (ip) injection of sodium pentobarbital (60mg/kg) and muscle samples were taken.

Citrate Synthase Assay
Muscles devoid of connective tissue (5mg) were homogenized in extraction buffer [5mM HEPES, 1mM EGTA, 0.10% (v:v) Triton X-100] and incubated 60 min on ice. 1mM DTT was extemporaneously added. The assay mixture contained measurement buffer [1M Tris-HCl, 10mM 5,5’-dithiobis (2-nitrobenzoic acid), 10mM acetyl-Co, milli-Q water] and diluted sample (100µg of soluble proteins per milliliter of total assay). Sample absorbance at 412nm was monitored in 1.5mL cuvettes of spectrophotometer with thermostat 30°C (3 min). Citrate synthase activity was measured by addition of 10mM oxaloacetate during 3 min. Data are expressed in IU.g⁻¹ fresh weight.

Maximal aerobic speed and endurance
MAS can be used as an indicator of performance, owing to the linear relationship between oxygen consumption and exercise intensity (8, 49, 59). MAS was individually determined using an incremental exercise protocol at 0° inclination, consisting of a 6 minutes warm-up at 13m/min; then speed was increased by 3m/min every 2 minutes until rat was unable to run. MAS is the speed corresponding to the last entire stage completed by the animal.
Endurance is defined in this experiment as the running time to exhaustion at 75% MAS. After a 6 minute warm-up at 13-18m/min; speed was increased to reach an intensity of 75% MAS. Exercise was stopped when rats were no longer able to run.
**Intermittent hypoxia protocol**

Animals were exposed daily to 8 hours of intermittent hypoxia (IH) or normoxia (N) during their daytime sleep period, for 21 days. The IH stimulus was performed using a specifically designed device, as previously described (2). It consisted of 1-min cycles alternating 30s of hypoxia (5% inspired oxygen fraction (FiO₂)) and 30s of normoxia (21% FiO₂). FiO₂ was monitored throughout the experiment with a gas analyzer (ML206, ADInstruments, Oxford, United Kingdom). Normoxic rats were exposed to air streams to reproduce equivalent levels of noise and turbulences related to gas circulation than IH, without hypoxia. At the end of the exposure, rats were anesthetized with intraperitoneal injection of sodium pentobarbital (60 mg/kg). Arterial blood pressure (BP) was recorded, blood was collected and hearts were rapidly excised and either rapidly frozen (for western blot and qPCR analysis) or used for Langendorff technique.

**Arterial blood pressure measurement**

Temperature of anesthetized rats was maintained at 37°C, adjusted using a rectal probe connected to a thermal pad (Harvard apparatus, Les Ulis, France). Arterial BP was measured using an arterial carotid catheter linked to a mecanotransducer. Systolic BP, diastolic BP, mean BP and heart rate were recorded using the PowerLab data acquisition system (Powerlab, ADInstruments, Oxford, United Kingdom).

**Blood samples**

Following arterial blood pressure measurement, animals were heparinized (500U/kg). Blood was collected in capillary tubes and centrifuged (13000rpm/7min/21°C) to measure hematocrit.

Venous blood was also collected in inferior cava vein on EDTA and antioxidant solution (EGTA 950mg, glutathione 600mg, pH6-7, H₂O 10mL, 10µl/ml) and was rapidly centrifuged (13000rpm/10min/4°C) to collect plasma and perform catecholamine assay.

**Catecholamine assay**

Adrenaline and noradrenaline were measured in venous plasmas using the CatCombi ELISA kit (IBL international, Hamburg, Germany). Data are expressed in ng/ml.
Western-blot analysis

Frozen hearts were homogenized (Precellys 24, 6500rpm, 3X20s-5s; Bertin Technology, Montigny le Bretonneux, France) in order to extract total proteins (Sample lysis buffer: 5mM EDTA, 1mM Na<sub>3</sub>VO<sub>4</sub>, 20mM NaF, 1mM DTT, proteases inhibitor cocktail). Protein concentration was calculated using a Bradford assay (Bradford reagent, Sigma-Aldrich, Saint-Quentin Fallavier, France). Depending on proteins analyzed, 30 to 100µg of protein were separated by SDS polyacrylamide gels (8-12%) and transferred to polyvinylidene difluoride (PVDF) membranes. Next, membranes were blocked with 5% non-fat milk or bovine serum albumin (BSA) in Tris-buffered saline (TBS) with Tween 20 (0.1%). Then, membranes were incubated overnight at 4°C with primary antibodies in TBS-Tween 20-5% BSA or non-fat milk: phospho-eIF2α, eIF2α, ATF4 and cleaved caspase-3 (1/500, Cell Signaling Technology, Hitchin, United Kington); ATF6 (1/200, Santa Cruz Biotechnology, Heidelberg, Germany), phospho-PERK, PERK, CHOP and Grp78 (1/500, Santa Cruz Biotechnology, Heidelberg, Germany) and actin (1/2000, Sigma-Aldrich, St Quentin Fallavier, France). The following day, membranes were incubated for 1h at room temperature with horseradish peroxidase-conjugated appropriate anti-IgG (1/5000, Santa Cruz Biotechnology, Heidelberg, Germany). Enhanced chemiluminescence was performed with the Western-Blot ECL substrate (Clarity, Bio-Rad, Marnes-la Coquette, France) according to the manufacturer’s instructions and video acquisition (chemidoc-xrs-system, Bio-Rad, Marnes-la Coquette, France). The relative amount of protein was quantified by densitometry (Image Lab, Bio-Rad, Marnes-la Coquette, France) and expressed as the ratio of loading control. Phospho-proteins were expressed relative to total proteins and not phospho-proteins were expressed relative to actin. Finally, protein expressions of IH/Sed, N/HIT and IH/HIT were expressed relative to the N/Sed group normalized at 1.

Quantitative real-time RT-PCR analysis

Total RNA was isolated from whole heart by using the TRI reagent (Sigma-Aldrich, St Quentin Fallavier, France) according to the manufacture’s specifications. 0.5µg of total RNA was reversely transcribed to complementary DNA (cDNA) using iScript Reverse Transcription Supermix (C-1000 Thermal Cycler, Bio-Rad, Marnes-la Coquette, France). Real-time quantitative PCR was performed by using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Marnes-la Coquette, France) and PCR primers (Sigma-Aldrich, St Quentin Fallavier, France) for rat ER stress-related genes: spliced XBP1(XBP1(s)) (forward (GAGTCCGACGAGGTTG); reverse (GCGTCAGAATCCATGGGA)) and total XBP1
(XBP1(t)) (forward: GTGCAGGCCCAGTTGTCACC; reverse: TCTGGGTAGACCTCTGGGAG). Cycling parameters were as follows: 95°C, 30 seconds; 95°C, 5 seconds; 58°C, 10 seconds and increment 0.5°C for 5 seconds, from 65°C to 95°C. All PCR assays were performed in triplicate (CFX96 Touch RT PCR, Bio-Rad, Marnes-la Coquette, France). The PCR fluorescent signals for XBP1(s) and XBP1(t) were standardized to PCR fluorescent signals obtained from endogenous reference genes (actin and cyca) (CFX Manager 3.1, Biorad, Marnes-la Coquette, France). Comparative and relative quantification of XBP1(s) product were normalized to XBP1(t) and were calculated by the $2^{ΔΔCt}$ method.

**Ex-vivo ischemia-reperfusion protocol**

*Langendorff perfusion:* hearts of anaesthetized rats were rapidly excised and immediately immersed in 4°C Krebs-Henseleit (KH) buffer solution (in mM: NaCl 118, KCl 4.7, CaCl$_2$ 1.8, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, NaHCO$_3$ 25.2, and glucose 11mM). The aortic stump was cannulated, and the heart was perfused using the Langendorff technique at a constant pressure (75mmHg) with oxygenated KH buffer. Myocardial temperature was maintained at 37°C. A water-filled latex balloon, coupled with a pressure transducer, was inserted into the left ventricular cavity via the left atrium for pressure recording. Left ventricular end-diastolic pressure (LVEDP) was adjusted to about 7mmHg. After 20min of stabilization, a no-flow global and total ischemia was induced by stopping the perfusion for 30min. Thereafter, the heart was reperfused for 120min. Hemodynamic variables were continuously recorded (LVEDP, developed pressure (LVDP), cardiac contractility (dP/dt) and heart rate; Labchart, ADinstrument, Oxford, United Kingdom) (35).

*Infarct size determination:* at the end of ischemia-reperfusion protocol, atria were removed and the heart was frozen at -20°C for 10min. It was then cut into 2-mm transverse sections from apex to base (6–7 slices/heart). Once thawed, the slices were incubated at 37°C with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) for 20 min and fixed one day in 10% formaldehyde to clearly distinguish stained viable tissue from unstained unviable tissue. Infarct size was determined by a computerized planimetric technique (Image J software, NIH, USA) and expressed as a percentage of the ventricular size (35).

**Statistical analysis**

Statistics were performed using GraphPad Prism 6 Software (San Diego, California, USA). Data are expressed as mean ± SEM. Differences between groups over time were determined
by two-way ANOVA), with a subsequent Tukey’s post-hoc test for multiple group comparisons. A p value <0.05 was considered statistically significant.
Results

HIT versus MIT effects on performance and mitochondrial adaptations

We assessed metabolic and functional variables to determine which protocol (moderate-intensity aerobic endurance training (MIT) or high-intensity aerobic endurance training (HIT)) of equal volume (i.e. total energy expenditure although different intensity) promoted more beneficial outcomes after short-term training. Citrate synthase (CS) is a pacemaking enzyme of the Krebs cycle, used as an indicator of cellular aerobic metabolism (43). HIT significantly increased maximal CS activity in soleus muscle (56.7±4.1 vs 37.9±4.8U/l, in HIT and Sed groups respectively, p<0.05) (Figure 1A), whereas MIT had no significant effect. Functional capacity was assessed as maximal aerobic speed (MAS) and endurance at 75% MAS. HIT induced a greater increase in MAS than MIT (50.8±1.5 vs 35.7±3.2m/min, in HIT and PRE/HIT respectively, p<0.05, n=4; and 44.3±4.3 vs 34.4±2.6m/min in MIT and PRE/MIT respectively, p<0.15, n=4) (Figure 1B). Finally, endurance at 75% MAS tended to be higher in the HIT compared to the MIT group (69.3±8.6 vs 48.2±10.1minutes, in HIT and MIT respectively, p=0.08) (Figure 1C). Based on the greater short-term effect of HIT on performance (MAS, endurance) and mitochondrial adaptations (citrate synthase activity), we retained this protocol for the trained groups of the present study.

HIT prevents the IH-induced cardiac pro-apoptotic ER stress

Chronic IH triggered a cardiac ER stress, characterized by a significant enhancement of the ER chaperone Grp78 expression (2.6±0.6 fold increase, p<0.01) (Figure 3A) and an increased phosphorylation of PERK (1.7±0.2 fold increase, p<0.05) (Figure 3B) and eIF2α (1.5±0.1 fold increase, p=0.058) (Figure 3C). Whereas ATF6 expression and XBP1 mRNA splicing were not increased by IH (Figures 3E and 3F), IH also induced the ER-initiated pro-apoptotic signaling with a significant raise in ATF4 expression (1.6±0.2 fold increase, p<0.05) (Figure 3D) and a trend to enhanced CHOP expression (1.5±0.4 fold increase) (Figure 3G), which were associated with an increased expression of cleaved caspase-3 (3.4±1.3 fold increase, p<0.05) (Figure 3H).

HIT prevented this IH-induced pro-apoptotic ER stress, as shown by the absence of significant increase in GRP78 expression, PERK and eIF2α phosphorylation and pro-apoptotic ATF4 and CHOP expression in the IH/HIT group. ATF6 expression was significantly reduced and splicing of XBP1 mRNA was not modified. Finally, HIT also prevented the IH-induced increase in cleaved caspase-3 (Figure 3).
In the normoxic group, HIT alone induced a slight ER stress, as shown by the significant increase in Grp78 expression, compared to N/Sed (2.3±0.3 fold increase, p<0.05) (Figure 3A). However, this HIT-induced ER stress was different from those induced by IH, as HIT did not activate the UPR (i.e. no significant effect of HIT on PERK and eIF2α phosphorylation, on ATF4, CHOP and ATF6 expression and XBP1 mRNA splicing), nor the cleaved caspase-3 (Figure 3).

**HIT prevents the IH-induced increase in blood pressure and infarct size**

IH was associated with a significant increase in heart rate (Figure 3A) and mean BP (134±3.8 vs 119±3.6mmHg, p<0.05) (Figure 3B). This IH-induced elevation of mean BP was demonstrated by significant increases in both systolic and diastolic BP (systolic BP: 155±5.1 vs 138±3.6mmHg, and diastolic BP: 123±3.3 vs 109±3.7mmHg, in IH/Sed and N/Sed groups respectively, p<0.05) and was associated with a trend to increase in sympathetic activity that was assessed indirectly through measurement of plasma catecholamine levels. In sedentary animals, noradrenaline concentrations tended to be greater in hypoxic rats (27.7±5.4 vs 13.3±1.8ng/ml, in IH/Sed and N/Sed groups respectively, p=0.08) (Figure 3C). HIT prevented both the IH-induced increases in heart rate and blood pressures (mean BP: 122±5.0 and 113±4.3mmHg, systolic BP: 145±6.0 and 135±5.1mmHg and diastolic BP: 111±5.2 and 103±4.1mmHg, IH/HIT and N/HIT groups respectively) (Figure 3) and the IH-associated increased plasma noradrenaline levels (17.5±5.9 and 11.96±2.7ng/ml, in IH/HIT and N/HIT respectively) (Figure 3C). Finally, in sedentary conditions, infarct size was significantly higher in hypoxic than in normoxic group (34.5±2.9% vs 22.7±1.7%, in IH/sed and N/sed groups respectively, p<0.05). This IH-induced increase in infarct size was prevented by HIT (28.8±3.9% and 21.0±5.1%, in IH/HIT and N/HIT groups respectively), whereas HIT alone had no effect in normoxic animals (Figure 4). Hemodynamic Langendorff variables were not different between the 4 groups at any time of the experiment (data not shown Figure S1).
Discussion
In this study, we demonstrated that chronic exposure to intermittent hypoxia induces a sustained cardiac pro-apoptotic ER stress, an elevation of arterial blood pressure and an increased myocardial infarct size following ischemia-reperfusion, which were all prevented by short-term, high-intensity aerobic training. Therefore, to limit myocardial damage following an ischemic event, early diagnosis and treatment of sleep apnea is particularly relevant. Furthermore, specific aerobic training might represent a potent preventive strategy to improve prognosis in OSA patients presenting with myocardial infarction.

Duality of the IH stimulus
It is well known that hypoxia is an ambivalent stimulus that can exert both beneficial and detrimental effects, and in particular in the myocardium. We previously demonstrated that the duration, the depth and the pattern of the hypoxic stimulus is crucial to determine whether the effect is protective or detrimental. On one hand, we showed that 4 hours of IH (1-min cycle, 10% FiO₂) reduced myocardial infarct size in rats, whereas 30 min of IH or 4 hours of continuous hypoxia at 10% FiO₂ did not (5), suggesting that myocardial preconditioning can be achieved with a very specific protocol of acute IH. On the other hand, a more severe IH stimulus (FiO₂ 5%) is detrimental for the myocardium (i.e. increases myocardial infarct size), when applied either in an acute (4 hours) or chronic (several weeks) protocol (5, 35, 54). The specific IH stimulus used in the present study (1-min cycle, 5% FiO₂, for several weeks) is now commonly used to emulate OSA-like IH (16).

Chronic exposure to IH promotes a cardiac pro-apoptotic ER stress
In the present study, we have shown that chronic exposure to IH induces a sustained cardiac ER stress. This ER stress was characterized by the significant up-regulation of the chaperones Grp78, increased phosphorylation of both PERK and eIF2α and a significant increase in ATF4 expression and a trend to increase in ATF6 and CHOP expressions. Finally, this IH-induced ER stress was associated with myocardial apoptosis, as we further demonstrated an increased expression of cleaved caspase-3 in rats exposed to IH. These results are consistent with recent studies in rats and mice, demonstrating that chronic IH induces a pro-apoptotic ER stress in both myocardium (18, 70) and neurons (11, 71). Furthermore, the link between ER stress and apoptosis is now well described. In particular, CHOP seems to be the main ER actor involved in apoptosis regulation, as it reduces the expression of the anti-apoptotic factor Bcl2 (46) and upregulates the pro-apoptotic factor BIM (69), subsequently activating caspase
signaling. Supporting this, CHOP deficiency is protective against ER stress-induced apoptosis and myocardial dysfunction (52). In our model, we cannot exclude that IH could directly induce apoptosis independently of ER stress, through the classical extrinsic or intrinsic mitochondrial apoptotic pathways. In fact, sympathetic activation and oxidative stress, both activated by IH, can also induce myocardial apoptosis, subsequently to ER stress activation or in an ER stress-independent manner (13, 15, 33, 58, 63).

**IH-induced increase in blood pressure and infarct size: potential role of ER stress**

We confirmed results of previous studies indicating that IH was associated with a chronic sympathetic activation (for a review, see (21)) and an increase in arterial blood pressure (6, 35, 57, 62). As ER stress inhibitors have been shown to prevent both angiotensin-2-induced systemic hypertension (37) and hypoxia-induced pulmonary arterial hypertension (42) in mice, our data suggest that prolonged ER stress induced by IH could contribute to the IH-induced elevation of arterial blood pressure. We further confirmed that chronic exposure to IH was associated with an increased response to myocardial ischemia-reperfusion (i.e. increased infarct size), which is consistent with previous studies in both rodents (6, 35, 54, 57, 62) and humans (9). This result is particularly relevant as it suggests that a subject previously exposed to chronic IH will have a poor prognosis following an acute myocardial event. This raises the need to better understand mechanisms that could be activated by chronic IH and involved in the IH-increased infarct size following myocardial ischemia-reperfusion, in order to identify and treat at-risk patients. Again, ER stress could represent a good intermediate mechanism as its role in the pathophysiology of cardiovascular diseases is well documented (26, 47). CHOP-deficient mice failed to increase infarct size following myocardial ischemia-reperfusion (48), and, in the context of chronic IH, previous studies have demonstrated the role of IH-induced ER stress on myocardial apoptosis and subsequent contractile dysfunction (18, 70). The results of the present study support this and suggest that the IH-induced pro-apoptotic ER stress could explain the IH-associated increased in myocardial infarction.

**HIT prevents IH-associated myocardial ER stress and increased infarct size**

Interestingly, we further demonstrated that high intensity training inhibits the IH-induced ER stress and also prevents the IH-induced increase in arterial blood pressure and infarct size. The beneficial effect of HIT in decreasing pro-apoptotic ER stress has been previously described in the skeletal muscle of rats and was associated with a decrease in fasting plasmatic glucose and insulin (39). Exercise training has also been shown to reduce ER stress-
induced apoptosis in Alzheimer’s disease in mice (36). As previously mentioned, a pro-apoptotic ER stress has been correlated with myocardial damages in several pathophysiological conditions, such as ischemia-reperfusion, myocardial infarction and heart failure (47). Therefore, in our study, the normalization of myocardial ER stress by HIT could explain the limitation of IH-induced increase in infarct size. Accordingly, two studies have recently demonstrated that other cardioprotective interventions (i.e. metallothionein and adiponectin) were also efficient against IH-induced ER stress and associated cardiomyocyte death (18, 70), independently of any ischemia-reperfusion protocol.

**High-intensity aerobic training activates an adaptive ER stress**

We chose high-intensity aerobic training as a preventive strategy to limit both IH-induced myocardial ER stress and increased infarct size, as only 2 weeks has the potential to exert beneficial effects on performance and mitochondrial adaptations. Interestingly, in the normoxic group, HIT induced a slight ER stress, different from the stress induced by IH. These data suggest that HIT may activate the adaptive pathway of UPR, which maintains ER homeostasis upon luminal stress, and allows myocardium adaptations to exercise. In accordance, exercise-induced ER stress has been well documented in different organs, such as skeletal muscles (67), liver (27) and brain (40), and is considered as a pro-survival, and not a pro-apoptotic, response of the UPR. However, in our study, HIT-induced ER stress was not associated with any beneficial or deleterious effect on cardiovascular parameters (i.e. arterial blood pressure and infarct size).

**HIT as a preventive strategy against OSA-related cardiovascular consequences**

Exercise is described as an efficient strategy for the primary and secondary prevention of cardiovascular disease. It is associated with a decrease in cardiovascular mortality, through a lowering of resting heart rate, improved vascular endothelial function, increased vasculogenesis and several metabolic adaptations of the myocardium which result in improved tolerance to ischemia-reperfusion. Indeed, the ability of exercise to protect the heart against ischemia-reperfusion injuries in both human and animal models is well documented (22). Although it is generally admitted that moderate-intensity training is sufficient to reduce the risk and recurrence of cardiovascular diseases, beneficial effects of exercise may vary according to its intensity and duration (22). Moreover, high-intensity exercise brings greater short-term cardiovascular effects than moderate-intensity training (28). In the context of a 21-day exposure to IH, current animal models of long-term moderate aerobic training were not
suitable. Thus, we opted for an efficient short-term intense protocol aimed at improving cardiovascular variables and that could be applied together with IH exposure. We demonstrated that only 10 sessions of HIT elicited greater effects than traditional moderate-intensity training on performance (i.e. maximal aerobic speed and endurance), muscle adaptation (i.e. citrate synthase activity) and was also associated with improvement of cardiovascular parameters (i.e. arterial blood pressure and infarct size). These results are consistent with previous studies demonstrating that HIT can decrease sympathetic activation and arterial blood pressure in humans (12, 53). Concerning animal studies, HIT has been shown to decrease arterial blood pressure, preserve endothelial function (30), and improve myocardial contractile function both in basal conditions (38) and in a model of post-infarction heart failure (34, 49) in rats.

Conclusion

High-intensity training prevents the IH-dependent increase in myocardial infarction following ischemia-reperfusion, possibly through a down-regulation of the pro-apoptotic ER stress pathway (Figure 6). Thus, our results suggest that high-intensity training could represent a very promising preventive strategy to limit IH-induced myocardial ischemia reperfusion-related damages in OSA patients and improve their prognosis. The severity of sleep apnea can also be reduced by exercise only (41), therefore rehabilitation programs should be implemented and evaluated in OSA patients presenting with high cardiovascular risk.
References


Figure legends

Figure 1: Experimental design. Wistar rats were randomly submitted to normoxia (N) or intermittent hypoxia (IH) during 21 days and they were subjected to 2 different conditions, sedentary (Sed) or high-intensity interval training (HIT) during the last 10 days of exposure. Thus, 4 subgroups of animals were used in the present study: N/Sed, N/HIT, IH/Sed and IH/HIT. Two sets of measurement were realized to evaluate the infarct size and investigate the involvement of ER stress for each condition.

Figure 2. High-intensity aerobic training effects. A) Maximal activity of mitochondrial enzyme citrate synthase, B) maximal aerobic speed (MAS) and C) post-training endurance at 75% MAS in sedentary rats (Sed), traditional moderate-intensity training (MIT) or high-intensity training (HIT) conditions; before (PRE) and after (POST) endurance trainings (n=4-8 per conditions). Data are means ± SEM. *p < 0.05 vs Sed; $p < 0.05 vs PRE/HIT.

Figure 3. High-intensity aerobic training prevents the IH-induced cardiac endoplasmic reticulum stress. (A) Grp78 relative to actin, (B) phospho-PERK relative to PERK, (C) phospho-eIF2α relative to eIF2α, (D) ATF4 relative to actin, (E) ATF6 relative to actin, (F) XBP1(s) mRNA relative to XBP1(t) mRNA, (G) CHOP relative to actin and (H) Cleaved caspase-3 relative to actin, in animals exposed for 21 days to either intermittent hypoxia (IH) or normoxia (N) and submitted to sedentary (Sed) or high-intensity training (HIT) conditions (n=6 in each group). Data are means ± SEM, **p<0.01 vs N/Sed; * p<0.05 vs N/Sed; #p<0.05 vs IH/Sed; §p<0.05 vs N/HIT.

Figure 4. High-intensity aerobic training prevents the IH-induced increase in heart rate and blood pressure. (A) Heart rate (bpm), (B) mean arterial blood pressure (BP), (C) Plasma noradrenaline and (D) adrenaline concentrations, in animals exposed for 21 days to either intermittent hypoxia (IH) or normoxia (N) and submitted to sedentary (Sed) or high-intensity training (HIT) conditions (n=12-22 rats per group). Data are means ± SEM,*p<0.05 vs N/Sed.

Figure 5. High-intensity aerobic training prevents the IH-induced increase in infarct size. (A) Representative images of myocardial infarct expansion. (B) Infarct size expressed as a percentage of ventricles size, in animals exposed for 21 days to either intermittent hypoxia...
(IH) or normoxia (N) and subjected to sedentary (Sed) or high-intensity training (HIT) conditions (n=11-15 rats per group). Data are means ± SEM, *p<0.05 vs N/Sed.

Figure 6. IH-induced myocardial pro-apoptotic ER stress. A) Several stimuli (i.e. oxidative stress, calcium perturbations, ischemia…) can induce an ER stress. The generation of unfolded proteins induces Grp78 release from IRE1, ATF6 and PERK and activation of the 3 pathways of the unfolded protein response (UPR). Upon intense stresses, UPR cannot control the level of unfolded proteins and ER initiates pro-apoptotic pathways, leading to the upregulation of CHOP and activation of caspase pathway. Chronic intermittent hypoxia (IH) also induces a pro-apoptotic ER stress, characterized by the activation of the PERK/ATF4/CHOP pathway and the expression of cleaved caspase-3. →: Common ER stress response; ➔: IH-induced ER stress and apoptosis. B) Through inhibition of IH-induced pro-apoptotic ER stress, short-term high-intensity training (HIT) prevented myocardial apoptosis and therefore may prevent IH-induced cardiovascular alterations.
### Supplemental Data

#### Table S1: Hemodynamic variables in Langendorff rat hearts.**

Heart rate, coronary flow, left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDP) and cardiac contractility (dP/dt), determined after 20 minutes stabulation (Stab), 30 minutes reperfusion (R30), 60 minutes reperfusion (R60) and 120 minutes reperfusion (R120) in animals exposed for 21 days to either intermittent hypoxia (IH) or normoxia (N) and submitted to sedentary (Sed) or high-intensity training (HIT) conditions (n=11-15 rats per group). Data are means ± SEM,*p<0.05 vs N/Sed and N/HIT.

<table>
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<tr>
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<th>Stab</th>
<th>R30</th>
<th>R60</th>
<th>R120</th>
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<tr>
<td><strong>Heart rate (bpm)</strong></td>
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<tr>
<td>N/Sed</td>
<td>273 ± 9</td>
<td>175 ± 22</td>
<td>173 ± 48</td>
<td>181 ± 26</td>
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<tr>
<td>IH/Sed</td>
<td>273 ± 10</td>
<td>150 ± 28</td>
<td>198 ± 17</td>
<td>204 ± 20</td>
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<tr>
<td>N/HIT</td>
<td>272 ± 8</td>
<td>162 ± 17</td>
<td>221 ± 34</td>
<td>192 ± 12</td>
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<tr>
<td>IH/HIT</td>
<td>256 ± 8</td>
<td>201 ± 21</td>
<td>199 ± 14</td>
<td>193 ± 15</td>
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<td><strong>Coronary flow (ml/min)</strong></td>
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<td>N/Sed</td>
<td>14.7 ± 1.0</td>
<td>5.2 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>3.2 ± 0.2</td>
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<td>IH/Sed</td>
<td>16.3 ± 1.2</td>
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<td>N/HIT</td>
<td>14.4 ± 0.6</td>
<td>5.3 ± 0.9</td>
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<td>IH/HIT</td>
<td>14.3 ± 1.3</td>
<td>6.6 ± 0.9</td>
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<td><strong>LVEDP (mmHg)</strong></td>
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<td>IH/Sed</td>
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<td>N/HIT</td>
<td>7.0 ± 0.9</td>
<td>66.3 ± 2.7</td>
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<td>IH/HIT</td>
<td>6.9 ± 1.1</td>
<td>69.1 ± 4.9</td>
<td>56.6 ± 4.3</td>
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<td><strong>LVDP (mmHg)</strong></td>
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<td>IH/Sed</td>
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<td>N/HIT</td>
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<td><strong>dP/dtmin (mmHg/min)</strong></td>
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<td>−431 ± 69</td>
<td>−602 ± 78</td>
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<td>785 ± 115</td>
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<tr>
<td>IH/Sed</td>
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<td>IH/HIT</td>
<td>2939 ± 303*</td>
<td>567 ± 110</td>
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* p<0.05 vs N/Sed and N/HIT.
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- Blood pressure measurement
- Infarct size assessment or ER-stress evaluation

n=21
n=20
n=17
n=21
Figure 2

A. CS activity (U/g·f fresh weight)

B. Maximal aerobic speed (m/min)

C. Endurance at 75% MAS (min)
Figure 3

A) Grp78/Actin

B) pPERK/PERK

C) peIF2a/peIF2a

D) ATF4/Actin

E) ATF6/Actin

F) XBP1(s) (fold increase)

G) CHOP/Actin

H) c-Casp-3/Actin
Figure 4

A

Heart rate (bpm)

B

Mean BP (mmHg)

C

Plasma noradrenaline (ng/ml)

D

Plasma adrenaline (ng/ml)

* p=0.08
Figure 5

A

N/Sed  IH/Sed  N/HIT  IH/HIT

B

Infarct size (% of ventricles)

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* Indicates significant difference.
Figure 6

**A**

Homeostasis perturbations (oxydative stress, calcium dysregulation, ...)

Unfolded proteins

Grp78

Grp78

IRE1

Cleaved ATF6

Spliced XBP1

Grp78

ATF6

Grp78

PERK

P

P

elf2α

ATF4

Cleaved caspase 3↑

CHOP

APOPTOSIS

**B**

IH

ER stress

- Increased blood pressure
- Myocardial apoptosis
- Increased myocardial ischemia-reperfusion injuries

HIT

HIT