Università degli Studi di Cagliari

PHD DEGREE

Innovation Sciences and Technologies

Cycle XXXII

TITLE OF THE PHD THESIS

THE EFFECT OF NORMOBARIC HYPOXIA AND METABOREFLEX IN THE CARDIOVASCULAR ADJUSTMENTS TO EXERCISE

Scientific Disciplinary Sector(s)

BIO/09

PhD Student: GABRIELE MULLIRI

Coordinator of the PhD Programme PROF. ROBERTO ORRU'

Supervisor PROF. ANTONIO CRISAFULLI

Final exam. Academic Year 2018 – 2019

Thesis defence: January-February 2020 Session

INDEX

INTRODUCTION

Hypoxia can be defined as a condition in which the body, or a part of it, is deprived of adequate oxygen supply.

Human beings can experience hypoxia as a result of insufficient ventilation (such as during apnoea, acute or chronic respiratory failure, breathing low-oxygen content air – for example at high altitude), insufficient oxygen-binding/oxygen-carrying capacity of blood (such as during anaemia and carbon monoxide or cyanuric acid poisoning), or insufficient blood flow to peripheral tissues (such as in heart failure, shock, and arterial occlusion).

In physiological conditions, hypoxia is experienced by ascending at high altitudes. At sea level, air pressure (P_{atm}) is 760 mmHg with a fixed percentage distribution of gases [oxygen (O_2) 20.93%, carbon dioxide (CO_2) 0.03%, and Nitrogen (N_2) 79.04%]. At higher altitudes, air pressure decreases and reaches 380 mmHg at 5840 mt and 250 mmHg on the summit of Mount Everest (8878mt), but the percentage distribution of gases does not change. According to Dalton's Law, this fact implies a reduction in the partial pressure of O_2 from 159 mmHg at sea level (i.e. 20.93% of 760 mmHg), to 79 mmHg at 5840 m (i.e. 20.93% of 380 mmHg), and 52 mmHg at the top of Mount Everest (i.e. 20.93% of 250 mmHg). Moreover, as we breathe the inspired air is moistened by the upper airways with water vapor at a pressure (P_{H2O}) of 47 mmHg that doesn't change at high altitudes.

Considering that the partial pressure of CO_2 inside the alveoli ($P_{alv}CO_2$) is around 40 mmHg and that in physiologic conditions it depends on ventilation and CO_2 production regardless of the altitude, the partial pressure of O_2 at the alveolar level ($P_{alv}O_2$) can be indirectly calculated from the following alveolar gas equation:

$$P_{\text{alv}}O_2 = \text{Fi}O_2 x\left(P_{\text{atm}} - P_{H2o}\right) - P_{\text{alv}}\text{CO}_2/\text{RQ}$$

where FiO_2 is the fraction of O_2 in inspired air and RQ is the Respiratory Quotient (Cruickshank et al. 2004; Sharma et al. 2019).

According to this equation, $P_{alv}O_2$ is equal to 99 mmHg at sea level. Considering that the partial pressure of O_2 in pulmonary capillaries is equal to 40 mmHg, there is a 60 mmHg gradient that produces a shift of O_2 from the alveoli to the capillary bed, thereby allowing blood oxygenation. Starting from these considerations, it's easy to calculate $P_{alv}O_2$ at 5840 mt and at the top of Mount Everest, which is 19.69 mmHg and -7.51 mmHg respectively. These levels of hypoxia are expected to impair the oxygen exchange between blood and alveolar air and rapidly lead to death.

Fortunately, when the human body undergoes acute or chronic hypoxia, it responds with a series of adjustments and adaptations both at the cellular and systemic levels. These mechanisms allowed

Reinold Meissner to reach the peak of Mount Everest (8878 mt) in 1978 without oxygen supplementation.

Cellular response to hypoxia.

Hypoxia is a very challenging condition for cellular metabolism because it impairs oxidative phosphorilation, the main metabolic pathway that synthesizes adenosine triphosphate (ATP) utilizing O_2. A low ATP concentration leads to a reduced Na+/K+ ATPase activity, impeding the homeostatic control of cellular membrane potential. Partial depolarization opens voltage-gated Ca^{2+} channels, increasing the net influx of this ion that activates the apoptotic enzymatic cascade leading to cellular death (Michiels 2004).

Cells respond to this condition in different ways. One important mechanism is the inhibition of the most ATP consuming activities, like RNA/DNA and protein synthesis, prioritizing Na^{+}/K^{+} ATPase activity and Ca^{2+} homeostasis (Buttergeit et al. 1995). Another fundamental adaptation is the enhancement of anaerobic glycolysis by Phosphofructokinase-1 (PFK-1) activation, a key enzyme of this energetic pathway. PFK-1 is allosterically activated by adenosine monophosphate (AMP), adenosine diphosphate (ADP), and fructose 2,6 biphosphate. In particular, fructose 2,6 biphosphate is synthesized by Phosphofructokinase-2 (PFK-2) which is mainly regulated by AMP kinase (AMPK) through phosphorylation (Hue et al. 1987; Marsin et al. 2000). AMPK can be considered the most important cellular energy sensor because it can activate catabolic pathways that produce ATP and inhibit anabolic ATP-consuming pathways (Hardie and Hawley 2001; Hardie 2001; Kemp et al. 1999). These effects are achieved by phosphorylation of enzymes directly involved in these metabolic reactions or regulating gene expression through phosphorylation of transcription factors. For example, AMPK can increase the translocation of Glut-4 (the insulin-mediated glucose transporter) to the cellular membrane, increasing glucose inflow into the cell. Moreover, it inhibits fatty acid, triglycerides and sterol synthesis and reduces the expression of genes that code for enzymes of gluconeogenesis and fatty acid synthesis (Hardie and Hawley 2001). These actions make AMPK a key regulator of cell metabolism in low energy conditions like hypoxia.

Another fundamental actor in cellular homeostasis under hypoxia is the Hypoxia Inducible Factor 1 (HIF-1), a transcription factor that promotes the expression of genes involved in oxygen delivery and anaerobic metabolism, exerting its effect on cellular as well as on systemic level (Lundby 2013). HIF-1 is a heterodimer formed by two different proteins, HIF-1α and HIF-1β, which are constitutively expressed in human cells. Instead of being in a stable form as HIF-1β, HIF-1α has a brief half-life of 5-8 minutes in normoxic conditions, thanks to continuous hydroxylation by two different enzymes, PHD and FIH-1 which use oxygen as the substrate of their activity. Hydroxylated HIF-1α is degraded by the ubiquitin-proteasome system and prevented from binding

with HIF-1β.

In hypoxia, PHD and FIH-1 lower their hydroxylation activity and HIF-1α can dimerize with HIF-1β, enters the cellular nucleus and works as a transcription factor, interacting with the transcriptional co-activators P300 and CBP. This heterodimer can bind to Hypoxia Response Elements (HRE) at target gene loci thereby promoting the expression of numerous genes involved in the cellular and systemic response to hypoxia (Koyasu et al 2018, Sousa et al 2019).

HIF can increase the expression of enzymes involved in the anaerobic glycolytic pathway [such as phosphoglycerate kinase,(PKG) and lactate dehydrogenase A (LDHA)], in glucose transport from plasma to intracellular space (such as GLUT1), in neoangiogenesis [(such as the Vascular Endothelial Growth Factor (VEGF)] (Hu et al. 2007), and in new erythrocytes production, [such as erythropoietin (EPO)]. (Lundby 2013)

The level of HIF-1α starts to rise rapidly and peaks after a few hours of hypoxia, progressively declining towards basal levels in the next hours-days (Lundby 2009). This behavior is probably related to acclimatization that stops the hypoxic stimulus at the cellular level, decreasing HIF-1α stabilization (Lundby 2009).

Local Vascular tone regulation and hypoxia.

The delivery of oxygen to peripheral tissues depends on blood flow and blood oxygen content. As blood oxygen content lowers during hypoxia, the peripheral arteries vasodilate to increase local blood flow. In contrast, pulmonary arteries vasoconstrict in response to hypoxia, representing the only exception to this mechanism as we'll explain later.

Hypoxia can produce vasodilation thanks to direct and indirect-paracrine mechanisms. The main direct effects are potassium ATP-sensitive membrane channels (K-ATP) opening and endothelial Nitric Oxide Synthase (eNOS) activation. K-ATP is highly represented on smooth muscle cells membrane and opens in response to low ATP intracellular concentrations, as happens in hypoxic conditions. The increase of K^+ outflow hyperpolarizes the cell membrane, closing the Ca^{2+} voltage-gated membrane channels, preventing Ca^{2+} from entering into the intracellular space and ultimately reducing smooth muscle contraction (Dart et al. 1995). eNOS synthesizes Nitric Oxide (NO) from oxygen and L-arginine and gives L-citrulline as a byproduct. NO diffuses to near vascular smooth muscle cells and increases intracellular production of cyclic guanosine monophosphate (cGMP) (Pearce at al. 1990) and this in turn activates several protein kinases involved in Ca^{2+} homeostasis and K^+ conductance, thereby inhibiting smooth muscle contraction (Surks et al. 1999; Bolotina et al. 1994). The indirect-paracrine mechanism is mediated by erythrocytes and involves different paracrine molecules like NO, prostaglandins (in particular PGI_2 and PGE_2), adenosine and Epoxyeicosatrienoic acids (EET). (Hoiland et al. 2016). In fact, erythrocytes can generate NO from

circulating nitrites (NO_2^-) and deoxyhemoglobin, which acts as nitrite reductase in low-oxygen conditions (Kim-Shapiro et al. 2006). Moreover, hemoglobin can bind to S-Nitrosothiol (SNO) in the lungs and form a complex called S-nitrosohemoglobin, which can work as a NO donor in the peripheral tissues inducing vasodilation (Jia et al. 1996). Another important erythrocyte-mediated mechanism is ATP release induced by hypoxia (Bergfeld et al. 1992; Ellsworth et al. 1995; Ellsworth et al. 2009). ATP binds to endothelial $P2Y_2$ purinoreceptor and starts a series of intracellular signal cascades that culminate in the liberation of NO, adenosine, prostaglandins, and EET (Hoiland et al 2016). Adenosine, Prostaglandins and EET bind to specific endothelial and smooth muscle cells receptors resulting in reduced Ca^{2+} sensitivity, reduced Ca^{2+} influx, increased K^+ outflow and increased local production of NO. The role of these molecules in hypoxia-mediated vascular tone regulation is well documented in animal models, but further studies are required to confirm the same action in humans (Hoiland et al. 2016).

All these mechanisms had been advocated to be responsible for hypoxia-induced vasodilation in different organs like the brain (Hoiland et al. 2016, Pearce et al 1990), the skeletal muscle (Dinenno et al. 2016), and the coronary circulation (Tune 2007). Regarding coronary circulation, since O_2 extraction is very high in normal resting conditions, the only mechanism available to increase O_2 delivery in acute hypoxia is vasodilation, which has been shown to occur in healthy individuals exposed to acute normobaric hypoxia simulating an altitude of 4500 mt both in resting conditions as well as after exercise (Wyss et al. 2003).

In contrast, pulmonary arteries respond to hypoxia with vasoconstriction. This mechanism is very important in ventilation/perfusion (V/Q) matching because it shifts blood from poorly ventilated and hypoxic alveoli (for example it happens during atelectasis) towards better-ventilated lung districts. Unfortunately, this local response to hypoxia involves the lungs globally when a low-oxygen content gas mixture is breathed, as happens at high altitudes, increasing pulmonary arterial pressure.

The peculiar pulmonary arteries response is likely due to vascular smooth muscle cells depolarization, which activates Ca^{2+} voltage-gated membrane channels and increases Ca^{2+} inflow (Michiels) This depolarization is connected to the inactivation of specific K^+ channels like Kv1.5, Kv2.1 and Kv9.3 (Archer et al. 1998; Hulme et al. 1999). Another source of intracellular Ca^{2+} is the sarcoplasmic reticulum that seems to release Ca^{2+} into the cytosol in hypoxic conditions (Swenson 2013).

All these changes are probably initiated by the impairment of redox state into the smooth muscle cells and the increase of reactive oxygen species (ROS) production that activates different intracellular signaling pathways involved in Ca^{2+} homeostasis and sensitivity (Schumacker 2011). Moreover, it seems that near endothelial cells may be a source of ROS and could release

Endothelin-1 in response to hypoxia. Endothelin-1 binds to Endothelin A receptors and induces vascular smooth cell contraction (Aaronson et al. 2002). Finally, Wang et al. illustrated an alternative mechanism that can contribute to pulmonary vasoconstriction. They proposed that capillary vascular endothelial cells that reside in proximity to alveoli depolarize in response to hypoxia and propagate the signal via inter-endothelial connexion 40-dependent gap junctions, working as an "oxygen sensor" that communicate to proximal arteries the oxygenation state of alveolar space inducing vasoconstriction (Wang et al. 2012).

Blood response to hypoxia.

Arterial oxygen content per 100 ml (C_aO_2) defines the total oxygen-carrying capacity of the blood. Its value can be calculated from the following equation:

$$C_aO_2 = (1,34 \times SpO_2 \times Hb) + (0,0031 \times P_aO_2)$$

where SpO_2 is the oxygen saturation of hemoglobin, Hb is the hemoglobin concentration in blood and P_aO_2 is the partial pressure of oxygen in arterial blood.

It follows that an increase in Hb brings to an increase in C_aO_2 and oxygen-carrying capacity of the blood.

One of the most important effects of the HIF complex as a hypoxia-sensible transcription factor is the increase of Erythropoietin (EPO) expression at the kidney level. EPO is the hormone responsible for new red blood cell production, which is the key factor in increasing hematocrit and hemoglobin in response to hypoxia (Ploszczyca et al. 2018). EPO production increases after minutes to hours following acute hypoxia exposure (Eckardt et al. 1989) and reaches a peak after 1-3 days (Berglund et al. 1992) if hypoxia persists. Even if the hypoxic stimulus continues in the subsequent days, EPO level starts to decline and it reaches a plateau slightly above sea-level concentrations; this behavior mimics HIF-1α time course, being likely both the expression of good acclimatization (Ploszczyca et al. 2018). EPO takes few weeks to exert its effects, but hematocrit can rise after a few hours of hypoxia exposure. This phenomenon is linked to a shift of plasma and extracellular fluids to intracellular space, with a decrease in plasma volume that can reach 20% of total volume (Hannon et al. 1969). The mechanisms responsible for this response are still unclear, but it seems that they depend on various volume-regulating hormones like the renin-angiotensin system and atrial natriuretic peptide (ANP), which has been shown to be increased after acute hypoxia (Albert et al. 1989).

Another fundamental response to hypoxia is the change of hemoglobin P_{50}, the partial pressure of oxygen at which oxygen saturation is 50%. P_{50} can be considered a measure of hemoglobin affinity for oxygen: a low P_{50} indicates a high-affinity state that facilitates hemoglobin oxygen loading in

pulmonary capillaries but impairs unloading at tissue level; a high P_{50} indicates the opposite. P_{50} is influenced by several factors like blood pH, carbon dioxide tension, the concentration of 2,3-diphosphoglyceric acid, magnesium, ATP, chloride, blood temperature and the amount of hemoglobin bound to carbon monoxide. It was demonstrated that hypoxia increases the blood content of 2,3-diphosphoglyceric acid, magnesium, ATP and chloride (Peronnet et al. 1991; Friedmann et al. 2005) inducing an increase in P_{50} of 0,3- 0,7 mmHg (Lundby et al 2004; Wagner t al. 2002). Thus, it seems that a low-affinity state is advantageous in hypoxic conditions at the cost of a lower ability of hemoglobin to bind oxygen in lung capillaries (Lundby 2013).

Ventilation and hypoxia.

According to the alveolar gas equation, $P_{alv}O_2$ depends on the amount of O_2 in inspired air and the $P_{alv}CO_2$. As we ascend to high altitudes, the partial pressure of inspired O_2 decreases while $P_{alv}CO_2$ doesn't change, so pulmonary ventilation (Ve) has to increase in order to increment the amount of O_2 arriving at the alveolar space and to eliminate CO_2.

At rest, acute exposure to hypoxia induces an initial increase (seconds to minutes) in both Tidal volume (TV) and respiratory frequency (RF). After 10 minutes, both variables decline until 30 minutes after initial exposure, when Ve stabilizes above normoxic levels. If the hypoxic stimulus persists, Ve gradually increases in the subsequent hours/days until it reaches a plateau (Lundbty 2013).

These adaptations allow $P_{alv}O_2$ and blood oxygen saturation (SpO_2) to increase and $P_{alv}CO_2$ to decrease within about 14 days before the effects of EPO become evident at the cost of respiratory alkalosis that is in turn compensated by the action of kidneys (Lundby 2013). The increase in Ve in chronic adaptation to hypoxia seems to be related to an increase in carotid chemoreceptors sensitivity to O_2. In support of this concept, it has been shown that after one day of hypoxia, gene expression increases in carotid body cells enhancing cell number (Wang et al. 2008). Moreover, in a goat model Bisgard et al. isolated the carotid bodies from systemic circulation and perfused them with hypoxic blood obtaining an increase in Ve, thus demonstrating the importance of carotid chemoreceptors in hypoxic-mediated hyperventilation (Bisgard et al. 1986).

Another source of blood oxygenation impairment in hypoxic conditions is the ventilation/perfusion (V/Q) mismatch. In normoxic conditions, as $P_{alv}O_2$ decreases in poorly ventilated alveoli, proximal pulmonary arterioles vasoconstrict shifting blood to better-oxygenated alveoli. In hypoxic conditions $P_{alv}O_2$ globally drops, thus inducing diffuse vasoconstriction which impairs V/Q and reduces blood oxygenation (Wagner et al. 1987;see "*Local vascular tone regulation and hypoxia"* chapter). This effect is greatly amplified by the increased cardiac output that occurs in hypoxia which accelerates blood flow in lung vessels thus reducing the time available for gas exchange

between capillaries and alveolar space (Lundby 2013).

Furthermore, it is worth mentioning the interstitial pulmonary edema caused by an excessive hypoxic pulmonary vasoconstriction (HPV) that impairs oxygen diffusion at the alveolar-capillary interface amplifying deoxygenation, a situation that could precipitate in life-threatening pulmonary edema (Luks et al. 2017).

Central Hemodynamic changes in hypoxia.

Since oxygen blood content decreases in hypoxic conditions, regulation of cardiac output (CO) becomes fundamental to guarantee a sufficient oxygen delivery to peripheral tissues. As expected, at rest after acute exposure to hypoxia CO increases. This adjustment is related to an increase in heart rate (HR), while stroke volume (SV) has been reported to be unchanged (Talbot et al. 2005) or decreased (Stembridge et al 2015).

Blood pressure (BP) remains unchanged or slightly increases after acute hypoxia exposure (Naeije 2010) whereas systemic vascular resistance (SVR) decreases. The effect on SVR is the result of the balance between the hypoxia-mediated local vasodilation (see "*Local Vascular tone regulation and Hypoxia*" chapter) and the vasoconstriction due to the sympathetic nervous system (SNA) activation, with the former prevailing on the latter.

If hypoxic stimulus persists for different days/weeks, CO returns to normoxic levels, HR remains higher and SV lower with respect to normoxia (Klausen 1966; Vogel et al 1967). Considering that left ventricular (LV) contractility seems to be preserved or enhanced in chronic hypoxia (Reeves et al. 1987; Suarez et al 1987), SV reduction has been considered to be related to plasma volume contraction (see "*Blood response to Hypoxia*" chapter), increased right ventricle (RV) afterload due to pulmonary arteries vasoconstriction, and impaired diastolic function (Stembridge et al. 2015a). Plasma volume restoration by isotonic Dextran infusion has been reported to improve SV in some investigations (Siebenmann et al. 2013), while other research did not report any improvement (Calbet et al. 2004).

Recently, echocardiographic studies (Stembridge et al. 2014; Stembridge et al. 2015b) contributed to shed light on the SV reduction after 10 days of exposure to high altitude (5050 mt). The authors reported a decreased left ventricular end-diastolic volume (LV EDV), an increased pulmonary systolic pressure (PSP), an enhanced LV systolic function [assessed as ejection fraction (EF) obtained with echocardiography, and as strain, rotation, and twist obtained by tissue Doppler imaging], a decreased RV systolic function [assessed by tricuspid annular plane systolic excursion (TAPSE) and RV longitudinal strain], a preserved LV diastolic function (assessed by left ventricular untwist velocity and apical diastolic rotational velocity analysis) with a decreased mitral inflow E/A ratio (a load-dependent index of diastolic function) probably related to LV EDV

reduction. These results seem to indicate that the main responsible for the SV reduction is an afterload dependent depression in RV function that reduces LV filling.

Maximal HR in hypoxia has been reported to be reduced (Lundby et al. 2001). This effect is related to an increased parasympathetic drive since maximal heart rate is restored after administration of glycopyrrolate, a parasympathetic blocking agent. The increased HR is accompanied by a decrease in maximal SV, so that CO is unchanged in this experimental setup. (Boushel et al. 2001). This result suggests that the regulated variable at maximal effort is CO which is likely adjusted by reducing SV in order to allow an appropriate transit time of red blood cells in the pulmonary circulation and more efficient blood oxygenation (Boushel et al. 2001).

Autonomic regulation of the cardiovascular system in hypoxia.

The Autonomic Nervous System plays a pivotal role in systemic cardiovascular adjustment to hypoxia. The increase in HR that occurs in acute hypoxic conditions depends on both sympathoexcitation and vagal withdrawal. Indeed, it has been demonstrated that muscle sympathetic nerve activity (mSNA), a marker of sympathetic nervous system activation, is increased after hypoxia exposure (Saito et al. 1988). The vagal contribution to HR in hypoxia has been studied only indirectly by using HR variability (HRV) analysis, which seems to indicate a reduced vagal tone at rest after acute hypoxia exposure (Perini et al. 1996; Princi et al. 2008).

Pharmacological blockade has been used to assess the relative contribution of sympathetic and parasympathetic branches of the autonomic nervous system to HR under acute hypoxia. The isolated inhibition of sympathetic (using propanolol) or parasympathetic (using atropine) nervous system do not affect the tachycardic effect of hypoxia, while simultaneous inhibition abolishes it (Koller et al. 1988). This indicates that both branches are involved in HR response during acute exposure to hypoxia.

There are many putative contributors to these changes in the autonomic nervous system activity. Carotid and aortic chemoreceptors are the main peripheral sensors of hypoxia. Carotid chemoreceptors are primarily sensitive to P_aO_2, while aortic chemoreceptors are responsive to C_aO_2 (Lahiri et al. 1981). It seems that carotid chemoreceptors are the main responsible of hyperventilation and vasoconstriction response to hypoxia, while aortic chemoreceptors are the main determinant of hypoxia-mediated tachycardia (Niewinski et al. 2014). However, other studies indicate that carotid chemoreceptors activation exerts an important indirect effect on HR, since hyperventilation triggers the pulmonary stretch receptors and reduces the vagal traffic to the heart (Kato et al. 1988). Moreover, carotid chemoreceptors increase the level of circulating catecholamines, further influencing HR when activated (Siebenmann et al. 2015).

Furthermore, acute hypoxia induces a shift of the baroreflex set-point to a higher pressure level,

thus causing an increase in sympathetic tone and a vagal withdrawal (Halliwill et al. 2003; Querido et al. 2011). The precise mechanism of this phenomenon remains unclear, but it seems related to peripheral chemoreceptors activation (Halliwill et al. 2003). Besides, hypoxia triggers baroreflex thanks to its vasodilator effect on peripheral arteries, and this further increases the sympathetic tone and inhibits the parasympathetic activity (Calbet et al. 2014).

If the hypoxia stimulus persists for days/weeks, C_aO_2 is partially restored by EPO-related increase in hematocrit and red blood cells, and this effect is further amplified by plasma volume contraction. Nonetheless, PaO_2 only slightly increases during chronic hypoxia. It derives that aortic chemoreceptors should be less active than carotid chemoreceptors in chronic hypoxic conditions, with a consequent reduction in tachycardia and a preserved hyperventilation and vasoconstriction. On the contrary, tachycardia persists after chronic hypoxia exposure (Siebenmann et al. 2015). This effect is likely related to a higher sensitivity of carotid chemoreceptor to reduced PaO_2 which triggers sympathetic activation (Mazzeo et al. 1995; Hansen et al. 2003). Moreover, the baroreflex set-point is shifted to higher pressure and this enhances sympathetic traffic to the heart even in the presence of reduced aortic chemoreceptors activation (Siebenmann et al. 2015).

The effects of chronic hypoxia on the parasympathetic system are less clear. Pharmacological blockade of muscarinic receptors increases HR more after altitude acclimatization than at sea level (Wolfel et al. 1998; Bao et al. 2002), but HRV analysis shows a persistent vagal withdrawal after 18 months at high altitude (Dhar et al. 2014). A possible explanation for these conflicting results is the augmentation of muscarinic receptor density after chronic hypoxia exposure, which has been demonstrated in rats (Kacimi et al. 1993).

Exercise in hypoxia.

In light of all the described effects of hypoxia on the human body, it's easy to imagine how exercise in hypoxia is challenging. Oxygen uptake (VO_2) is the amount of oxygen consumed by the body in one minute. VO_2 is directly related to workload and it increases linearly as exercise load increases. According to Fick's equation, VO_2 can be expressed as the product of CO and the difference in O_2 content between arterial and venous blood ($C_aO_2 - C_vO_2$) :

$$VO_2 = CO\, x\left(C_a O_2 - C_v O_2\right)$$

As a subject undergoes hypoxia, C_aO_2 diminishes while CO increases to compensate for it. A well-known measure of aerobic exercise performance is maximal oxygen uptake ($VO_{2\,max}$), which is the rate of oxygen uptake achieved at the end of a maximal incremental exercise test when VO_2 reaches a plateau and any load increment does not lead to any further VO_2 rise. Since during a maximal

incremental exercise test it's difficult to reach the true $VO_{2\ max}$, peak oxygen uptake ($VO_{2\ peak}$) at the end of the test is often used as a surrogate measure. Being $VO_{2\ max/peak}$ directly related to the weight of the subject, these measures are often expressed in $ml*min^{-1}*kg^{-1}$ in order to allow comparison among individuals with different body sizes.

As expected, hypoxia induces a reduction in $VO_{2\ max}$ which depends on the altitude or the partial pressure of inspired O_2. It has been demonstrated that above 1500 mt of altitude, $VO_{2\ peak}$ is reduced by 1% for every 100mt of altitude (Fulco et al. 1998). At the summit of Mount Everest, the reduction reaches 80%, thus leaving very little exercise capacity left. This VO_{2max} reduction is amplified in elite athletes. Indeed, it has been shown that in these subjects VO_{2max} starts to decline at 580 mt of altitude (Gore et al. 1996) and the reduction is higher compared to untrained individuals at the same altitude (Fulco et al. 1998). These evidences are likely related to the enhanced muscle extraction of O_2 in athletes during exercise, which is preserved even when P_aO_2 drops in hypoxic conditions, resulting in a greater oxygen desaturation and exercise capacity impairment (Van Thienen et al. 2016).

Acclimatization increases C_aO_2 by increasing the amount of circulating red blood cells and hematocrit, a potentially positive effect on VO_{2max} according to Fick's equation. Actually, it has been demonstrated that after 1-2 months of EPO treatment VO_{2max} improves up to 3500 mt, without affording any additional positive effect above this altitude (Robach et al. 2008).

Submaximal exercise in hypoxia showed a level of impairment which is comparable to the maximal one. The energetic cost of an exercise bout at constant power is similar between sea level and high altitude, but the relative exercise intensity (expressed as a percentage of VO_{2max}) is increased as VO_{2max} declines with hypoxia exposure (Lundby 2013). Nonetheless, it has been shown that the time to exhaustion for submaximal exercise at a constant load performed at 4300 mt of altitude improves after 16 days of acclimatization, thereby suggesting that acclimatization can be beneficial for submaximal exercise performance at higher altitudes too (Horstman et al. 1980).

Differently from aerobic performance, anaerobic exercise seems not to be affected at high altitude. Indeed, activities that depend on air friction (like 100mt running or throwing sports) are advantaged because of the reduced air density. Nonetheless, hypoxia greatly impairs the regeneration of ATP and glycogen after aerobic exercise requiring longer resting time to fully recover after an exercise bout (Lundby 2013).

Considering the effects of hypoxia and the consequent potential benefit on aerobic exercise, several investigators tried to asses whether training at high altitudes improved athletes' performance. Different training regimens have been tested (live low/train high, live high/train high, live high/train low), but the only one that seems to give a beneficial effect is the live high/train low (LHTL) protocol. LHTL consists in leaving and sleeping at moderate altitudes (3000 mt) and training at

lower altitudes or sea-level. This regimen seems to be beneficial because it evokes the positive effect of hypoxia and at the same time it allows the athlete to train at intensities that couldn't be maintained under oxygen deprivation (Levine et al. 1997).

Metaboreflex and hypoxia.

An important mechanism for cardiovascular adaptation to exercise is the so-called "muscle metaboreflex". When the muscles are activated during exercise they produce several metabolic byproducts such as lactate, adenosine, potassium, and phosphate. These substances are sensed by type IV nerve endings in the muscles which in turn convey the information to the cardiovascular control centers located in the caudal and rostral ventrolateral medulla. This information is about the degree of metabolic activation and the presence of any mismatch between the local circulation and the contracting muscle thereby evoking a cardiovascular reflex, i.e. the metaboreflex. This mechanism is mediated by the sympathetic autonomic nervous system and consists in an increase in SVR and an increase in SV, the latter being related to enhanced cardiac contractility and an increased cardiac pre-load deriving from a venoconstriction that recruits the Frank-Starling mechanism (Nobrega et al. 2014). The vasoconstrict effect of the metaboreflex-induced increase in sympathetic activity is counteracted in active muscles by the vasodilatory effect of molecules produced during exercise, like nitric oxide, adenosine, and prostacyclin. This phenomenon is called "functional sympatholysis" and allows a shift of blood flow from inactive tissues to exercising muscles in order to match their metabolic demand (Tschakovsky et al. 2002). All these modifications produce an increase in BP that is directly related to exercise intensity and that allows optimal muscle perfusion without impairing blood delivery to other noble tissues such as the brain and the heart (Crisafulli et al. 2015).

Metaboreflex acts during exercise in concert with other two important reflex mechanisms, the "central command" and the "mechanoreflex". The central command consists in the direct cardiovascular response to the activation of the motor area of the cerebral cortex during exercise. This phenomenon is possible thanks to a direct neural connection of these cortex areas to the brain stem and its nuclei involved in cardiovascular regulation. The central command triggers an initial hemodynamic adjustment to exercise that is then finely tuned by the activation of the metaboreflex and the mechanoreflex (Crisafulli et al. 2015).

The mechanoreflex behaves similarly to the metaboreflex, but it conveys information about the mechanical status of the contracting muscle. Mechanoreflex works thanks to type III nerve endings within the muscles and tendons that inform the cardiovascular control areas about the stretching and tension that develops in active muscles (Hollander et al. 1975; Fischer et al. 2004). Investigators often refer to mechanoreflex and metaboreflex together as the forming parts of the "exercise pressor

reflex".

These 3 mechanisms interact and influence each other during exercise and some level of redundancy exists. All of them exert a similar effect on the cardiovascular system by modulating the autonomic nervous system but with some differences. Indeed, while the central command and mechanoreflex seem to induce an increase in sympathetic drive and a vagal withdrawal, metaboreflex exerts its effect only on the sympathetic branch (Crisafulli et al. 2015).

Moreover, they exert direct effects on baroreflex as they produce a shift in the baroreflex operating-point to higher values while preserving baroreflex sensitivity (Iellamo et al. 1997; Iellamo et al. 2001; Potts et al. 1993; Ogoh et al. 1993). These modifications are important to buffer any sympathetic overdrive and to avoid any excessive peripheral vasoconstriction (Raven et al. 2006; Joyner 2006).

An experimental method often used to isolate metaboreflex from both mechanoreflex and central command is the post-exercise muscle ischemia (PEMI), which consists in the occlusion of blood flow on a limb that has just ceased to exercise. This maneuver impedes the washout of metabolites produced during exercise and amplifies the activation of the metaboreflex, avoiding the influence of the motor areas of the cortex and mechanoreflex on the cardiovascular control areas while the metaboreflex is active (Crisafulli et al. 2003; Crisafulli et al. 2008). PEMI has been extensively used to evaluate the cardiovascular response to metaboreflex activation. Despite the changes in hemodynamic parameters evoked by this method, PEMI showed no effect on HR (Piepoli et al. 1995; Iellamo et al. 1999). This result is likely related to the increased parasympathetic influence on the heart evoked by the baroreflex activation that counterbalances the metaboreflex-mediated sympathetic drive that occurs during this maneuver (Crisafulli et al. 2015).

Some investigators tried to shed light on the effects of acute hypoxia on the metaboreflex. Houssiere and colleagues (Houssiere et al. 2005) evaluated the effect of 3 minutes of hypoxia alone, 3 minutes of isometric handgrip exercise alone, and 3 minutes of both intervention combined on mean BP, HR, mSNA, and Ve. The same variables were also evaluated for 3 minutes after these interventions applying a forearm circulation arrest maneuver, a protocol similar to PEMI. Their results showed that hypoxia alone increased mSNA, HR, Ve, with no change in mean BP while exercise alone increased all the measured variables. The combination of hypoxia and exercise increased all the variables studied, with an enhanced Ve and HR response. During the forearm circulation arrest, all variables returned to baseline level in 3 minutes after hypoxia alone, while Ve and mSNA remained elevated after exercise alone and exercise in hypoxia, and mean BP stayed elevated only after exercise in hypoxia.

Gujic et al. (Gujic et al. 2007) performed a similar experimental protocol that consisted in 4 interventions: normoxic rest (used as control), normoxic isometric handgrip exercise, hypoxia alone

and hypoxic isometric handgrip exercise for 3 minutes, each one followed by 3 minutes of forearm circulation arrest. Furthermore, they assessed the baroreflex control of sympathetic outflow and R-R intervals inducing variations on BP pharmacologically. The studied variables were systolic BP, diastolic BP, HR, Ve, and mSNA. Furthermore, %mSNA/mmHg was studied as a measure of baroreflex sensitivity of sympathetic outflow, while ms/mmHg was evaluated as a measure of baroreflex sensitivity of R-R intervals. Finally, the operating point of baroreflex regarding systolic BP, diastolic BP, mSNA and R-R intervals were evaluated to complete the baroreflex analysis. Their results demonstrated that when chemoreflex and metaboreflex are activated simultaneously, as it happened when the forearm circulation arrest was applied after exercise in hypoxia, all the examined variables remained elevated after 3 minutes, with a complementary effect on systolic BP (that was elevated after exercise alone and not after hypoxia alone) and on HR (that was elevated after hypoxia alone and not after exercise alone), with no significant differences in Ve and mSNA, that were similarly influenced after hypoxia alone and normoxic exercise.

Regarding baroreflex control of sympathetic outflow, the investigators reported that baroreflex sensitivity (expressed as %mSNA/mmHg) was increased during forearm circulation arrest after hypoxia alone without any modification in the operating point of diastolic BP and mSNA, while it was unchanged after normoxic and hypoxic exercise with an increased operating point of diastolic BP and mSNA. Moreover, results from baroreflex control of R-R interval analysis showed a decreased cardiac baroreflex sensitivity (expressed as ms/mmHg) during forearm circulation arrest after hypoxia alone and hypoxic exercise, while it remained unchanged after normoxic exercise. These results were correlated to a reduced end-tidal pressure of CO_2 that occurs during hypoxia exposure as a consequence of an increased Ve, so they can not be considered directly related to the hypoxic stimulus. Concerning the operating point of systolic BP and R-R interval, the former was shifted to higher values after normoxic and hypoxic exercise while the latter was shifted to lowers values after hypoxia alone and hypoxic exercise.

From these results, the investigators concluded that metaboreflex and hypoxia-induced chemoreflex evoke partially overlapped responses on the cardiovascular system and ventilation, without summation when activated simultaneously. Furthermore, they exert a different effect on baroreflex control of sympathetic outflow and R-R intervals, with the metaboreflex being more active on the operating point of BP and mSNA and the chemoreflex having a greater effect on the operating point of R-R intervals. It is worth to mention that regarding baroreflex sensitivity on the sympathetic outflow, metaboreflex seems to prevail on chemoreflex since the augmentation of %mSNA/mmHg induced by chemoreflex was abolished by the concomitant activation of metaboreflex. The authors suggested that these results were likely related to a partial overlap of chemoreflex and metaboreflex central projection in the brain stem (in particular in the nucleus of tractus solitarii and the

paramedian reticular nuclei), where different interactions between the two stimuli could take place. Unfortunately, these studies did not include hemodynamic parameters like SV and SVR making the cardiovascular system analysis still incomplete.

AIM OF THE RESEARCH

Unlike the extensive bibliography published on the effects of hypoxia at the cellular level that was worth the 2019 Nobel Prize in Medicine, the effects at the systemic level are partly unclear and require new studies to better elucidate the mechanisms behind them. My work was aimed at following this strand of research.

In particular, the main focus of my study was to better elucidate the interaction between hypoxia and metaboreflex activation, focusing on hemodynamic parameters (such as SV and SVR) that have never been studied in the past. To reach this goal, I designed and performed two series of experiments in which the effects of a previous dynamic exercise bout in hypoxia (Experiment 1) and the simultaneous exposure to hypoxia (Experiment 2) on metaboreflex activation were evaluated.

Considering the effects of hypoxia on SV and SVR illustrated so far, we hypothesized that it could impair the ability of metaboreflex to increase SV and prevent SVR to increase as a compensatory mechanism, leading to a blunted BP response.

In my opinion, these studies could provide new useful insights regarding the cardiovascular adaptations to extreme environments, a topic that is receiving growing interest in aerospace medicine. In fact, hypoxic exercise has been recently proposed as a valuable training method to increase physical condition prior, during and after returning from spaceflight missions (Willis et al. 2019) This training protocol could help to reduce the effects of prolonged microgravity on human body like a decreased aerobic fitness, muscle and bone atrophy and an overall cardiovascular dysfunction (Demontis et al. 2017). Moreover, good acclimatization to the hypoxic environment could help the astronauts to better face any loss of pressure that could lower the partial pressure of oxygen in the space habitat (Lewis 2018). In light of the above, a better understanding of cardiovascular adaptation to hypoxia is needed in the perspective of new space missions to explore Mars and the recent development of space tourism, that could expose the challenging conditions of space travel to people without adequate training.

MATERIALS AND METHODS

Population

Experiment 1

Seventeen healthy Caucasian subjects (including 7 females) aged 22–50 years agreed to participate in the first study. All were well-trained athletes regularly involved in endurance competitions (cycling, triathlon, and marathon running). The protocol was not completed by all the subjects as seven of them complained of unbearable fatigue during the EH test. Thus, they were excluded from results, which included the remaining ten subjects (four females and six males). Their age, body mass, and height were 35.6 ± 8.4 years, 66.8 ± 12.6 kg, and 174.6 ± 9.3 cm, respectively.

Experiment 2

Eleven healthy Caucasian male subjects aged 22–46 years were recruited to participate in the second study. All were athletes regularly involved in leisure-time sports activities at least 3 times/week. Their age, body mass, and height were 35.6 ± 8.4 years, 71.8 ± 10.3 kg, and 174.5 ± 5. cm, respectively.

The subjects involved in both experimental protocols were considered healthy on the basis of a preliminary medical examination (see experimental design). None had any history of cardiac or respiratory disease or were taking any medication at the time of the experiment.

All the subjects were normotensive and nonsmokers and were unaware of the nature of the study. Subjects were asked to refrain from alcoholic beverages and caffeine for at least 24 h before the experimental sessions. The study was performed according to the Declaration of Helsinki and was approved by the ethics committee of the University of Cagliari. Written informed consent was obtained from all the participants included in the study.

Experimental design

Preliminary test (Experiment 1 and 2)

All the subjects involved in the two experiments underwent a general medical visit. Afterward, a cardiopulmonary test (CPX) with a gas analyzer (VO2000, MedGraphics St. Paul, MN, USA), calibrated immediately before each CPX, was conducted on a mechanically braked cycle ergometer (Monark 828E, Vansbro, Sweden).

The test consisted in a linear increase of workload (30 W min^{-1}), starting at 30 W, at a pedaling

frequency of 60 rpm, until exhaustion, which was taken as the point at which the subject was unable to maintain a pedaling rate of at least 50 rpm. During the CPX, anaerobic threshold (AT), maximum workload (W_{max}), and maximum oxygen uptake (VO_{2max}) were measured. Achievement of $VO_{2\ max}$ was considered as the attainment of at least two of the following criteria: (1) a plateau in VO_2 despite increasing workload (< 80 ml min−1); (2) respiratory exchange ratio (RER) above 1.10; and (3) heart rate (HR) ± 10 beats min−1 of predicted maximum HR calculated as 220-age (Howley et al. 1995). The anaerobic threshold was determined using the *V*-slope method, which detects AT using a computerized regression analysis of VO_2 slopes vs. the carbon dioxide production (VCO_2) plot during exercise (Beaver et al. 1986). VO_{2max} was calculated as the average VO_2 during the final 30 s of the incremental test. During the preliminary test, participants familiarized with the laboratory equipment and staff. This allowed habituation to the environment and the ergometer that was otherwise identical to the exercise under hypoxia sessions.

Experiment 1 protocol

Exercise under hypoxia sessions

After the preliminary visit (interval 4–7 days), each subject completed, in separate days (interval 3–5 days), three randomly assigned exercise sessions: (1) an exercise session in normoxia (EN); (2) an exercise session in normobaric hypoxia with a FiO2 of 15.5% (EH15.5%), which simulated the partial pressure of oxygen at an altitude of 2500 m; (3) and finally, an exercise session in normobaric hypoxia with a FiO2 of 13.5% (EH13.5%), which simulated an altitude of 3500 m. Exercise sessions were conducted on the same bicycle employed for the CPX test. During each session, the subject exercised for 10 min at 80% of the AT previously assessed during the CPX. Throughout the sessions, the subject was connected with a facemask to a hypoxic gas generator (Everest Summit II Generator, Hypoxico, New York, USA). This device utilizes a molecular sieve system that uses zeolites to separate nitrogen from O_2 in the air, and consequently provides a nitrogen-rich gas mixture, to purge the atmosphere within the container. Connected to the container, and in conjunction with a one-way pressure relief valve, the hypoxic generators can reduce the concentration of O_2 up to about 12.5%, which corresponds approximately to the O_2 pressure at 4000 m altitude. The level of oxygen concentration was adjusted by the operator on the basis of an oxygen analyzer provided with the device (Maxtec, Handi+, Salt Lake City, UT, USA). The applied generator setting was blinded to the athlete and controlled every minute by the operator. This device is commonly used for training in normobaric hypoxia (Schega et al. 2016). Before commencing sessions, the athlete was connected to the hypoxic generator and rested for 3 min to reach a stable condition in respiratory gas exchange. Throughout sessions, subjects were also monitored for

peripheral blood O_2 saturation (SO_2), measured by finger pulse oximetry. To further detect the real presence of body hypoxemia, cerebral tissue oxygenation (COX) was assessed with near-infrared spectroscopy (NIRS) (Nonin, SenSmart X-100, Plymouth, MN, USA). Briefly, one NIRS sensor was placed on the left forehead above the eyebrow. The probe was covered and maintained with a headband and taped to reduce the intrusion of extraneous light. Care was taken to ensure that the attached probe did not constrict the head and did not block the circulation.

Test for metaboreflex function assessment

A brief recovery was allowed after each exercise session (10 min). Then (within 15 min), the participants were subjected to the following protocol, randomly assigned to study the effects of hypoxic exercise on hemodynamic responses during the metaboreflex:

- *Post-exercise muscle ischemia (PEMI):* This session was composed of 3 min of resting, followed by 3 min of exercise, consisting of cycling at mild intensity (30% of *W*max measured during the CPX) at 60 rpm. The cycling period was followed by 3 min of PEMI on the right leg. Ischemia was induced by rapidly (in less than 3 s) inflating, at the cessation of exercise, a thigh cuff, to 50 mmHg above peak exercise systolic pressure. The cuff was kept inflated for 3 min. A further 3 min of recovery was allowed after the cuff was deflated, for a total of 6 min of recovery. Thus, the total PEMI session duration was 12 min. This protocol, although conducted at mild intensity, has demonstrated the ability to "trap" muscle metabolites in the exercising limb and to stimulate the metaboreceptors (Crisafulli et al. 2008; Scott et al. 2002). PEMI is effective in eliciting substantial hemodynamic responses in terms of cardiac contractility, pre-load, and after-load (Crisafulli et al. 2008; Marongiu et al. 2013; Milia et al. 2015; Roberto et al. 2012; Scott et al. 2002). This response is suggested to be mediated only by the metaboreflex, since in this setting, the central command and mechanoreflex activation are no longer present (Bastos et al. 2000; Crisafulli et al. 2011; Nobrega et al. 2014);
- *Control exercise recovery (CER) session:* The same rest-exercise protocol used for PEMI was used, but the recovery was conducted without cuff inflation.

The PEMI and the CER tests were randomized and recovery was allowed between sessions (approximately 15 min). Recovery was considered complete when HR was not higher than 5 bpm compared with the pre-exercise level. The study design is summarised by Fig. 1. All experiments were carried out in a temperature-controlled, air-conditioned room (22 °C—relative humidity 50%). To eliminate any potential effect of hormonal changes on hemodynamics, all women were tested in the follicular phase of the menstrual cycle (i.e., within 10 days from the start of menstruation) as self-reported. Please refer to Figure 1 for a visual explanation of the test.

Experiment 2 protocol

Test for metaboreflex function assessment with simultaneous hypoxia exposure

After a few days (interval 4-7) from the preliminary test, the subjects involved underwent a randomly assigned normoxic (NORMO) and hypoxic (HYPO) CER/PEMI sessions in two separate days. After 1 minute of rest, the subjects were connected to the hypoxic gas generator mask previously described and performed CER and PEMI protocols wearing it throughout the tests both in NORMO and HYPO sessions. During HYPO sessions, a gas mixture with a FiO_2 of 13,5%, was delivered to the subjects, simulating an altitude of about 3500 mt. To assess whether the hypoxic stimulus was effective, all subjects were monitored for SO_2 and COX as happened in Experiment 1. See Fig 2. and 3. for a visual explanation of the test.

Hemodynamic assessment during metaboreflex activation (Experiment 1 and 2)

Throughout all sessions of metaboreflex activation, subjects' hemodynamics were collected by impedance cardiography (NCCOM 3, BoMed Inc., Irvine, CA), which has been previously utilized in similar experimental settings (Crisafulli et al. 2008, 2009, 2011). This device allows for continuous, non-invasive, hemodynamic evaluation. The rationale of its use is based on the fact that pulsatile aortic blood flow, due to ventricular systole, induces proportional fluctuation in electrical conductivity. Therefore, changes in thoracic impedance (Z_0) are representative of the blood volume ejected during systole. By employing standard formulas, the SV can be estimated from Z_0 variations (Bernstein 1986). The data acquisition procedure is described in detail in previous research (Crisafulli et al. 2008, 2009, 2011). Briefly, analog traces provided by impedance cardiography along with ECG were collected and stored by means of a digital chart recorder (ADInstruments, PowerLab 8sp, Castle Hill, Australia) at a sampling rate of 500 Hz. Z_0 and its first derivative (dZ/dt) were collected, stored, and analyzed afterward offline. Stored traces were also used to calculate beat-to-beat HR, which was estimated as the reciprocal of the electrocardiogram R–R interval. The pre-ejection period (PEP) and the left ventricular ejection time (VET) were also measured from impedance traces. PEP corresponds to the time interval between the onset of QRS complex of ECG and the beginning of the systolic deflexion of dZ/dt, while VET was measured as the interval between the beginning of systolic dZ/dt deflection and the minimum of dZ/dt curve relative to the same cardiac cycle. Diastolic time (DT) was measured by subtracting the sum of PEP and VET from the cardiac cycle total period, measured as R-R interval. The ventricular filling rate (VFR), which is a measure of the mean rate of diastolic blood flux, was obtained by dividing SV by DT

(Gledhill et al. 1994; Marongiu et al. 2013; Milia et al. 2015). The mean ventricular ejection rate (VER), an index of myocardial performance, was obtained by calculating the SV/VET ratio (Gledhill et al. 1994; Sanna et al. 2017). CO was obtained as the product of SV by HR. A standard manual sphygmomanometer was employed for systolic (SBP) and diastolic (DBP) blood pressure assessment, which was performed on the non-dominant arm by the same physician throughout all protocol sessions. Mean arterial blood pressure (MAP) was calculated with the formula by Moran (Moran et al. 1995; Sainas et al. 2016), which takes into account changes in the diastolic and systolic time due to exercise-induced tachycardia in calculating MAP. To have a measure of global vascular resistance, SVR was calculated by multiplying the MAP/CO ratio by 80, where 80 is a conversion factor to change units to standard resistance units.

Data Analysis (Experiment 1 and 2)

Data are shown as mean ± SD. All collected data were averaged over 1 min. Differences in SO_2 and COX were assessed using a two-way analysis of variance (ANOVA) (factors of time and condition: EN, EH15.5%, and EH13.5% for Experiment 1, NORMO and HYPO for Experiment 2) followed by Bonferroni post hoc where appropriate. Hemodynamic values at rest, at the third minute of exercise, and at the third minute of PEMI (when a steady-state in metaboreflex activity was expected to be reached) during the metaboreflex tests were assessed. Two-way ANOVA was utilized to compare hemodynamic data for the effects of test (PEMI and CER) and condition (EN, EH15.5%, and EH13.5% sessions for Experiment 1 and NORMO and HYPO for Experiment 2) followed by Bonferroni post hoc when appropriate.

To further assess the metaboreflex activity, the following procedure was employed: the difference in the level of variables between the post-exercise ischemia phases of the PEMI and the CER test at the third minute of recovery was calculated. This procedure enabled metaboreflex response to be assessed, i.e., the response due to the metaboreflex activity (Crisafulli et al. 2013; Milia et al. 2015). Unpaired samples T-Test was used to assess any differences between Experiment 1 and 2 populations CPX variables. Differences in measured variables due to metaboreflex response were assessed by means of the one-way repeated measures ANOVA test, followed by Bonferroni post hoc when appropriate for Experiment 1 and Paired samples T-Test for Experiment 2. Statistical analysis was performed by utilizing commercially available software (GraphPad Prism). Statistical significance was established as a p-value of < 0.05 in all the cases.

RESULTS

Experiment 1

The results of the CPX test are shown in Table 1. During exercise sessions, subjects cycled at a workload corresponding to 187.2 ± 3.4 W, i.e., 80% of AT. The time course of SO_2 and COX is illustrated in Fig. 4. There was a progressive reduction in SO_2 throughout the EH15.5% and EH13.5% sessions with respect to EN (panel A). Even though the EH13.5% appeared to induce a stronger SO_2 reduction than the EH15.5% test, no statistical difference was demonstrated between sessions, with the exception of the second minute of exercise. Panel B of Fig. 3 shows that COX (reported as % changes from rest) progressively decreased during the EH15.5% and EH13.5% sessions as compared to EN, without any significant difference between the two exercise sessions conducted in hypoxia.

Table 2 shows the mean values of hemodynamic parameters gathered during rest periods preceding the PEMI and the CER tests. The SV was the only parameter affected by condition, as it was on average reduced by the EH15.5% and the EH13.5% sessions, as compared to EN.

Table 3 shows that at the third minute of exercise, preceding the PEMI and the CER maneuvers, none of the hemodynamic parameters were influenced by test or condition.

Figures 5 and 6 depict hemodynamic variables obtained during the third minute of recovery of the PEMI and the CER tests. Panel A of Fig. 5 demonstrates that HR was on average higher after the EH15.5% and EH13.5% sessions, with respect to EN (p= 0.042 for condition). No significant difference was found for HR response between conditions (panel B). The SV was significantly reduced by exercise in hypoxia (p = 0.042 for condition, panel C). Moreover, exercise in hypoxia significantly reduced SV response (panel D) with respect to EN. The CO was unchanged by exercise in hypoxia, neither in absolute values (panel E), nor in terms of its response (panel F). Figure 6a illustrates that the absolute values of VFR were unaffected by exercise in hypoxia. However, its response was significantly lower after EH15.5% and EH13.5% as compared to EN (panel B). Similarly, VER was not significantly different among treatment with hypoxic gases (panel C), but its response was reduced in EH15.5% and EH13.5% sessions in comparison with EN (panel D). The MAP was significantly influenced by test, as this parameter was higher during the PEMI than during the CER maneuvers (p = 0.0005 for test effect, panel E) without any detectable difference in its response (panel F). Finally, panel G of Fig. 6 demonstrates that the PEMI tests induced higher SVR than the CER tests (p = 0.039 for test effect). Furthermore, the response in this parameter was more elevated after both sessions of exercise in hypoxia than in normoxia (panel H).

Experiment 2

Results of the CPX are reported in Table 4. During Experiment 2 subjects showed a lower VO_2 max than Experiment 1 individuals (p=0.008). Figure 7 illustrates the behavior of SO_2 (panel A) and COX (panel B) during NORMO CER, NORMO PEMI, HYPO CER, and HYPO PEMI. There was a statistically significant reduction in both variables during the hypoxic sessions that started at third minute of rest in HYPO PEMI and fourth minute of rest in HYPO CER, continuing for the entire duration of the protocols.

Table 5 shows the mean values of hemodynamic parameters gathered during the fourth minute of rest preceding the PEMI and the CER tests. No differences were detected between NORMO and HYPO sessions. Table 6 shows that at the third minute of exercise preceding the PEMI and the CER maneuvers, none of the hemodynamic parameters were influenced by test or condition as happened in Experiment 1. Figures 8 and 9 illustrate hemodynamic variables measured during the third minute of recovery of the PEMI and the CER tests.

Panel A of Fig. 8 shows that HR was unaffected by hypoxia and no significant difference was found for HR response between conditions either (panel B). SV was not significantly affected by exercise in hypoxia, but SV response showed a significant reduction in HYPO (panel D, p= 0.0205) with respect to NORMO. The CO was affected by test (p= 0.013) with a significant increase in PEMI only during NORMO tests (panel E), with CO response being increased during NORMO tests with respect to HYPO tests (panel F, p=0.021).

Panel A of figure 9 illustrates that the absolute values of VFR were unchanged during NORMO and HYPO tests. However, its response was significantly lower during HYPO sessions (panel B, p=0.0034). Regarding VER, there was not significant difference among treatments with hypoxic gas (panel C), and its response seemed not to be affected by condition (panel D). MAP was not significantly influenced by test (panel E), with no significant differences in response analysis. Finally, panel G and H of Fig. 9 demonstrates that there were no differences in SVR values between NORMO and HYPO (panel G), but there was an almost significant (p=0.070) difference in SVR response, with SVR being higher during PEMI in hypoxia (panel H).

DISCUSSION

The main aim of the present study was to discover whether hypoxia could alter the cardiovascular response during the metaboreflex activation in two different experimental set-ups: a previous exercise session in hypoxia and simultaneous hypoxia exposure. Our hypothesis was that the capacity to globally vasoconstrict both the arteriolar and the venous beds was impaired due to the production of vasodilating metabolites able to restrain the metaboreflex-induced vasoconstriction (Casey and Joyner 2012). It has, in fact, been demonstrated that during the metaboreflex, both arteriolar and venous circulation is constricted by the augmented sympathetic tone (Crisafulli et al. 2017; Marongiu et al. 2013; Nobrega et al. 2014; Sheriff et al. 1998; Shoemaker et al. 2005).

Although no direct measure of venous constriction was gathered, results show that hypoxia led to an impairment in the capacity to enhance venous return, as testified by the reduction in the VFR response. This parameter has already been utilized in recent research on the metaboreflex and has been able to detect reductions in venous return and diastolic functions during the metaboreflex (Crisafulli et al. 2009; Magnani et al. 2018; Marongiu et al. 2013; Mulliri et al. 2016; Roberto et al. 2017). The lack of any VFR response indicates that the capacity to centralize blood volume was negatively affected by hypoxia and that this occurrence led to a reduction in the SV response. Indeed, SV response was lower after EH and during HYPO sessions, with SV absolute values also being affected after EH.

Several researchers have demonstrated that the capacity to increase cardiac pre-load is pivotal to achieving normal hemodynamics during the metaboreflex (Bastos et al. 2000; Crisafulli et al. 2009; Marongiu et al. 2013; Milia et al. 2015). Studies have reported that the capacity to centralize blood volumes, by means of visceral and venous constriction, supports ventricular performance, by recruiting the Frank–Starling mechanism. This allows SV to increase during the metaboreflex (Bastos et al. 2000; Crisafulli et al. 2009; Marongiu et al. 2013; Milia et al. 2015; Sheriff et al. 1998; Shoemaker et al. 2005). It is to be noted that the impaired SV was not compensated by any HR response. Although HR was on average higher during both the PEMI and the CER test after EH sessions, the response in this variable (i.e., the difference between the PEMI and the CER test) was unaffected by condition in both EH and HYPO sessions. This is in good accordance with the concept that HR does not usually participate in the hemodynamic adjustments during the metaboreflex obtained by PEMI (Crisafulli et al. 2011, 2015; Fisher et al. 2013; Iellamo et al. 1999; Nishiyasu et al. 1994).

This finding supports the concept that during PEMI, reductions in cardiac pre-load are compensated mainly by arteriolar constriction, rather than by chronotropic increments (Crisafulli et al. 2011; Mulliri et al. 2016; Roberto et al. 2017). Another consequence of the impaired VFR response was

the reduced capacity to increase VER during the metaboreflex after EH. This parameter is considered to be directly related to myocardial performance and it is sensible to make modifications in both cardiac inotropism and pre-load (Gledhill et al. 1994; Sanna et al. 2017). Indeed, VER response was on average positive after EN and negative after both sessions of EH.

It is worth mentioning that during Experiment 2, no differences were detected in HR and VER response during NORMO and HYPO sessions. These results seems to be in contrast with Experiment 1 outcomes. This different finding is likely related to the intensity of the hypoxic stimulus and the level of athletic fitness of the population involved in Experiment 1 and 2. In fact, the hypoxic exercise sessions in Experiment 1 were longer in duration (10 minutes vs 3 minutes) and could have elicited a stronger chemoreflex activation and hypoxia-related metabolites production even if it preceded the metaboreflex activation by several minutes. Moreover, it was recently demonstrated that exercise in hypoxic conditions at a fixed load induces a stronger level of hypoxemia in athletes with a greater aerobic fitness than in less trained subjects (Van Thienen et al. 2016). This fact probably led the well-trained Experiment 1 population to undergo a greater hypoxic stimulus with respect to less-trained Experiment 2 subjects.

As far as SVR is concerned, this parameter was increased after the hypoxic test when compared to normoxic ones in both experiments, with SVR response being close to significance in Experiment 2. This indicates that the capacity to vasoconstrict the arteriolar bed was not impaired by hypoxia. Rather, it was enhanced, and this occurrence speaks against our initial hypothesis that hypoxia could reduce gross vascular resistance due to vasodilating metabolite accumulation.

A possible explanation for the increased SVR response found after hypoxic stimulation may be that the baroreflex activity was effective in counteracting the reduced CO response by recruiting the after-load reserve and inducing arteriolar constriction. This phenomenon explains the unchanged MAP found during the metaboreflex after and during hypoxia exposure. Hence, the baroreflex successfully maintained the MAP response, despite the reduced SV and CO response.

It remains to be explained why hypoxia affected the venous bed without modifying arteriolar vasoconstriction. It was likely due to hypoxia producing a variety of vasodilating metabolites, such as NO, adenosine, and prostaglandin-derived factors (Dinenno 2016; Marshall 2015). In this regard, it is to be noted that oral nitrates were found to exert a venodilator effect with a quantitatively lesser effect on arteriolar resistance vessels. In particular, it has been demonstrated that exercise sessions in the administration of NO-donors before the metaboreflex activation impairs cardiac pre-load by inducing venous dilation, whereas effects on SVR were less evident (Koole et al. 2000; Marongiu et al. 2013). Moreover, recent research showed that preconditioning maneuvers, able to augment the NO-production, resulted in an impaired possibility to induce venous constriction and to increase SV during the metaboreflex in healthy subjects (Mulliri et al. 2016). It is then possible, in the current

study, that the hypoxia increased the production of NO (likely the main vasodilatory contributor) and—to a lesser extent—other metabolites (prostaglandins?) which in turn restrained the sympathetic- induced venous constriction during the metaboreflex (Casey and Joyner 2012). This occurrence prevented the recruitment of the Frank-Starling mechanism and reduced SV response, although it must be acknowledged that this hypothesis remains speculative since the present study did not measure metabolite production.

Another possible explanation for the SV reduction found in our study is the hypoxia-mediated arteriolar vasoconstriction of the pulmonary vascular bed. Indeed, it is has been demonstrated that hypoxia exposure leads to an increase in pulmonary arteries vasoconstriction (Michiels 2004) that has been recently shown to produce an increased pulmonary artery systolic pressure (Stembridge et al. 2014; Stembridge et al. 2015b). It can be hypothesized that the augmented pulmonary vascular resistance reduced the volume of blood ejected by the RV into the pulmonary vascular bed, leading to a reduced LV filling and a consequent reduction of LV preload. This in turn resulted in an impaired SV. Echocardiographic studies are required to elucidate this possible explanation of SV reduction after and during acute exposure to hypoxia.

To summarize, our study proposed that hypoxia-induced metabolite production which restrained the sympathetic-induced venous constriction induced by the metaboreflex activation. This effect could have been further amplified by pulmonary vasoconstriction, leading to a reduced volume of blood filling the LV. This resulted in an impaired capacity to recruit the pre-load reserve and, in turn, prevented the possibility to increase SV during the metaboreflex.

It is to be highlighted that baroreflex activation successfully defended MAP by increasing gross vascular resistance notwithstanding the reduced SV. That is, there was a functional shift from a flow- to a vasoconstriction-mediated mechanism for maintaining the target blood pressure during the metaboreflex.

To the best of our knowledge, the present study is the first to report on the hemodynamics during the metaboreflex elicited immediately after a single bout of hypoxic exercise. In the past, metaboreflex was studied during hypoxia, but without the assessment of central hemodynamics (Gujic et al. 2007; Houssiere et al. 2005). Instead, only blood pressure and HR were collected along with SNA. Thus, there are no studies to compare the present results with. The paucity of research dealing with the hemodynamic consequences of intermittent hypoxia is somewhat surprising considering that the use of normobaric hypoxic devices has become popular in athletes and patients (Millet et al. 2016). However, the pros and cons of these practices have yet to be demonstrated. In particular, the transition from hypoxia to normoxia may be critical since the cardiovascular homeostasis is challenged. It should also be considered that mild hypoxia may be protective from ischemic events by inducing the phenomenon known as ischemic preconditioning and by promoting

neurogenesis (Marongiu and Crisafulli 2014; Tsai et al. 2013). Hence, the weight of scientific evidence about the effects of hypoxia on the cardiovascular apparatus remains equivocal since both detrimental and positive effects have been reported. Further study on the cardiovascular effects of hypoxia is then warranted.

Limitations of the study

One potential limitation of the present investigation was that we did not measure the production of any metabolite during the two hypoxic tests. This kind of assessment would have been useful as hypoxia is known to induce the production of a variety of vasodilating metabolites, such as NO, adenosine, and prostaglandin-derived factors (Dinenno 2016). Unfortunately, these measurements are complex, expensive, and somewhat invasive. However, results of SO_2 and COX confirm that a substantial hypoxia was induced by hypoxic tests in with respect to normoxic ones. Inasmuch as hypoxia is known to increase metabolites production (Casey and Joyner 2012), it was then likely that hypoxic bouts induced greater metabolic by-products accumulation than normoxic ones. Moreover, we could not measure VO_2 during hypoxic bouts since the gas analyzer was not set up to be used simultaneously with the hypoxic gas generator because of technical reasons.

Another limit was that the rise in MAP found during PEMI could potentially be due to the mechanical effect of the circulatory occlusion and not to metaboreflex activation. However, in our opinion, it is very unlikely that mechanical circulatory occlusion in one leg *per se* would cause substantial hemodynamic changes. Early as well as more recent findings reported that mechanical occlusion alone in one or two legs/arms could not induce any detectable hemodynamic effect. For example, already in 1976, Rowell and co-workers (Rowell et al. 1976) reported that total circulatory occlusion of both resting legs for 15 min had little or no effect on HR or MAP. They further concluded that a *work factor* (i.e., metabolites production) seems necessary for any significant cardiovascular response to be generated by muscle ischemia. Therefore, mechanical stimulation of muscle is not a major factor in generating pressor responses. Moreover, in the recent past some experiments conducted in our lab did not discover any hemodynamic effect due to regional circulatory occlusion (in one leg) not preceded by exercise (Crisafulli et al. 2008). Thus, results from these studies do not support the thesis that circulatory occlusion *per se* can cause significant hemodynamic changes.

A final potential limitation of the present investigation was that the study population was limited to young, healthy, Caucasian trained subjects and results can not be extended either to a general population. In conclusion, data from the present research demonstrated that exercise in acute hypoxia impairs the capacity to enhance venous return during the metaboreflex elicited in

normoxia, immediately after EH sessions. The reduced capacity to increase venous return negatively affects SV, which can not participate in the hemodynamic response during the metaboreflex. This in turn causes a functional shift from a flow- to a vasoconstriction-mediated mechanism by which the target blood pressure is achieved during the metaboreflex. Importantly, the described dysregulation was successfully counteracted by cardiovascular controlling mechanisms (probably the baroreflex), which maintained MAP notwithstanding the reduced SV.

CONCLUSIONS AND FUTURE DIRECTIONS

The results of my research illustrated the effects of exposure of both previous and concomitant hypoxia on the hemodynamic responses induced by metaboreflex activation. The hypoxia-mediated impairment of SV response to metaboreflex may explain, at least in part, the impaired exercise capacity that human beings experience when they are exposed to enviroments with reduced O2 content.

Considering that hypoxia is a challenge commonly faced in the aerospace environment, my research could give new useful hemodynamc information for aviation and space medicine. Further studies are required to better elucidate the mechanisms behind the hemdoynamic scenario depicted by my experiments. In particular, Echocardography could give direct measurements of venous return and arterial pulmonary pressure to confirm our hypothesis on what causes SV reduction.

TABLES AND FIGURES

	AT	W_{max}
Workload (W)	234 ± 38	297 ± 58
VO_2 (ml kg $^{-1}$ min $^{-1}$)	40.1 ± 10.9	45.8 ± 11.9
VO_2 (ml min $^{-1}$)	2537 ± 556	2903 ± 626
VCO_2 (ml kg $^{-1}$ min $^{-1}$)	2817 ± 650	3697 ± 960
RQ	1.11 ± 0.09	1.27 ± 0.14
Ve (l min $^{-1}$)	68.8 ± 15.5	98.1 ± 27.9
HR (bpm)	155 ± 7	168 ± 6

Table 1. Metabolic data values at the anaerobic threshold (AT) and at maximum workload (Wmax) collected during cardiopulmonary test of the subjects involved in Experiment 1.

	EN	EH 15,5%	EH 13,5%	*p* value condition effect	*p* value test effect
HR (bpm)	PEMI 77 ±11 CER 81 ± 10	PEMI 84 ± 9 CER 79 ± 8	PEMI 88 ± 9 CER 83 ± 9	0.102	0.338
SV (ml)	PEMI 62.2 ± 12.7 CER 64.5 ± 8.7	PEMI 53.1 ± 10.7 CER 54.8 ± 12.6	PEMI 52.9 ± 9.7 CER 59.0 ± 15.6	0.048	0.327
CO (l min^{-1})	PEMI 4.76 ± 0.9 CER 5.25 ± 1.00	PEMI 4.35 ± 1.13 CER 4.59 ± 1.19	PEMI 4.74 ± 1.15 CER 4.86 ± 1.19	0.306	0.322
VFR (ml s^{-1})	PEMI 161.0 ± 38.7 CER 194.0 ± 56.5	PEMI 174.2 ± 57.5 CER 152.1 ± 52.4	PEMI 177.2 ± 54.4 CER 174.4 ± 49.6	0.636	0.841
VER (ml s^{-1})	PEMI 259.8 ± 59.4 CER 268.9 ± 45.6	PEMI 240.0 ± 45.0 CER 241.8 ± 60.3	PEMI 242.9 ± 52.7 CER 255.7 ± 59.1	0.387	0.573
MAP (mmHg)	PEMI 81.6 ± 9.1 CER 83.1 ± 8.4	PEMI 81.9 ± 7.8 CER 84.6 ± 8.5	PEMI 85.6 ± 7.6 CER 83.5 ± 6.8	0.683	0.735
SVR (dynes s cm^{-5})	PEMI 1429.3 ± 379.3 CER 1310.8 ± 282.9	PEMI 1480.9 ± 289.7 CER 1649.6 ± 440.3	PEMI 1530.6 ± 424.2 CER 1474.9 ± 471.4	0.275	0.985

Table 2. Hemodynamic values during rest periods preceding the postexercise muscle ischemia (PEMI) and the control exercise recovery (CER) tests in the three conditions: *EN* exercise in normoxia; *EH15.5%* exercise in hypoxia with FiO2 = 15.5%; and *EH13.5%* exercise in hypoxia with FiO2 = 13.5%.

	EN	EH 15,5%	EH 13,5%	*p* value condition effect	*p* value test effect
HR (bpm)	PEMI 117 ± 14 CER 119 ± 13	PEMI 117 ± 10 CER 116 ± 10	PEMI 117 ± 10 CER 119 ± 12	0.823	0.831
SV (ml)	PEMI 99.7 ± 32.9 CER 124.4 ± 42.8	PEMI 115.6 ± 35.7 CER 125.3 ± 37.4	PEMI 105.1 ± 27.3 CER 109.7 ± 28.7	0.455	0.134
CO (l min^{-1})	PEMI 11.83 ± 4.3 CER 14.75 ± 4.9	PEMI 13.64 ± 5.06 CER 14.67 ± 5.08	PEMI 12.52 ± 4.30 CER 13.27 ± 4.63	0.694	0.206
VFR (ml s^{-1})	PEMI 488.4 ± 195.8 CER 640.4 ± 204.9	PEMI 556.4 ± 221.6 CER 621.0 ± 240.8	PEMI 531.7 ± 239.7 CER 572.9 ± 221.1	0.869	0.138
VER (ml s^{-1})	PEMI 510.1 ± 180.4 CER 585.8 ± 199	PEMI 572.2 ± 191.7 CER 586.7 ± 169.9	PEMI 513.5 ± 135.8 CER 544.8 ± 149.6	0.650	0.367
MAP (mmHg)	PEMI 97.9 ± 11.7 CER 97.9 ± 9.2	PEMI 96.3 ± 6.3 CER 94.6 ± 5.4	PEMI 95.9 ± 8.6 CER 93.1 ± 7.6	0.522	0.193
SVR (dynes s cm^{-5})	PEMI 768.5 ± 432.3 CER 609.3 ± 272.0	PEMI 623.3 ± 193.9 CER 578.6 ± 210.5	PEMI 664.4 ± 176.5 CER 620.6 ± 221.4	0.424	0.492

Table 3. Hemodynamic values at the third minute of exercise of the post-exercise muscle ischemia (PEMI) and the control exercise recovery (CER) tests in the three conditions: *EN* exercise in normoxia; *EH15.5%* exercise in hypoxia with FiO2 = 15.5%; and *EH13.5%* exercise in hypoxia with FiO2 = 13.5%.

	AT	W_{max}
Workload (W)	140.90 ± 31.68	252,27 ± 41,25
VO_2 (ml kg^{-1} min^{-1})	21.7.1 ± 3.26	34.69 ± 3.70
VO_2 (ml min^{-1})	1569 ± 420	2527 ± 466
VCO_2 (ml kg^{-1} min^{-1})	1771 ± 440	3533 ± 616
RQ	1.10 ± 0.1	1.40 ± 0.08
Ve (l min^{-1})	38.82 ± 10.9	94.02 ± 21.75
HR (bpm)	140 ± 12	174 ± 9

Table 4. Metabolic data values at the anaerobic threshold (AT) and at maximum workload (Wmax) collected during cardiopulmonary test of the subjects involved in Experiment 2.

	NORMO	HYPO	*p* value condition effect	*p* value test effect
HR (bpm)	PEMI 94.41 ± 12.76 CER 92.90 ± 11.63	PEMI 88.67 ± 8.80 CER 88.65 ± 11.63	0.828	0.163
SV (ml)	PEMI 60.45 ± 12.39 CER 63.77 ± 13.99	PEMI 60.45 ± 9.69 CER 69.10 ± 17.26	0.520	0.152
CO (l min^{-1})	PEMI 5.64 ± 1.23 CER 5.85 ± 1.28	PEMI 5.35 ± 0.97 CER 6.14 ± 1.89	0.998	0.240
VFR (ml s^{-1})	PEMI 230.52 ± 56.03 CER 246.47 ± 102.4	PEMI 195. 47 ± 59.44 CER 240.88 ± 116.96	0.444	0.251
VER (ml s^{-1})	PEMI 263.04 ± 54.62 CER 271.77 ± 46.29	PEMI 261.09 ± 50.30 CER 286.30 ± 71.46	0.714	0.325
MAP (mmHg)	PEMI 84.69 ± 11.37 CER 88.03 ± 7.55	PEMI 82,72 ± 11.03 CER 85.45 ± 11.37	0.475	0.342
SVR (dynes s cm^{-5})	PEMI 1249.06 ± 259.15 CER 1249.09 ± 311.23	PEMI 1285.95 ± 348.74 CER 1178.54 ± 265.89	0.853	0.554

Table 5. Hemodynamic values during the fourth minute of rest of postexercise muscle ischemia (PEMI) and the control exercise recovery (CER) tests in the two conditions: normoxia (NORMO) exercise in hypoxia with FiO2 = 13.5% (HYPO).

	NORMO	HYPO	*p* value condition effect	*p* value test effect
HR (bpm)	PEMI 146.19 ± 11.42 CER 139.58 ± 16.89	PEMI 146.08 ± 13.59 CER 144.91 ± 14.60	0.548	0.371
SV (ml)	PEMI 143.14 ± 44.95 CER 135.49 ± 54.02	PEMI 129.42 ± 47.70 CER 154.15 ± 48.97	0,868	0,567
CO (l min^{-1})	PEMI 21.21 ± 5.87 CER 18.91 ± 7.71	PEMI 18.96 ± 7.51 CER 22.72 ± 6.77	0.713	0.732
VFR (ml s^{-1})	PEMI 1180.72 ± 546.28 CER 978.12 ± 482.98	PEMI 850.27 ± 350.95 CER 1071.12 ± 464.89	0.404	0.949
VER (ml s^{-1})	PEMI 796.75 ± 239.71 CER 760.29 ± 215.00	PEMI 774.56 ± 313.81 CER 792.75 ± 274.21	0.949	0.909
MAP (mmHg)	PEMI 102.12 ± 11.86 CER 100.30 ± 13.94	PEMI 101.21 ± 9.69 CER 103.18 ± 11.26	0.783	0.983
SVR (dynes s cm^{-5})	PEMI 486.16 ± 221.48 CER 412.22 ± 123.96	PEMI 473.36 ± 140.85 CER 389.72 ± 116.33	0.949	0.909

Table 6. Hemodynamic values at the third minute of exercise of postexercise muscle ischemia (PEMI) and the control exercise recovery (CER) tests in the two conditions: normoxia (NORMO) exercise in hypoxia with FiO2 = 13.5% (HYPO).

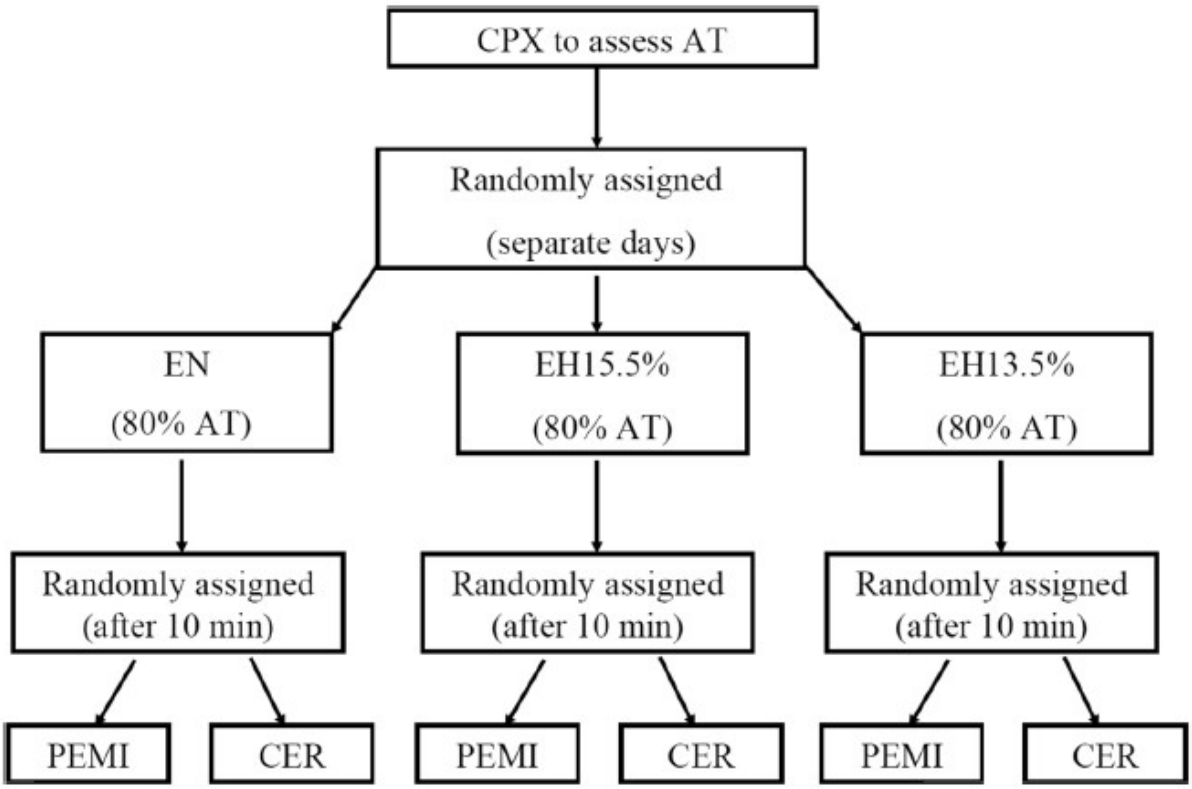

Fig. 1. Experiment 1 study design. After the cardiopulmonary test (CPX, interval 4–7 days), subjects underwent in separate days (interval 3–5 days), three randomly assigned exercise sessions, each lasting 10 min at a workload corresponding to 80% of the AT previously measured during the CPX. (1) Exercise session in normoxia (EN); (2) exercise session in normobaric hypoxia with a FiO_2 of 15.5% (EH15.5%); (3) and finally, an exercise session in normobaric hypoxia with a FiO_2 of 13.5% (EH13.5%). After each exercise session, participants underwent randomly assigned the post-exercise muscle ischemia (PEMI) and the control exercise recovery (CER) test to study the metaboreflex.

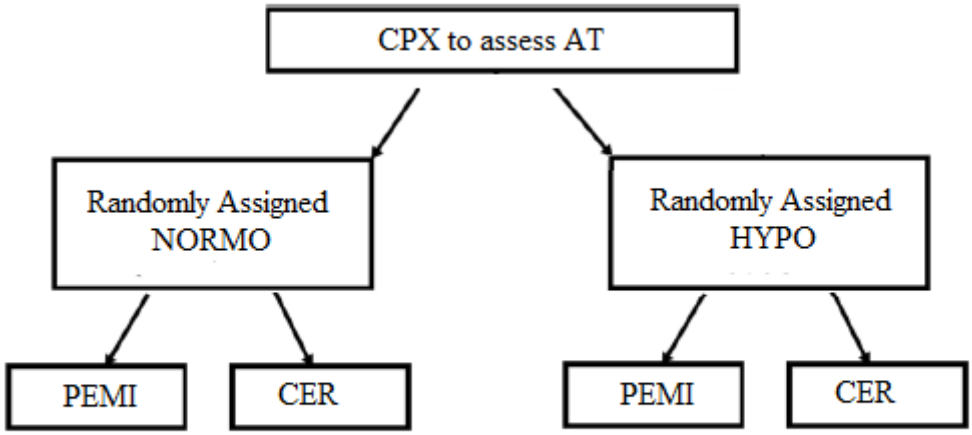

Fig. 2. Experiment 2 study design. After the CPX (4-7 days interval), the subjects underwent 2 randomly assigned metaboreflex activation protocols (PEMI/CER) in separate days (interval 3-5 days) in normoxia (NORMO) and normobaric hypoxia (HYPO). The hypoxic stimulus was induced by letting the subjects breathe a gas mixture with 13.5 % of FiO_2.

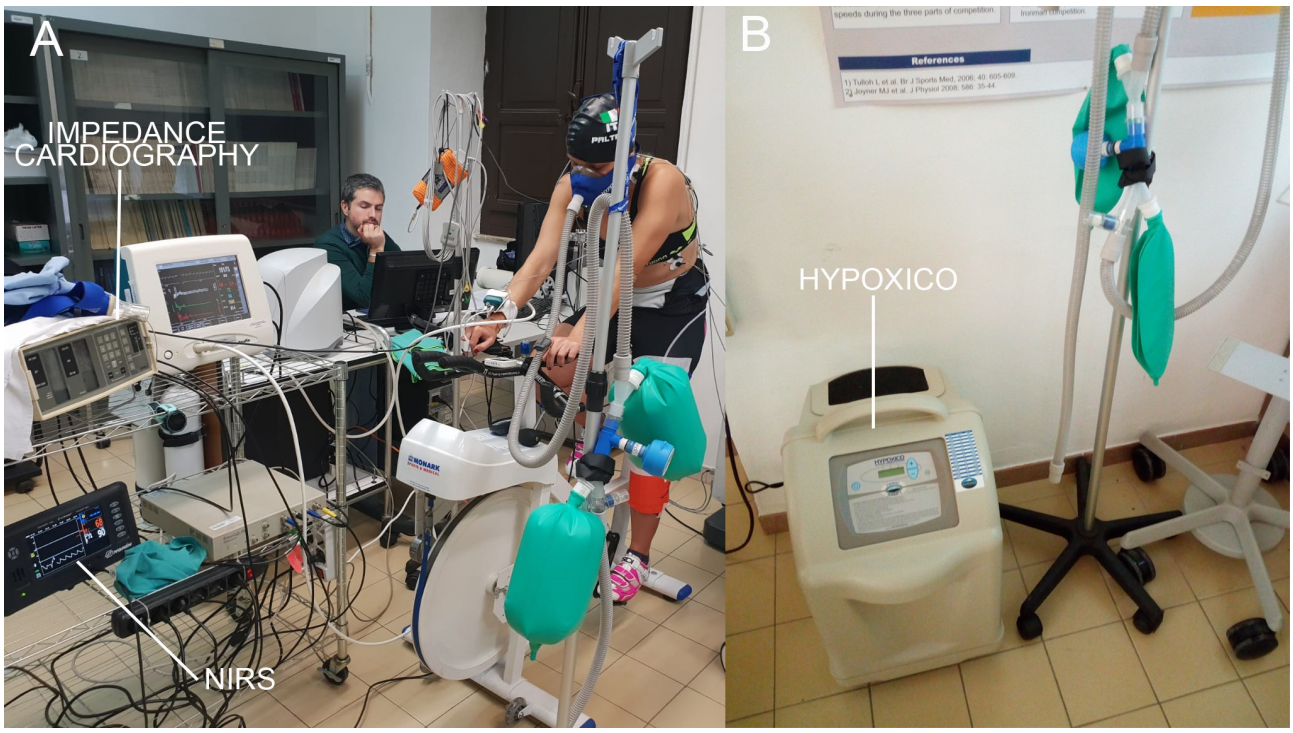

Fig 3. Experimental setup. **A**. The figure shows a subject instrumented with Impedance Cardiography, NIRS (near-infrared spectroscopy) and the mask of the hypoxic gas generator Hypoxico. **B**. Hypoxico main unit.

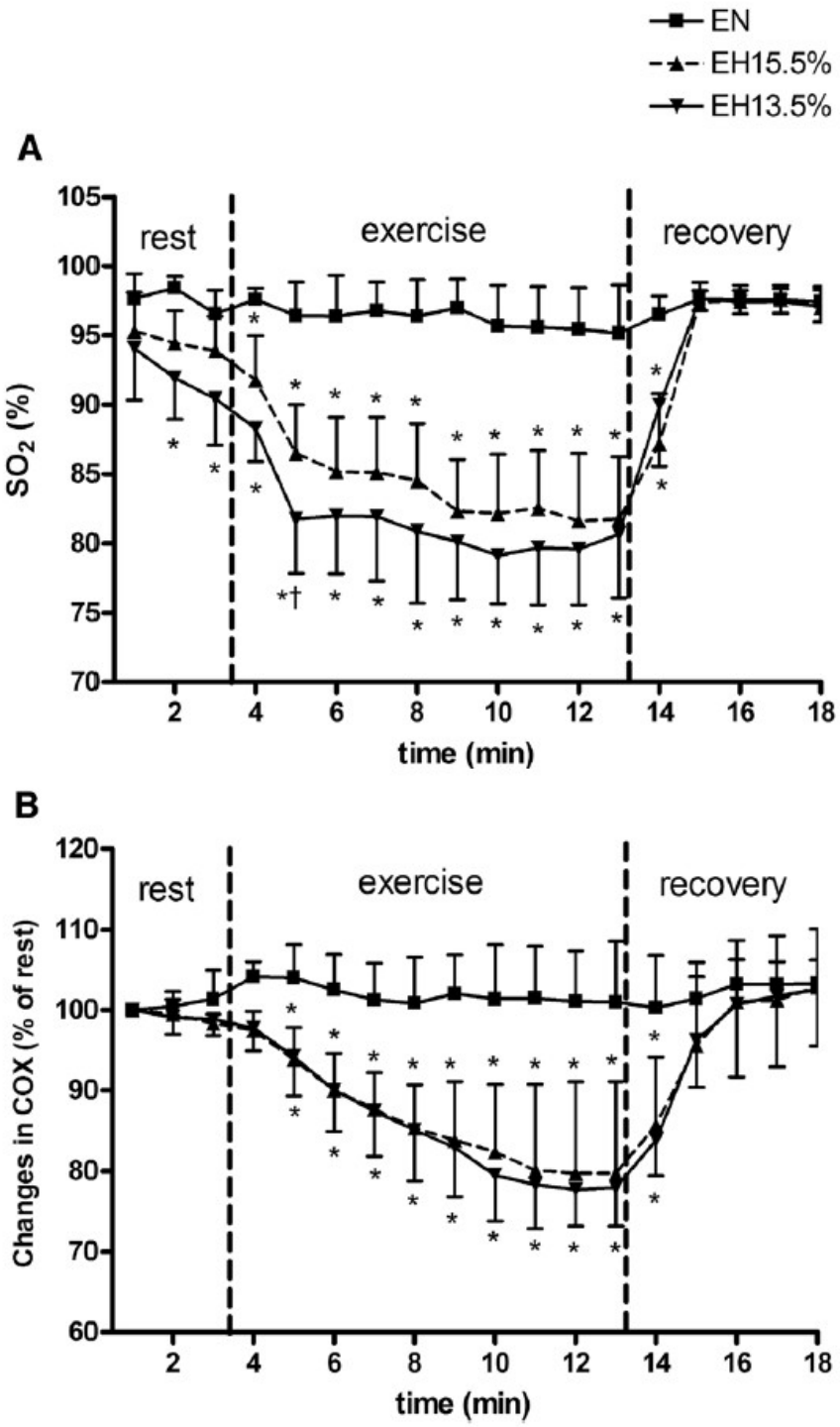

Fig. 4. A. Changes in the level of peripheral blood O_2 saturation (SO_2) during the sessions of exercise in normoxia (EN), in normobaric hypoxia with a FiO_2 of 15.5% (EH15.5%), and in normobaric hypoxia with FiO_2 of 13.5% (EH13.5%). **B** Shows changes in cerebral oxygenation (COX) during the same tests. Values are mean ± SD. $N = 10$. *$p < 0.05$ vs. EN; †$p < 0.05$ vs. EH15.5%

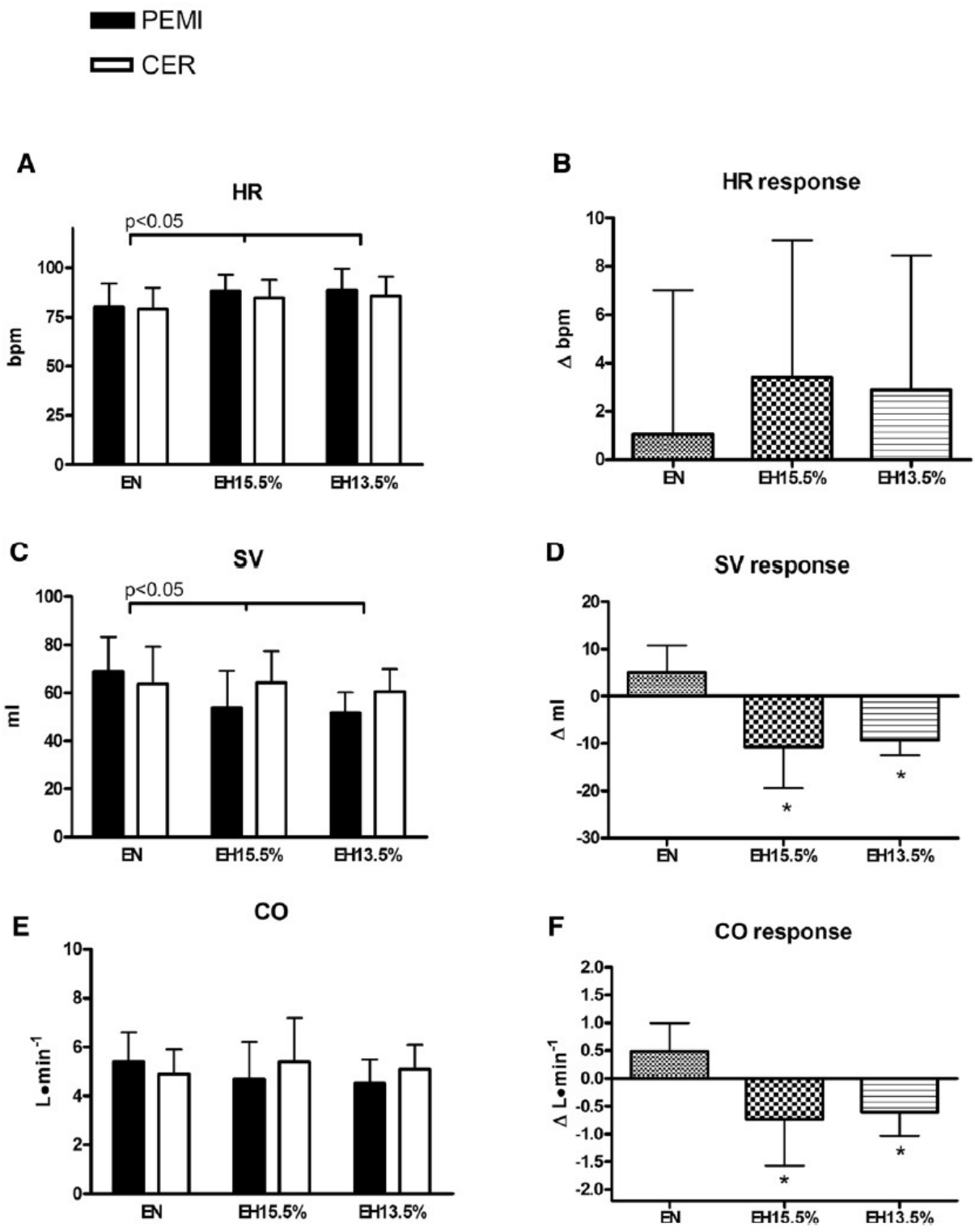

Fig. 5 Absolute values and responses of cardiovascular parameters during the postexercise muscle ischemia (PEMI) and the control exercise recovery (CER) test, conducted after exercise in normoxia (EN), in normobaric hypoxia with a FiO2 of 15.5% (EH15.5%), and in normobaric hypoxia with FiO2 of 13.5% (EH13.5%). *HR* Heart rate (**A,B**), *SV* stroke volume (**C,D**), and *CO* cardiac output (**E,F**). Responses were calculated as the difference between the PEMI and the CER test at the third minute of recovery. Values are mean ± SD. $N = 10$. Horizontal brackets indicate the significant ($p < 0.05$) overall main effect of condition. There were no interaction effects. *$p < 0.05$ vs. EN

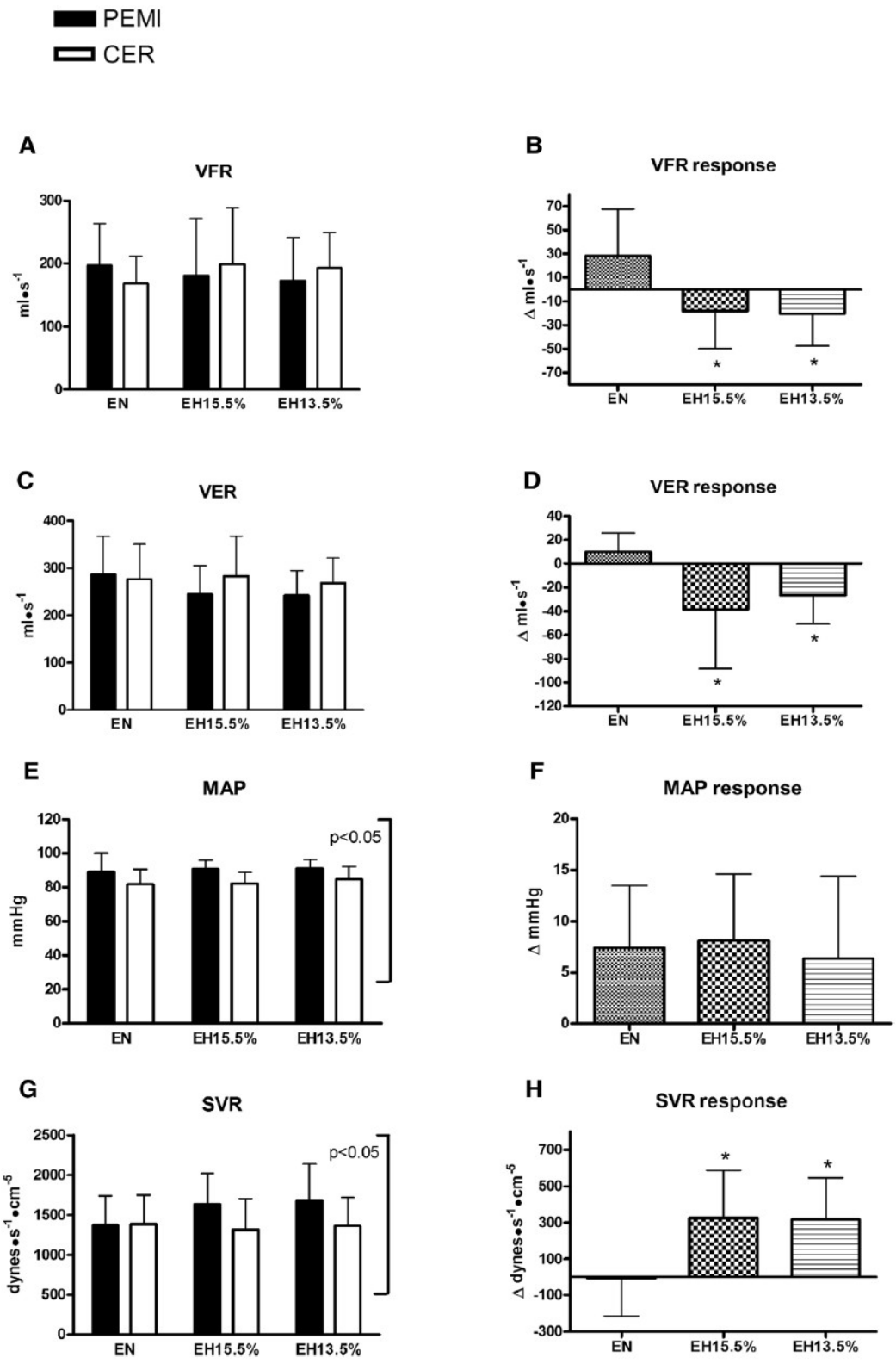

Fig. 6. Absolute values and responses of cardiovascular parameters during the postexercise muscle ischemia (PEMI) and the control exercise recovery (CER) test conducted after exercise in normoxia (EN), in normobaric hypoxia with a FiO2 of 15.5% (EH15.5%), and in normobaric hypoxia with FiO2 of 13.5% (EH13.5%). *VFR* Ventricular filling rate (**A,B**), *VER* ventricular ejection rate (**C,D**), *MAP* mean arterial pressure (**E,F**), and *SVR* systemic vascular resistance (**G,H**). Responses were calculated as the difference between the PEMI and the CER test at the third minute of recovery (see text for further details). Values are mean ± SD. $N = 10$. Vertical brackets indicate the significant ($p < 0.05$) overall main effect of test. There were no interaction effects. *$p < 0.05$ vs. EN.

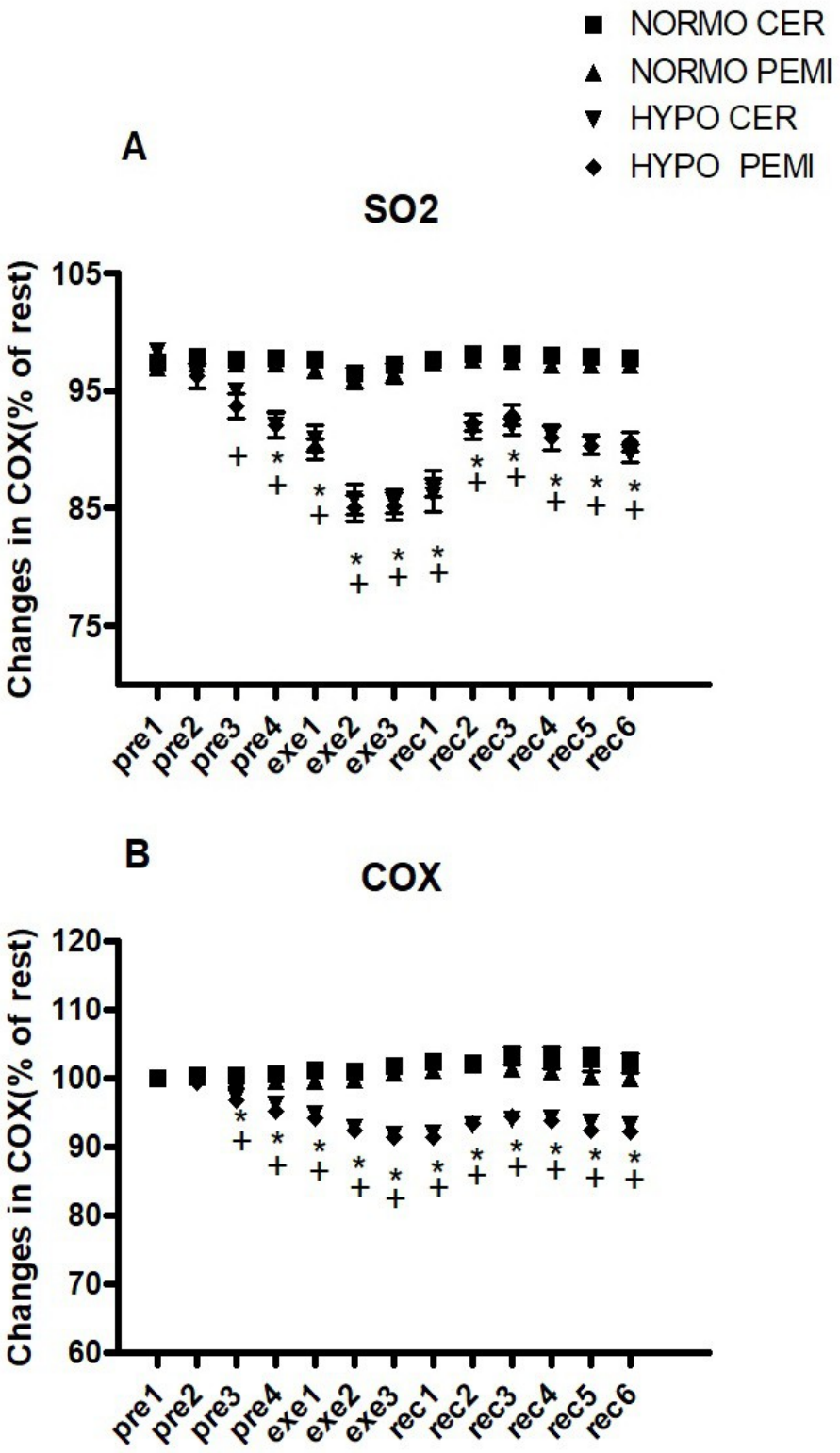

Fig. 7 A. Changes in the level of peripheral blood O_2 saturation (SO_2) during the PEMI and CER sessions in normoxia (NORMO), and hypoxia (HYPO) with a FiO_2 of 13.5%). **B** Shows changes in cerebral oxygenation (COX) during the same tests. Values are mean ± SD. $N = 11$. * $p < 0.05$ vs. HYPO CER vs. NORMO CER. + $p < 0.05$ vs. HYPO PEMI vs NORMO CER.

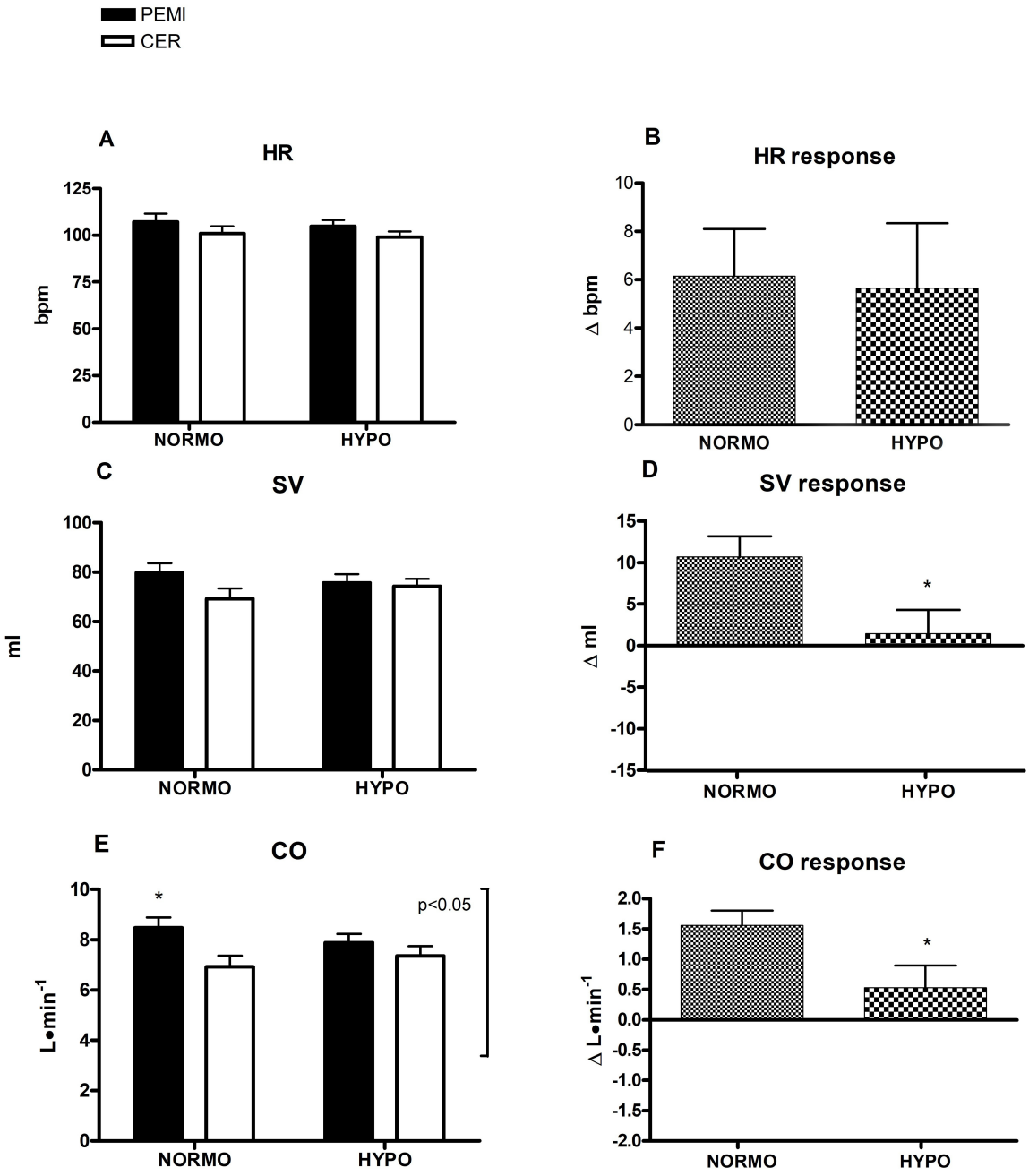

Fig. 8. Absolute values and responses of cardiovascular parameters during the postexercise muscle ischemia (PEMI) and the control exercise recovery (CER) test, conducted in normoxia (NORMO), and in normobaric hypoxia with FiO2 of 13.5% (HYPO). *HR* Heart rate (**A,B**), *SV* stroke volume (**C,D**), and *CO* cardiac output (**E,F**). Responses were calculated as the difference between the PEMI and the CER test at the third minute of recovery. Values are mean ± SD. $N = 11$. Vertical brackets indicate the significant ($p < 0.05$) overall main effect of test. There were no interaction effects. *$p <$ 0.05 NORMO PEMI vs. NORMO CER for the absolute values analysis, vs NORMO for the response analysis.

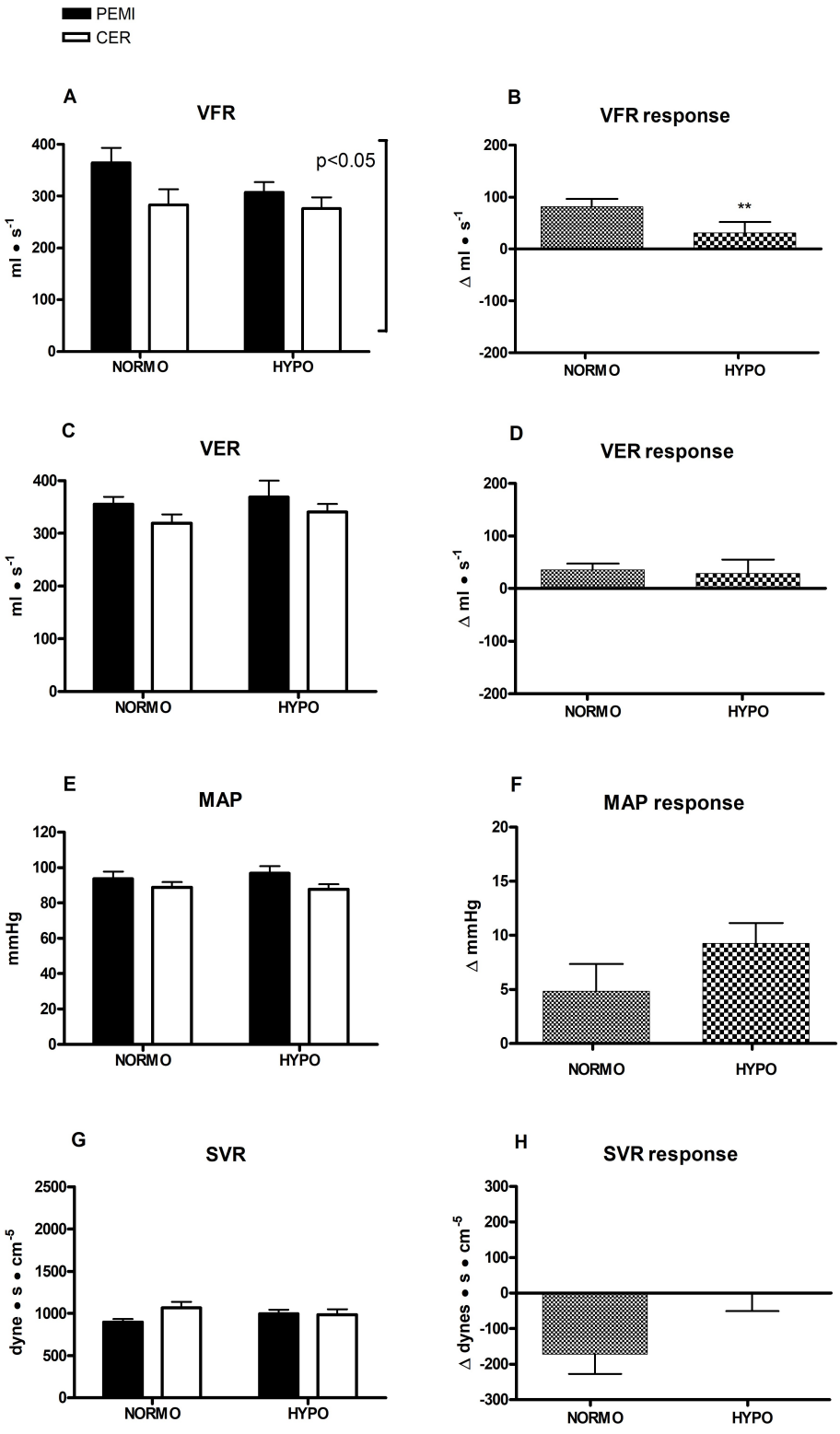

Fig. 9. Absolute values and responses of cardiovascular parameters during the postexercise muscle ischemia (PEMI) and the control exercise recovery (CER) test conducted in normoxia (NORMO), and in normobaric hypoxia with FiO2 of 13.5% (HYPO). *VFR* Ventricular filling rate (**A,B**), *VER* ventricular ejection rate (**C,D**), *MAP* mean arterial pressure (**E,F**), and *SVR* systemic vascular resistance (**G,H**). Responses were calculated as the difference between the PEMI and the CER test at the third minute of recovery (see text for further details). Values are mean ± SD. $N = 11$. Vertical brackets indicate the significant ($p < 0.05$) overall main effect of test. There were no interaction effects. $^{**}p < 0.01$ vs. NORMO.

REFERENCES

1. Aaronson PI, Robertson TP, and Ward JP. Endotheliumderived mediators and hypoxic pulmonary vasoconstriction. Respir Physiol Neurobiol 2002, 132:107–120.
2. Albert TS, Tucker VL, Renkin EM. Atrial natriuretic peptide levels and plasma volume contraction in acute alveolar hypoxia. J Appl Physiol. 1997, 82:102–110;
3. Archer SL, Souil E, Dinh-Xuan AT, et al. Molecular identification of the role of voltage-gated K+ channels, Kv1.5 and Kv2.1, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. J Clin Invest 1998, 101:2319–2330.
4. Bao X, Kennedy BP, Hopkins SR, Bogaard HJ, Wagner PD, Ziegler MG. Human autonomic activity and its response to acute oxygen supplement after high altitude acclimatization. Auton Neurosci. 2002; 102: 54–59;
5. Bastos BG, Williamson JW, Harrelson T, Nôbrega ACL. Left ventricular volumes and hemodynamic responses to postexercise ischemia in healthy humans. Med Sci Sports Exerc. 2000; 32: 1114–1118;
6. Beaver WL, Wasserman K, Whipp BJ.A new method for detecting anaerobic threshold by gas exchange. J Appl Physiol. 1986; 60:2020–2027;
7. Bergfeld GR, Forrester T. Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. Cardiovasc Res 1992, 26: 40–47,
8. Berglund B., Fleck S. J., Kearney J. T., Wide L. Serum erythropoietin in athletes at moderate altitude. Scand. J. Med. Sci. Sports. 1992 2:21–25
9. Bernstein DP. A new stroke volume equation for Thoracic Electrical Bioimpedance: theory and rationale. Crit Care Med. 1986; 14:904–909;
10. Bisgard GE, Busch MA, Forster HV. Ventilatory acclimatization to hypoxia is not dependent on cerebral hypocapnic alkalosis. J Appl Physiol. 1986;60:1011–1015;
11. Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA. Nitric oxide directly activates calciumdependent potassium channels in vascular smooth muscle. Nature 1994, 368: 850–853;
12. Boushel R, Calbet JA, Radegran G, et al. Parasympathetic neural activity accounts for the lowering of exercise heart rate at high altitude. Circulation. 2001;104:1785–1791;
13. Buttergeit F, Brand MD. A hierarchy of ATP-consuming processes in mammalian cells. Biochem J 1995. 312; 163-167;
14. Calbet JA, Boushel R, Robach P,Hellsten Y, Saltin B, Lundby C.Chronic hypoxia increases arterialblood pressure and reducesadenosine and ATP inducedvasodilatation in skeletal

muscle inhealthy humans. Acta Physiol (Oxf). 2014; 211: 574–584;
15. Calbet JA, Radegran G, Boushel R, Sondergaard H, Saltin B, Wagner PD. Plasma volume expansion does not increase maximal cardiac output or VO2 max in lowlanders acclimatized to altitude. Am J Physiol Heart Circ Physiol. 2004; 287: H1214–H1224;
16. Casey DP, Joyner MJ. Compensatory vasodilatation during hypoxic exercise: mechanisms responsible for matching oxygen supply to demand. J Physiol. 2012 590:6321–6326;
17. Crisafulli A, Marongiu E, Ogoh S. Cardiovascular reflexes activity and the interaction during exercise. Biomed Res Int. 2015; 2015:394183;
18. Crisafulli A, Milia R, Lobina A, Caddeo M, Tocco F, Concu A, Melis F. Haemodynamic effect of metaboreflex activation in men after running above and below the velocity of the anaerobic threshold. Exp Physiol. 2008; 93(4): 447-57;
19. Crisafulli A, Milia R, Lobina A, Caddeo M, Tocco F, Concu A, Melis F. Hemodynamic effect of metaboreflex activation in men after running above and below the velocity of the anaerobic threshold. Exp Physiol. 2008; 93:447–457;
20. Crisafulli A, Milia R, Vitelli S, Caddeo M, Tocco F, Melis F, Concu A. Hemodynamic responses to metaboreflex activation: insights from spinal cord-injured humans. Eur J Appl Physiol. 2009; 106:525–533;
21. Crisafulli A, Piras F, Filippi M, Piredda C, Chiappori P, Melis F, Milia R, Tocco F, Concu A. Role of heart rate and stroke volume during muscle metaboreflex-induced cardiac output increase: differences between activation during and after exercise. J Physiol Sci. 2011; 61:385–394;
22. Crisafulli A, Scott AC, Wensel R, Davos CH, Francis DP, Pagliaro P, Coats AJ, Piepoli MF. Muscle metaboreflex-induced increases in stroke volume. Med Sci Sports Exerc. 2003; 35(2): 221-8;
23. Crisafulli A, Tocco F, Milia R, Angius L, Pinna M, Olla S, Roberto S, Marongiu E, Porcu M, Concu A. Progressive improvement in hemodynamic response to muscle metaboreflex in heart transplant recipients. J Appl Physiol. 2013; 114:421–427;
24. Crisafulli A. The impact of cardiovascular diseases on cardiovascular regulation during exercise in humans: studies on metaboreflex activation elicited by the post-exercise muscle ischemia method. Curr Cardiol Rev. 2017;13:293–300;
25. Cruicksahnk S, Hirschauer N. The alveolar gas equation. Continuing education in Anesthesia, Critical Care & Pain. 2004; volume 4 Num 1;
26. Dart C, Standen NB. Activation of ATP-dependent K+ channels by hypoxia in smooth muscle cells isolated from the pig coronary artery. J Physiol 1995, 483: 29-39
27. Demontis GC, Germani MM, Caiani EG, Barravecchia I, Passino C, Angeloni D. Human

pathophysiological adaptations to the space environment. Front. Physiol. 2017: 8:547. doi: 10.3389/fphys.2017.00547;

28. Dhar P, Sharma VK, Hota KB, Das SK, Hota SK, Srivastava RB, Singh SB. Autonomic cardiovascular responses in acclimatized lowlanders on prolonged stay at high altitude: a longitudinal follow up study. PLoS ONE. 2014; 9: e84274;
29. Dinenno FA. Skeletal muscle vasodilation during systemic hypoxia in humans. J Appl Physiol (1985) 2016 Jan 15, 120(2): 216-225;
30. Eckardt K. U., Boutellier U., Kurtz A., Schopen M., Koller E. A., Bauer C Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. J. Appl. Physiol. 1989 66: 1785–1788 ;
31. Ellsworth ML, Ellis CG, Goldman D, Stephenson AH, Dietrich HH, Sprague RS. Erythrocytes: oxygen sensors and modulators of vascular tone. Physiology (Bethesda) 2009, 24: 107–116,
32. Ellsworth ML, Forrester T, Ellis CG, Dietrich HH. The erythrocyte as a regulator of vascular tone. Am J Physiol Heart Circ Physiol 1995, 269: H2155–H2161,
33. Fischer JP, White MJ. Muscle afferent contributions to the cardiovascular response to isometric exercise. Exp Physiol. 2004; 89(6): 639-46;
34. Friedmann B, Frese F, Menold E, Bärtsch P. Individual variation in the reduction of heart rate and performance at lactate thresholds in acute normobaric hypoxia. Br J Sports Med. 2005, 39:148–153;
35. Fulco CS, Rock PB, Cymerman A. Maximal and submaximal exercise performance at altitude. Aviat Space Environ Med. 1998; 69:794–801;
36. Gledhill N, Cox D, Jamnik R. Endurance athletes' stroke volume does not plateau: major advantage is diastolic function. Med Sci Sports Exerc. 1994; 26:1116–1121;
37. Gore CJ, Hahn AG, Scroop GC, et al. Increased arterial desaturation in trained cyclists during maximal exercise at 580 m altitude. J Appl Physiol. 1996; 80:2204–2210;
38. Gujic M, Laude D, Houssiere A, Beloka S, Argacha JF, Adamopoulos D, Xhaët O, Elghoxi JL, Van de Borne P. Differential effects of metaborecptor and chemoreceptor activaion on sympathetic and cardiac baroreflex control following exercise in hypoxia in human. J Physiol. 2007; 585:165-174;
39. Halliwill JR, Morgan BJ, CharkoudianN. Peripheral chemoreflex andbaroreflex interactions incardiovascular regulation in humans. J Physiol. 2003; 552: 295–302;
40. Hannon JP, Shields JL, Harris CW. Effects of altitude acclimatization on blood composition of women. J Appl Physiol. 1969, 26:540–547;
41. Hansen J, Sander M. Sympathetic neural overactivity in healthy humans after prolonged

exposure to hypobaric hypoxia. J Physiol. 2003; 546: 921–929;
42. Hardie DG. Hawley SA. AMP-activated protein kinase: the energy charge hypothesis revised. Bioessays .2001; 23: 1112-1119;
43. Hoiland RL, Bain RA, Rieger MG, Bailey DM, Ainslie PN. Hypoxiemia, oxygen content and the regulation of cerebral blood flow. AM H Phsyiol Regul Integr Comp Physiol. 2016 Mar 1: 310 (5): R398-413;
44. Hollander AP, Bouman LN. Cardiac acceleration in manelicited by a muscle heart reflex. J Appl Physiol. 1975; 38 (2); 272-8;
45. Horstman D, Weiskopf R, Jackson RE. Work capacity during 3-wk sojourn at 4,300 m: effects of relative polycythemia. J Appl Physiol. 1980; 49:311–318:
46. Houssiere A, Najem B, Ciarka A, Velez-Roa S, Naeije R, Van de Borne P. Chemoreflex and metaboreflex control during static hypoxic exercise. Am J Physiol Heart Circ Physiol. 2005; 288: H1724-H1729;
47. Howley ET, Bassett DR, Welch HG. Criteria for maximal oxygen uptake: review and commentary. Med Sci Sports Exerc. 1995; 27:1292–1301;
48. Hu C, Sataur L, Wang L, Chen H, Simon M. The N-terminal transactivation domain confers target gene specificity of hypoxia inducible factors HIF-1α and HIF-2α. Mol Biol Cell 2007; 18: 4528-4542;
49. Hue L. Rider MH. Role of fructose 2,6 bisphosphate in the control of glycolisis in mammalian tissues. Biochem J. 1987; 245:313-324;
50. Hulme JT, Coppock EA, Felipe A, Martens JR, and Tamkun MM Oxygen sensitivity of cloned voltage-gated K(+) channels expressed in the pulmonary vasculature. Circ Res 1999, 85:489–497.
51. Iellamo F, Legramante JM, Raimondi G, Peruzzi G. Baroreflex control of sinus node during dynamic exercise in humans: effects of central command and muscle reflexes. Am J Physiol. 1997; 273 (3 pt 2): H 1157-64;
52. Iellamo F, Pizzinelli P, Massaro M, Raimondi G, Peruzzi G, Legramante JM. Muscle metaboreflex contribution to sinus node regulation during static exercise: insights from spectral analysis of heart rate variability. Circulation. 1999; 100(1): 27-32;
53. Iellamo F. Baroreflex control of heart during exercise: a topic of perennial conflict. J Appl Pysiol. 2001; 90 (3): 1184-5;
54. Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. Nature 1996 Mar 21, 380 (6571): 221-6
55. Joyner MJ. Baroreceptor function during exercise: resetting the record. Exp physiol. 2006; 91(1): 27-36;

56. Kacimi R, Richalet JP, Crozatier B. Hypoxia-induced differential modulation of adenosinergic and muscarinic receptors in rat heart. J Appl Physiol. 1993; 75: 1123– 1128;
57. Kato H, Menon AS, Slutsky AS.Mechanisms mediating the heart rateresponse to hypoxemia. Circulation. 1988; 77: 407–414;
58. Kim-Shapiro DB, Schecter AN, Gladwin MT. Unraveling the reactions of Nitric Oxide, Nitrite and Hemoglobin in Physiology and Therapeutics. Arterioscler Thromb Vasc Biol 2006 Apr; 26:697-605
59. Klausen K. Cardiac output in man at rest and work during and after acclimatization to 3,800 m. J Appl Physiol. 1966;21:609-616;
60. Koller EA, Drechsel S, Hess T, Macherel P, Boutellier U. Effects of atropine and propranolol on the respiratory, circulatory, and ECG responses to high altitude in man. Eur J Appl Physiol Occup Physiol 1988; 57: 163–172;
61. Koole MAC, Aerts A, Praet J, Franken P, Dendale P, Block P. Venous pooling during nitrate-stimulated tilt testing in patients with vasovagal syncope. Europace. 2000; 2:343–345;
62. Koyasu S, Kobayashi M, Goto Y, Hiroaka M, Harada H. Regulatory mechanisms of hypoxia inducible factor 1 activity: two decades of knowledge. Cancer Sci 2018 Mar; 109(3): 560-571;
63. Lahiri S, Mulligan E, Nishino T,Mokashi A, Davies RO. Relativeresponses of aortic body and carotidbody chemoreceptors tocarboxyhemoglobinemia. J ApplPhysiol Respir Environ ExercPhysiol. 1981, 50: 580–586;
64. Levine BD, Stray-Gundersen J. “Living high-training low”: effect of moderate-altitude acclimatization with low-altitude training on performance. J Appl Physiol. 1997;83:102–112;
65. Lewis P. Is hypoxia-induced skeletal muscle dysfunction lost in space or just a matter of a time? J. Physiol. 2018; 596, 2959–2960. doi: 10.1113/JP276033;
66. Luks AM, Swenson ER, Bartsch P. Acute high-altitude sickness. Eur Respir Rev. 2017; Jan 31 26(143);
67. Lundby C, Araoz M, van Hall G. Peak heart rate decreases with increasing severity of acute hypoxia. High Alt Med Biol. 2001;2:369–376;
68. Lundby C, Calbet JAL, van Hall G, et al. Pu lmonary gas exchange at maximal exercise in Danish lowlanders during 8 wk of acclimatization to 4,100 m and in high-altitude Aymara natives. Am J Physiol Regul Integr Comp Physiol. 2004, 287:R1202–R1208;
69. Lundby C. Physiologic systems and their responses to condition of Hypoxia. ACSM's Advanced Exercise Physiology 2nd edition. Lippincott Williams & Wilkins 2013;

70. Lundby C., Calbet J., Robach P. The response of human skeletal muscle tissue to hypoxia. Cell. Mol. Life Sci.. 2009: 66, 3615–3623;
71. Magnani S, Roberto S, Sainas G, Milia R, Palazzolo G, Cugusi L, Pinna V, Doneddu A, Hosseini Kakhak SA, Tocco F, Mercuro G, Crisafulli A. Metaboreflex-mediated hemodynamic abnormalities in individuals with coronary artery disease without overt signs or symptoms of heart failure. Am J Physiol (Heart Circ Physiol). 2018; 314(3):H452–H463;
72. Marongiu E, Crisafulli A. Cardioprotection acquired through exercise: the role of ischemic preconditioning. Curr Cardiol Rev. 2014; 10:336–348;
73. Marongiu E, Piepoli MF, Milia R, Angius L, Pinna M, Bassareo P, Roberto S, Tocco F, Concu A, Crisafulli A. Effects of acute vasodilation on the hemodynamic response to muscle metaboreflex. Am J Physiol (Heart Circ Physiol), 2013; 305:H1387–H1396;
74. Marshall JM. Interactions between local dilator and sympathetic vasoconstrictor influences in skeletal muscle in acute and chronic hypoxia. Exp Physiol. 2015; 100:1400–1411;
75. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L. Phosphorylation and activation of heart PFK-2 by AMPK has a role in stimulation of glycolisis during ischemia. Curr Biol 2000. 10; 124-1255;
76. Mazzeo RS, Brooks GA, Butterfield GE, Podolin DA, Wolfel EE, Reeves JT. Acclimatization to high altitude increase muscle sympathetic activity both at rest and during exercise. Am J Physiol. 1995; 269: R201–R207;
77. Michiels C. Physiological and pathological responses to Hypoxia. Am J Pathol. 2004 Jun; 164(6): 1875-82;
78. Michiels. Hardie DG. Metabolic control: a new solution to an old problem. Curr Biol, 2001; 10: R757-759;
79. Michiels. Kemp BE, Mitchellhill KI, Stapleton D. Michell BJ, Chen ZP, Witters LA. Dealing with energy demand: the AMP activated protein kinase. Trend Biochem Sci. 1999; 24: 22-25;
80. Milia R, Roberto S, Mulliri G, Loi A, Marcelli M, Sainas G, Milia N, Marongiu E, Crisafulli A. Effect of aging on hemodynamic response to metaboreflex activation. Eur J Appl Physiol. 2015; 115:1693–1703;
81. Millet GP, Debevec T, Brocherie F, Malatesta D, Girard O. Therapeutic use of exercising in hypoxia: promises and limitations. Front Physiol. 2016; 10:7:224;
82. Moran D, Epstein Y, Keren G, Laor A, Sherez J, Y Shapir. Calculation of mean arterial pressure during exercise as a function of heart rate. Appl Human Sci. 1995; 14:293–295;
83. Mulliri G, Sainas G, Magnani S, Palazzolo G, Milia N, Orrù A, Roberto S, Marongiu E, Milia R, Crisafulli A. Ischemic preconditioning reduces hemodynamic response during

metaboreflex activation. Am J Physiol (Regul Integr Comp Physiol). 2016; 310:R777–R787;

84. Naeije R. Physiological adaptation of the cardiovascular system to high altitude. Progress in Cardiovascular Diseases. 2010; 52: 456-466;
85. Niewinski P, Janczak D, Rucinski A,Tubek S, Engelman ZJ, Jazwiec P,Banasiak W, Sobotka PA, Hart EC,Paton JF, Ponikowski P.Dissociation between blood pressureand heart rate response to hypoxiaafter bilateral carotid body removalin men with systolic heart failure.Exp Physiol. 2014; 99: 552–561;
86. Nobrega ACL, O'Leary D, Silva BM, Marongiu E, Piepoli MF, Crisfulli A. Neural regulation of cardiovascular response to exercise. Role of the central command and peripheral afferents. Biomed Res Int. 2014; 2014:478965;
87. Ogoh S, Fisher JP, Dawson EA, White MJ, Secher NH, Raven PB. Autonomic nervous system influence on arterial baroreflex control of heart rate during exercise in humans. J Physiol. 2005; 566(pt 2): 599-611:
88. Pearce WJ, Reynier-Rebuffel AM, Lee J, Aubineau P, Ignarro L, Seylaz J. Effects of methylene blue on hypoxic cerebral vasodilatation in the rabbit. J Pharmacol Exp Ther 1990; 254: 616–625;
89. Perini R, Milesi S, Biancardi L,Veicsteinas A. Effects of highaltitude acclimatization on heart ratevariability in resting humans. Eur JAppl Physiol Occup Physiol. 1996; 73: 521–528;
90. Peronnet F, Thibault G, Cousineau DL. A theoretical analysis of the effect of altitude on running performance. J Appl Physiol. 1991, 70:404;
91. Piepoli M, Clark AL, Coats AJ. Muscle metaboreceptors in hemodynamic, autonomic and ventilatory responses to exercise in men. Am J Physiol. 1995; 269 (4 pt 2): h1428-36;
92. Ploszczyca K, Langfort J, Czuba M. The effects of altitude training on erytrhopoietic response and hematologial variables in adult athletes: a narrative review. Front Physiol. 2018 apr 11:9:375;
93. Potts JT, Shi XR, Raven PB. Carotid baroreflex responsiveness during dynamic exercise in humans. Am J Physiol. 1993; 265 (6 pt 2): H 1928-38;
94. Princi T, Zupet P, Finderle Z, AccardoA. Linear and nonlinear assessmentof heart rate variability innonacclimatized middleaged subjectsat different hypoxic levels. BiomedSci Instrum. 2008; 44: 380–385;
95. Querido JS, Wehrwein EA, Hart EC,Charkoudian N, Henderson WR,Sheel AW. Baroreflex control ofmuscle sympathetic nerve activity asa mechanism for persistentsympathoexcitation following acutehypoxia in humans. Am J PhysiolRegul Integr

Comp Physiol. 2011; 301: R1779–R1785;
96. Raven PB, Fadel PJ, Ogoh S. Arterial Baroreflex resetting during exercise: a current perspective. Exp Physiol. 2006; 91(1): 37-49;
97. Reeves JT, Groves BM, Sutton JR, Wagner PD, Cymerman A, Malconian MK, Rock PB, Young PM, Houston CS. Operation Everest II: preservation of cardiac function at extreme altitude. J Appl Physiol. 1987; 63: 531–539;
98. Robach P, Calbet JAL, Thomsen JJ, et al. The ergogenic effect of recombinant human erythropoietin on O 2max depends on the severity of arterial hypoxemia. PLos ONE. 2008; 3:e2996;
99. Roberto S, Marongiu E, Pinna M, Angius L, Olla S, Bassareo P, ToccoF, Concu A, Milia R, Crisafulli A. Altered hemodynamics during muscle metaboreflex in young, type 1 diabetes patients. J Appl Physiol. 2012;113:1323–1331;
100. Roberto S, Mulliri G, Milia R, Solinas R, Pinna V, Sainas G, Piepoli MF, Crisafulli A. Hemodynamic response to muscle reflex is abnormal in patients with heart failure with preserved ejection fraction. J Appl Physiol. 2017; 22(2):376–385;
101. Rowell LB, Blackmon JR. Human cardiovascular adjustments to acute hypoxaemia. Clin Physiol. 1976; 7:349–376;
102. Sainas G, Marongiu E, Milia R, Palazzolo G, Ibba G, Roberto S, Ghiani G, Tocco F, Crisafulli A. Mean blood pressure assessment during post-exercise: results from two different methods of calculation. J Sports Sci Med. 2016; 15:424–433;
103. Saito M, Mano T, Iwase S, Koga K,Abe H, Yamazaki Y. Responses inmuscle sympathetic activity to acutehypoxia in humans. J Appl Physiol. 1988, 65: 1548–1552;
104. Sanna I, Pinna V, Milia R, Roberto S, Olla S, Mulliri G, Crisafulli A. Hemodynamic responses during enduro-motorcycling performance. Front Physiol. 2017 8:1062. https ://doi.org/10.3389/fphys .2017.01062;
105. Schega L, Peter B, Brigadski T, Leßmann V, Isermann B, Hamacher D, Törpel A. Effect of intermittent normobaric hypoxia on aerobic capacity and cognitive function in older people. J Sci Med Sport. 2016; 19:941–945;
106. Schumacker PT. Lung cell hypoxia: Role of mitochondrial reactive oxygen species signaling in triggering responses. Proc Am Thorac Soc 2011, 8:477–484.
107. Scott AC, Wensel R, Davos CH, Kemp M, Kaczmarek A, Hooper J, Coats AJS, Piepoli MF. Chemical mediators of the muscle ergoreflex in chronic heart failure: a putative role for prostaglandins in reflex ventilatory control. Circulation. 2002; 106:214–220;
108. Sharma S, Burns B. Alveolar Gas Equation. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2019 Jan-. 2019 Apr 6;

109. Sheriff DD, Augstyniak RA, O'Leary DS. Muscle chemoreflexinduced increases in right atrial pressure. Am J Physiol (Heart Circ Physiol). 1998; 275:H767–H775;

110. Shoemaker JK, Mattar L, Kerbeci P, Trotter S, Arbeille P, Hughson RL. WISE 2005: stroke volume changes contribute to the pressor response during ischemic handgrip exercise in women. J Appl Physiol. 2005; 103:228–233;

111. Siebenmann C, Hug M, Keiser S, Muller A, van Lieshout J, Rasmussen P, Lundby C. Hypovolemia explains the reduced stroke volume at altitude. Physiol Rep. 2013; 1: e00094;

112. Siebenmann C, Lundby C. Regulation of cardiac output in hypoxia. Scand J Med Sci Sports. 2015; 25 (Suppl 4): 53-59;

113. Sousa Fialho MDL, Abd Jamil AH, Stannard GA, Heather LC. Hypoxia inducible factor 1 signalling, metabolism and its therapeutic potential in cardiovascular disease. Biochim Biophys Acta Mol Basis Dis. 2019 Apr 1:1865(4): 831-843

114. Stembridge M, Ainslie PN, Hughes MG, Stöhr EJ, Cotter JD, Nio AQX, Shave R. Ventricular structure, function and mechanics at high altitude: chronic remodeling in Sherpa vs. short term lowlander adaptation. J Appl Physiol. 2014; 117: 334-343;

115. Stembridge M, Ainslie PN, Hughes MG, Stöhr EJ, Cotter JD, Tymko MM, Day TA, Bakker A, Shave R. Impaired myocardial function does not explain reduced left ventricular filling and stroke volume at rest or during exercise at high altitude. J Appl Physiol. 2015; 119: 1219-1227;

116. Stembridge M, Ainslie PN, Shave R. Short term adaptation and chronic cardiac remodelling to high altitude in lowlander natives and Himalayan Sherpa. Exp Physiol. 2015; 100.11: 1242-1246;

117. Suarez J, Alexander JK, Houston CS. Enhanced left ventricular systolic performance at high altitude during Operation Everest II. Am J Cardiol.1987; 60: 137–142;

118. Surks HK, Mochizuki N, Kasai Y, Georgescu SP, Tang KM, Ito M, Lincoln TM, Mendelsohn ME. Regulation of myosin phosphatase by a specific interaction with cGMP-dependent protein kinase Ialpha. Science 1999 Nov, 286 (5444): 1583–7

119. Swenson ER. Hypoxic Pulmonary Vasoconstriction. High Alt Med Biol. 2013 Jun;14(2):101-10

120. Talbot NP, Balanos GM, DorringtonKL, Robbins PA. Two temporalcomponents within the humanpulmonary vascular response toapproximately 2 h of isocapnichypoxia. J Appl Physiol. 2005, 98:1125–1139;

121. Tsai YW, Yang YR, Sun SH, Liang KC, Wang RY. Post ischemia intermittent hypoxia induces hippocampal neurogenesis and synaptic alterations and alleviates long-term

memory impairment. J Cereb Blood Flow Metab. 2013; 33(5):764–773;

122. Tschakovsky ME, Sujirattanawimol K, Ruble SB, Valic Z, Joyner MJ. Is sympathetic neural vasoconstriction blunted in the vascular bed of exercising human muscle? J Physiol. 2002; 541 (pt 2) :623–635, 2002;

123. Tune JD. Control of coronary blood flow during Hypoxia. Hypoxia and the Circulation. Springer 2007

124. Van Thienen R, Hespel P. Enhanced muscolar oxygen extraction in athletes exaggerates hypoxemia during excercise in hypoxia. J Appl Physiol (1985). 2016; Feb 1 120 (3): 351-361;

125. Vogel JA, Harris CW: Cardiopulmonary responses of resting man during early exposure to high altitude. J Appl Physiol. 1967; 22: 1124-1128;

126. Wagner PD, Araoz M, Boushel R, et al. Pulmonary gas exchange and acid-base state at 5,260 m in high-altitude Bolivians and acclimatized lowlanders. J Appl Physiol. 2002, 92:1393–1400;

127. Wagner PD, Sutton JR, Reeves JT, et al. Operation Everest II: pulmonary gas exchange during a simulated ascent of Mt. Everest. J Appl Physiol. 1987;63:2348–2359;

128. Wang L, Yin J, Nickles HT. Hypoxic pulmonary vasoconstriction requires connexin 40-mediated endothelial signal conduction. J Clin Invest 2012, 122:4218–4230.

129. Wang ZY, Olson EB Jr, Bjorling DE, et al. Sustained hypoxiainduced proliferation of carotid body type I cells in rats. J Appl Physiol. 2008; 104:803–808;

130. Willis JS, Borrani F, Millet GP. High-Intensity Exercise With Blood Flow Restriction or in Hypoxia as Valuable Spaceflight Countermeasures? Front. Physiol. 2019; https://doi.org/10.3389/fphys.2019.01266;

131. Wolfel EE, Selland MA, Cymerman A, Brooks GA, Butterfield GE, Mazzeo RS, Grover RF, Reeves JT. O2 extraction maintains O2 uptake during submaximal exercise with beta-adrenergic blockade at 4,300 m. J Appl Physiol. 1998; 85: 1092–1102;

132. Wyss CA, Koepfli P, Fretz G, et al: Influence of altitude exposure on coronary flow reserve. Circulation. 2003; 108:1202-1207;