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Effects of high intensity exercise in hypoxia on vascular function in mouse models

Lavier Jessica

UNIL | Université de Lausanne
Faculté de biologie
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Jury

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pour le Doyen
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Prof. Nathalie Busso

Eventually all things fall into place. Until then, laugh at the confusion, live for the moments, and know EVERYTHING HAPPENS FOR A REASON.

– Albert Schweitzer

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List of Publications

1. Supramaximal Intensity Hypoxic Exercise and Vascular Function Assessment in Mice. **Lavier J.**, Beaumann M., Menetrey S., Mazzolai L., Peyter AC., Pellegrin M., Millet GP. *JoVE (Journal of Visualized Experiments)*. 2019;(145):e58708. https://doi.org/10.3791/58708

2. High-intensity exercise in hypoxia improves endothelial function via increased nitric oxide bioavailability in C57BL/6 mice. **Lavier J.**, Beaumann M., Menetrey S., Bouzourène K., Rosenblatt-Velin N., Pialoux V., Mazzolai L., Peyter AC., Pellegrin M., Millet GP. *Acta Physiologica*. 2021;n/a(n/a):e13700. https://doi.org/10.1111/apha.13700

3. High-Intensity Interval Training and Moderate-Intensity Continuous Training Attenuate Oxidative Damage and Promote Myokine Response in the Skeletal Muscle of ApoE KO Mice on High-Fat Diet. Wang L.*, **Lavier J.***, Hua W.., Wang Y., Gong L., Wei H., Wang J., Pellegrin M., Millet GP., Zhang Y. *Antioxidants.* 2021;10:992. https://doi.org/10.3390/antiox10070992
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4. Effects of three times versus five times per week treadmill exercise training on walking capacity and mRNA expression of genes involved in muscle energy metabolism in a mouse model of peripheral artery disease. **Lavier J.**, Bouzourène K., Mazzolai L., Millet GP., Pellegrin M. *In writing.*

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Abstract

Cardiovascular diseases are the main cause of death globally with an estimated 18.6 million deaths worldwide in 2019. Atherosclerosis-induced endothelial dysfunction is an independent risk factor for cardiovascular diseases, and therefore one of the main targets in primary and secondary prevention. Exercise training is highly recommended by international guidelines for the prevention of cardiovascular diseases, but also as the gold standard treatment in some pathologies (i.e., lower extremity peripheral artery disease). Recently, evidence showed promising results from combining exercise training and hypoxia to potentiate the vascular dilator function observed with the same level of exercise in normoxia. However, the optimal characteristics of exercise that would induce an improvement of endothelial function and/or regression of atherosclerosis, remain unclear. This work first demonstrated that hypoxic exercise training improved endothelial function compared to the same training in normoxia in healthy mice. Then, we found that the combination of high-intensity exercise and hypoxia improved endothelial function more than low-intensity exercise training in hypoxia. These first two results indicate a potential application for high-intensity exercise in hypoxia as a novel therapeutic strategy to improve and/or preserve endothelial function. Endothelial function is known to be modulated by the nitric oxide pathway, and a dysfunction in the production and/or bioavailability of nitric oxide gives ground for the development of endothelial dysfunction. Therefore, we ultimately investigated the nitric oxide pathway and showed that rather than being modulated by an increased nitric oxide production, the improved endothelial function was secondary to a systemic shift in the pro-/antioxidant balance towards an antioxidant profile, impacting nitric oxide bioavailability.

In conclusion, our work brought new perspective for the treatment of endothelial dysfunction and cardiovascular disease prevention. It also opens possibilities for testing this type of training on mouse models of cardiovascular diseases, in particular atherosclerosis and lower extremity peripheral artery disease. This will benefit patients and physicians looking to optimize physical activity prescription to prevent or treat cardiovascular diseases.

Résumé

Les maladies cardiovasculaires sont la première cause de mortalité dans le monde avec environ 18.6 millions de décès en 2019. La dysfonction endothéliale induite par l'athérosclérose est un facteur de risque indépendant des maladies cardiovasculaires, et est donc une des cibles principales des préventions primaires et secondaires. L'exercice physique est le traitement de référence pour certaines de ces pathologies selon les recommandations internationales. Récemment, l'exercice physique en hypoxie a montré des résultats prometteurs pour potentialiser la vasodilatation en comparaison avec le même type d'exercice en normoxie. Cependant, les caractéristiques optimales d'exercice permettant une amélioration de la fonction endothéliale et/ou une régression du développement de l'athérosclérose, demeures inconnues. Ce travail a tout d'abord démontré chez la souris saine qu'un exercice en hypoxie améliore davantage la fonction endothéliale que le même entraînement en normoxie ; et ce, d'autant plus avec un exercice à haute intensité qu'à intensité modérée. Ces premiers résultats suggèrent un potentiel effet thérapeutique de l'exercice de haute intensité en hypoxie pour améliorer et/ou préserver la fonction endothéliale. D'un point de vue mécanistique, le bénéfice observé serait le résultat non pas d'une augmentation de la production de monoxyde d'azote, mais d'une orientation de la balance pro-/antioxydante au niveau systémique vers le profil antioxydant, augmentant la biodisponibilité du monoxyde d'azote.

En conclusion, notre travail amène de nouvelles perspectives pour le traitement de la dysfonction endothéliale et la prévention des maladies cardiovasculaires. Il ouvre également la possibilité de tester ces types d'entraînement sur des modèles murins de pathologies humaines, en particulier d'athérosclérose et de la maladie artérielle périphérique. Ce travail apporte de nouvelles pistes aux patients et médecins cherchant à optimiser la prescription d'exercice pour prévenir traiter les maladies cardiovasculaires.

List of Abbreviations

α_1-AR
α_1-adrenergic receptor

3-NT
Nitrotyrosine

3MST
3-mercaptopyruvate

A-HOOH
Ascorbic acid

A-OO
Dehydroascorbic acid

ACh
Acetylcholine

ADRF
Adipocyte-derived relaxing factor

Akt
Protein kinase B

AOPP
Advanced oxidation protein products

$ApoE^{-/-}$
Apolipoprotein E deficient

ATP
Adenosine triphosphate

BH_2
7,8-dihydrobiopterin

BH_4
Tetrahydrobiopterin

Ca^{2+}
Calcium

cAMP
Cyclic adenosine monophosphate

CBS
β-synthase

cGMP
Cyclic guanosine monophosphate

CSE
γ-lyase

CVD
Cardiovascular disease

DEA/NO
Nitric oxide donor diethylamine

EDHF
Endothelium-derived hyperpolarizing factor

EDRF
Endothelium-derived relaxing factor

eNOS
Endothelium nitric oxide synthase

ExT
Exercise training

e^-
Electron

F_iO_2
Inspired fraction of oxygen

FMD
Flow mediated dilation

GPx
Glutathione peroxidase

GSH
Glutathione

GSSG
Glutathione oxidized formed

H^+
Proton

H_2O
Water

H_2O_2
Hydrogen peroxide

H_2S
Hydrogen sulfide

HIF-1
Hypoxia inducible factor 1

HIIT
High-intensity interval training

HR
Heart rate

HRR
Heart rate reserve

iNOS
Inducible nitric oxide synthase

L-NAME
L-NG-nitroarginine methyl ester

LDL
Low density lipoprotein

LowH
Low-intensity continuous training in hypoxia

LowN
Low-intensity continuous training in normoxia

MaxH
Maximal-intensity interval training in hypoxia

MaxN
Maximal-intensity interval training in normoxia

MICT
Moderate-intensity continuous training

MLCK
Myosin light chain kinase

NADPH
Nicotinamide adenine dinucleotide phosphate

nNOS
Neuronal nitric oxide synthase

NO
Nitric oxide

NO_2
Nitrite

NOS
Nitric oxide synthase

NOX
NADPH oxidase

NOx
Sum of nitrite and nitrate

O_2
Oxygen

$O_2^{\bullet -}$
Superoxide ion

$OH^{\bullet}$
Hydroxyl radical

$ONOO^-$
Peroxynitrite

p-eNOS
Phosphorylated endothelium nitric oxide

PAD
Lower extremity peripheral artery disease

Phe
Phenylephrine

P_iO_2
Inspired pressure of oxygen

PKG
Protein kinase G

PVAT
Perivascular adipose tissue

ROS
Reactive oxygen species

Ser^{1177}
Serine site 1177

sGC
Soluble guanylate cyclase

SOD
Superoxide dismutase

SupraH
Supramaximal-intensity sprint training in hypoxia

SupraN
Supramaximal-intensity sprint training in normoxia

Thr^{495}
Threonine site 495

TxA_2
Thromboxane A_2

$\dot{V}O_{2max}$
Maximum oxygen consumption

XO
Xanthine Oxidase

Chapter 1

Introduction

Introduction

Cardiovascular diseases (CVD) are the main cause of death globally with an estimated 18.6 million deaths worldwide in 2019, 17.1% more than in 2010 (Virani et al., 2021). The World Health Organization projected that by 2030, about 23.6 million people will die from CVD every year, meaning that despite countless efforts, the death rate of CVD is not showing any signs of slowing down (World Health Organization). What is even more concerning is that those statistics could not anticipate the 2020 SARS-CoV-2 pandemic. Social distancing, quarantines and lockdowns have induced dramatic changes in lifestyle, increasing unhealthy behaviors, including sedentary lifestyle and unhealthy diet (Mattioli et al., 2020), two important risk factors for CVD (Aboyans et al., 2017).

Impaired endothelial function is an independent risk factor for CVD (Widmer and Lerman, 2014). Endothelial function describes the capacity of vessels to vasoconstrict and vasodilate in response to chemical and mechanical stimuli, in order to regulate blood flow. Exercise training (ExT) is a highly efficient non-pharmacological approach for maintaining cardiovascular health and an important component of therapy for most CVD. Even though low-intensity ExT is associated with lower cardiovascular and all-cause mortality (Pelliccia et al., 2020) partially via its beneficial effects on endothelial function, there is no consensus regarding the potential beneficial effect of high-intensity ExT on endothelial function. Moreover, current evidences show that combining exercise with hypoxia potentiates the dilator function observed with the same level of exercise in normoxia (Montero and Lundby, 2016). However, the optimal characteristics of ExT needed to improve endothelial function remain unclear so far.

Improving endothelial function is of high importance in the fight against CVD. Which is why this thesis investigated three different ExT intensities, both in normoxia and in hypoxia, and their effects on endothelial function, as well as the mechanisms underlying those potential benefits.

I. Endothelial function

1. Definition

Blood vessels are composed of three layers: tunica adventitia, tunica media and tunica intima. The adventitia, the outer layer, is mostly made of fibro-elastic connective tissue. Its role is to hold and protect the structure. The media, the middle layer, primarily contains vascular smooth muscle cells and elastin fibers, which are essential for vasoconstriction and vasodilation. The intima, the inner layer, is the thinnest layer and is made of a single layer of endothelial cells, the endothelium, mounted on a basement membrane and an elastic layer, which provides stability and elasticity to the endothelium (Pugsley and Tabrizchi, 2000). With its privileged position as the direct link between the blood and the arterial wall, the endothelium is essential to maintain vessels homeostasis via endocrine, paracrine and autocrine signaling cascades (Moore and Ruska, 1957).

The endothelium is a dynamic structure, actively involved in a variety of metabolic and synthetic functions (Kumar et al., 2020). A functional endothelium acts as a barrier, restricting the passing of molecules from the circulation to the sub-endothelial space. Only essential molecules are being transported across the endothelium via specific mechanisms such as direct transporters, channels or vesicle-based transporters (Wettschureck et al., 2019). The endothelium also takes part in the host-defense system and inflammation by producing and reacting to mediators and chemokines (Kumar et al., 2020). Due to their key position, endothelial cells orchestrate the immune and inflammatory response by communicating with other cells through production and reaction to soluble cytokines (Galley and Webster, 2004). Secreted chemokines are presented to circulating leukocytes (neutrophils, eosinophils, T lymphocytes, natural killer cells and monocytes) and when secreted in large amounts, they participate in the systemic inflammatory response. Endothelial cells also regulate leukocyte movement and transfer towards tissues thanks to adhesion molecules, that are expressed on the surface of the endothelium and bind to specific receptors on the surface of leukocytes.

Those adhesion molecules are E-selectin, P-selectin, intercellular adhesion molecule-1 and vascular cell adhesion molecules. Activated endothelial cells also secrete platelet activating factor that mobilizes platelets. The integrity of the endothelium can therefore be maintained, and at the same time, activated inflammatory cells can be recruited out of the circulation and sent to the site of infection or injury (Galley and Webster, 2004). Another major role of the endothelium is hemostasis and coagulation. Endothelial cells and vascular smooth muscle cells express a multitude of proteins involved in hemostasis, which tightly control coagulation-related receptors on the surface of vascular cells and circulation coagulating proteins, whose role is to initiate a coagulation response in case of vascular injury. Under basal condition, the endothelium releases a protein called tissue factor pathway inhibitor, which inhibits tissue factor receptor, a receptor for the pro-coagulant factor VII. When the vessel wall in injured, tissue factor activates factor X, which combines to pro-coagulant factor Va, to convert prothrombin into thrombin. The latter is a protein with both pro- and anticoagulant effects due to its autoregulation by a negative loop feedback. By binding to thrombomodulin on the surface of endothelial cells, it prevents the tethering of pro-coagulant factors, blocking the pro-coagulant pathway, in favor of the anticoagulant one. The thrombin-thrombomodulin complex activates protein C pathway, leading to a dissociation of protein C from the endothelium protein C receptor. Protein C then can bind to protein S and function as an effective anticoagulant by inactivating factor Va (Galley and Webster, 2004).

Angiogenesis, the process of formation of new blood vessels, is also regulated by the endothelium, via the production of the angiogenic factor called vascular endothelial growth factor. This factor participates as well in the inflammatory response by prompting the discharge of adhesion molecules, metalloproteinases and nitric oxide (NO) (Cross et al., 2003).

The endothelium is a major regulator of vascular tone via the secretion of multiple vasodilator and vasoconstrictor substance. Since endothelial cells are so important for the regulation of the vascular tone, this particular role of the endothelium will be addressed in more depth in the next chapter.

2. Vasomotor control

2.1. Vasodilation

In order to maintain a balanced vascular tone, the vasodilation system must accurately compensate for the sympathetic vasoconstriction. In 1980, Furchgott and Zawadzki discovered that the endothelium was the key regulator of vasodilation and that thorough communication with vascular smooth muscle cells was needed in order to induce relaxation. Later, it was found that the actors implicated in this mechanism were endothelium-derived relaxing factors (EDRF), vasodilator prostaglandins and endothelium-derived hyperpolarizing factors (EDHF) (Feletou and Vanhoutte, 1988). The endothelium can either be activated by mechanical, physical or chemical stimuli. Shear stress and hypoxia are examples of mechanical and physical stimuli respectively, and their functioning will be described later. As for chemical stimuli, endogenous chemicals bind to their specific receptors on the surface of endothelial cells such as amines (i.e., catecholamine, serotonin, histamine), nucleotides (i.e., adenosine diphosphate, adenosine triphosphate ATP), peptides (i.e., bradykinin, angiotensin, endothelin, vasopressin), proteases (i.e., thrombin, trypsin), growth factors or cytokines (Vanhoutte and Mombouli, 1996). Once those chemicals bind to their receptors, they trigger an influx of calcium (Ca^{2+}) inside the endothelial cells, leading to the secretion of EDRF, prostaglandins and/or EDHF. EDFR and prostaglandins in turn act within the vascular smooth muscle cell by activating cyclic nucleotide messengers. EDHF hyperpolarizes the membrane of vascular smooth muscle cells, leading to an opening of Ca^{2+} sensitive potassium channels. Afterwards, vasorelaxation is achieved either via a recapture of Ca^{2+} by internal storage pockets (sarcoplasmic reticulum) or by export of Ca^{2+} to the extracellular medium (Vanhoutte and Mombouli, 1996) (Figure 1). If NO has been identified as the most important EDRF, the identity of EDHF remains unclear, despite recent suspicions that hydrogen sulfide (H_2S) might be a strong candidate (Tang et al., 2013). Pathways of NO, prostaglandins and H_2S will be described in more detail in chapter *3. Pathways regulating endothelium function*.

Shear stress is a notable modulator of vascular reactivity. When shear stress acts on endothelial cells, a mechanical signal has to be converted into a biochemical signal, i.e., mechanotransduction. This is achieved via sensors attached to/on the membrane of endothelial cells which transmit the signal to the interior of the cell (Ando and Yamamoto, 2013). Shear stress can directly activate **ion channels**. It has been shown that shear stress-induced current causes membrane hyperpolarization through an increased opening of potassium permeable ion channels. The membrane hyperpolarization acts as a driving force to pull extracellular Ca^{2+} inside the cell. But shear stress can also indirectly activate ion channels, such as the purinergic $P2X_4$ receptors, which are constitutively expressed at the surface of endothelial cells membrane. When endothelial cells are submitted to shear stress, they release endogenous ATP in an intensity-dependent manner. ATP activates the $P2X_4$ receptors, which leak Ca^{2+} in the intracellular space (Ando and Yamamoto, 2013). Endothelial cells **membrane receptors and G-protein coupled receptors** can also be activated by shear stress, in the absence of their ligands. Other membrane proteins that can translate the mechanical signal are **integrins**. Integrins are transmembrane glycoproteins that are important actors in shear stress mechanotransduction, with an extracellular subunit that binds to extracellular proteins (laminin, collagen, fibrinogen etc.), and an intracellular subunit that interacts with the cytoskeleton. This privileged situation makes it possible for integrins to directly sense changes in shear stress and respond by translating and transmitting the signal within the cell (Ando and Yamamoto, 2013). Another actor of mechanotransduction is the **glycocalyx**, which layers the inner surface of the endothelial cells. Changes in blood flow is known to alter the configuration of the glycocalyx and either its intracellular domain relays the signal to the cytoskeleton, or a mechanotransduction of the signal is operated via fluctuations in concentration gradients and transport of ions, amino acids and cell growth factors (Tarbell and Pahakis, 2006). Another element found on the surface of endothelial cells are **primary cilia**, which are rod-like organelles that protrude from the surface of cells. They react to shear stress by bending because of the current, leading either to a distortion of the cytoskeleton, or activation of an ion channel on the cilia that causes an influx of Ca^{2+} (Ando and Yamamoto, 2013). **Caveolae** are

another element present in abundance on the surface of endothelial cells. They are “omega shaped” plasma membrane invagination that are key actors of signal transduction, by forming a platform on which receptors, ion channels and signaling factors gather (Shaul and Anderson, 1998), making it the perfect configuration to propagate signals to subsequential pathways (Rizzo et al., 2003). Although the exact mechanisms are not entirely elucidated, what is known is that a release of ATP from caveolae induces an increase in Ca^{2+} in the surrounding of the invagination, leading to an activation of NO synthase (NOS) and eventually augmented NO production (Ando and Yamamoto, 2013; Yamamoto et al., 2007). Finally, the **cytoskeleton** of endothelial cells has also been shown to play a role in shear stress signal mechanotransduction and NO production (Knudsen and Frangos, 1997), as well as the **lipid bilayer membrane** making up the plasma membrane (Haidekker et al., 2000).

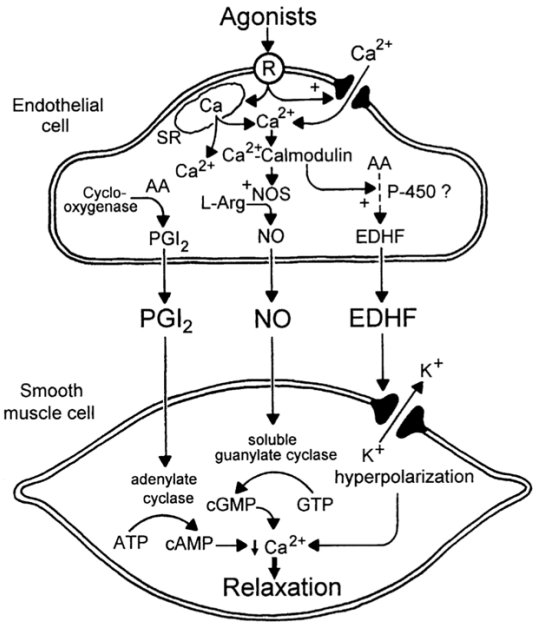

Figure 1 : Endothelium dependent vasorelaxation.

From Vanhoutte, 2004.

2.2. Vasoconstriction

To regulate vascular tone, the body needs a complex equilibrium of vasodilator and vasoconstrictor signals. The sympathetic nervous system exerts a vasoconstrictor influence on the vasculature, which is buffered by signals from vascular smooth muscle and endothelial cells (Kerr et al., 2012; Shoemaker et al., 2015). Sympathetic control of the vasculature illustrates an elemental characteristic of the autonomic nervous system's role in homeostatic adaptation to stress (Shoemaker et al., 2015). One of the sensing mechanisms of the sympathetic nervous system is the baroreflex (Eckberg et al., 1988), and it controls blood pressure through the cardiopulmonary and the arterial pathways. The prior has mechanosensors located in the right heart and pulmonary veins, whereas the latter has mechanosensors located in the aortic arch and carotid sinus. When a tissue stretch induced by increased transmural pressure is detected, a reflex is initiated that increases vagal blood outflow to the heart, and inhibits efferent sympathetic drive. In the same way, when the pressure decreases, the reflex is reduced and so is the cardiovagal outflow, and the sympathetic nerve activity to the periphery is increased (Shoemaker et al., 2015). Another sensing mechanism is the chemoreflex (Kara et al., 2003). Peripheral chemoreceptors are located in the carotid bodies (in the adventitia, at the bifurcation of the common carotid artery) and respond for the most part to hypoxemia. Central chemoreceptors are located in the region of the brainstem (connects the cerebellum to the spinal cord) and generally respond to hypercapnia. When either one of those reflexes is activated, hyperventilation and sympathetic response are triggered, along with a negative feedback loop, that lessens cardiovascular responses mediated by chemoreflex. In the same way, since chemoreflex induces an increased blood pressure, it is detected by the baroreflex that acts upon it, thus blunting the chemoreflex response (Kara et al., 2003). A final sensing mechanism that will be addressed here is the skeletal muscle metaboreflex. It explains a part of the pressor response to exercise and describes the capacity of metabolites released by fatigued muscles to set off a sympathetic response through the activation of specific muscle afferents (Grotle et al., 2020). The role of

this reflex is to ease augmentations of blood flow to the contracting skeletal muscle by increasing sympathetic outflow to the heart and the skeletal muscular vascular bed. That will in turn increase perfusion pressure and readjust blood flow towards the contracting muscle. Furthermore, the contracting muscle has a great capacity to vasodilate, which would create a drastic fall in arterial pressure were it not for a sympathetic restraint of active skeletal muscle blood flow (Grotle et al., 2020).

Another level of control of the sympathetic vasoconstriction is the enzymatic control, with the peri-arterial sympathetic nerves releasing neurotransmitters, cotransmitters and receptors at their junction with vascular smooth muscle cells (Wier et al., 2009). The major sympathetic neurotransmitters released in most peripheral vascular beds are norepinephrine, ATP and neuropeptide Y. While neurally released norepinephrine is known to activate slow, strong, and sustained contractions, the release of ATP activates a rapid, short-term component of smooth muscle contraction. These components of neurogenic contraction are referred to as “adrenergic” and “purinergic” respectively. The role of neuropeptide Y remains poorly understood, and rather than being a potent vasoconstrictor, it seems to modulate the actions of norepinephrine and possibly ATP (Wier et al., 2009). Each neurotransmitter is more or less present in vascular beds across the body. It has been shown that released ATP activates postjunctional purinergic ligand-gated Ca^{2+} channel $P2X_1$ receptors, located on the surface of vascular smooth muscle cells. Norepinephrine binds to α_1-adrenergic receptors (α_1-AR) that can activate a uniform change in Ca^{2+} concentration (Shoemaker et al., 2015). These α_1-AR are G-protein coupled receptors, however other G-protein coupled receptors are present on the surface of vascular smooth muscle cells, that do not react to neuropeptides, but react to hormones such as endothelin-1 (endothelin-1 receptor A) and angiotensin-II (angiotensin-II receptor 1), or even to eicosanoids like thromboxane A_2 (TxA_2; thromboxane-prostanoid receptor). Despite being activated by different types of messengers, these receptors all have a relatively similar functioning pattern (Wynne et al., 2009). After activation of the receptor, second messengers are produced, starting with phospholipase C activation. Phospholipase C

is a membrane-bound enzyme that cleaves membrane lipid phosphoinositide 4,5-bisphosphate, leading to the generation of inositol-trisphosphate and diacylglycerol (Hilgers and Webb, 2005). Then the pathway separates into two distinct chains of event leading to the release of Ca^{2+} into the cell (Docherty, 2019). On the one hand, inositol-trisphosphate mediates the delivery of Ca^{2+} from the sarcoplasmic reticulum via the ryanodine receptors. After their activation, the ryanodine receptors open the Ca^{2+} channels, leading to a fast influx of Ca^{2+}. The principal target of Ca^{2+} is calmodulin, which – once coupled with Ca^{2+} – will activate the myosin light chain kinase (MLCK) of the smooth muscle. On the other hand, diacylglycerol activates protein kinase C which directly phosphorylates MLCK. In both pathways, once MLCK is activated, it will in turn phosphorylate the myosin light chain allowing the interaction between the actin and myosin filaments, and finally leading to contraction (Docherty, 2019).

3. Pathways regulating endothelium function

3.1. NO bioavailability

3.1.1. NO production

NO is an endothelium-derived molecule at the center of cardiovascular homeostasis maintenance and a lack of its bioavailability has been shown to be directly linked to cardiovascular disorders (Costa et al., 2016).

The main source of NO in blood vessel walls comes from the conversion of amino acid L-arginine into L-citrulline by NOS (Chen et al., 2008). NOS has three isoforms, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelium NOS (eNOS). nNOS is mostly expressed in neurons, iNOS is induced by inflammatory cytokines and even though they can both be found in vessels, eNOS remains the dominant endogenous NOS in vessels (Vanhoutte et al., 2016).

Various stimuli can trigger/intensify the activity of eNOS, and in turn the generation of NO, such as shear stress, either in a Ca^{2+} dependent or independent way. Agonists such as acetylcholine (ACh), bradykinin and histamine act in a Ca^{2+} dependent manner and interact with a specific receptor on the surface of endothelial cells in order to increase intracellular concentration of Ca^{2+}, which links to calmodulin and activates the calmodulin-binding domain of eNOS (Zhao et al., 2015). In a Ca^{2+} independent way, eNOS can undergo phosphorylation, a post-transcriptional modification. Different phosphorylation sites can induce different reactions, for example, phosphorylation of the serine site Ser^{1177} by protein kinase B (Akt) activates eNOS, whereas the threonine site Thr^{495} inhibits the action of eNOS. Shear stress and bradykinin activate eNOS by increasing phosphorylation of Ser^{1177} alone or combining it with an enhanced dephosphorylation of Thr^{495} (Zhao et al., 2015).

The principal target of NO in the vascular smooth muscle cells is soluble guanylate cyclase (sGC). Once activated, this enzyme catalyzes the formation of cyclic guanosine monophosphate (cGMP), which activates protein kinase G (PKG). Not only does PKG inhibit the release of Ca^{2+} from the sarcoplasmic reticulum and its inflow from the extracellular space, it also promotes its reuptake by the sarcoplasmic reticulum (Gao, 2010). Since the concentration of Ca^{2+} decreases, MLCK deactivates and cannot phosphorylate myosin anymore, eventually leading to vascular smooth muscle cells relaxation (Zhao et al., 2015). NO can also act independently of the cGC-cGMP-PKG pathway and directly affect the sarcoplasmic reticulum Ca^{2+}-ATPase pump, reducing intracellular Ca^{2+} concentration even more, facilitating relaxation (Vanhoutte et al., 2016). Nitrite and nitrate are products of NO metabolism, but they also act as its reservoir, as various enzymes can catalyze the reduction of nitrate and nitrite (NO_2) into NO under certain conditions such as hypoxia or low pH (Chen et al., 2008). For instance, hemoglobin and myoglobin, which are NO foragers, can convert into NO producers in hypoxia and/or when pH is low (Huang et al., 2005; Umbrello et al., 2013). Contrary to hemoglobin and myoglobin, aldehyde dehydrogenase is an enzyme that does not need hypoxia in order to convert organic nitrate compounds into NO (Beretta et al., 2012). S-

nitrosothiols are intermediates in the transport and storage of NO, and they can be produced by endothelial cells either from endogenous or exogenous NO (Yang and Loscalzo, 2005). If S-nitrosothiols are stable under physiological conditions, they release NO in the presence of redox-sensitive metal ions (e.g., Fe^{2+} or Cu^{+}) or thiol-reducing agents (e.g., H_2S, reduced glutathione etc.) (Yang and Loscalzo, 2005).

3.1.2. NO production impairment and degradation

3.1.2.1. Oxidative stress

Oxidative stress and the concomitant production of reactive oxygen species (ROS) is the main reason for excessive NO degradation and thus decreased bioavailability, resulting in endothelial dysfunction. The main sources of ROS that are generating enough of them to impact NO bioavailability, are actors of the mitochondrial respiratory chain (complexes I, II and III), xanthine oxidase (XO), NADPH oxidase (NOX) and eNOS uncoupling (detailed in the next chapter *3.1.2.2. Insufficient supply of substrate and NOS uncoupling*) (Vanhoutte et al., 2017). The generation of ROS from oxygen (O_2) requires an interaction with electrons (e^-), usually provided by the mitochondrial electron-transport chain or NOX. When O_2 interacts with e^-, it forms the superoxide ion ($O_2^{\bullet -}$). $O_2^{\bullet -}$ being unstable, it either spontaneously or through enzyme-catalyzed dismutation transforms into hydrogen peroxide (H_2O_2): $O_2 + 2\ e^- \rightarrow O_2^{\bullet -} + O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$ (Thomas et al., 2008). The produced H_2O_2, despite not being a free radical, remains toxic due to its highly oxidative power (Winterbourn, 2013).

Mitochondrial respiratory chain. During the oxidative phosphorylation pathway, O_2 is normally reduced into water (H_2O) by four e^- via the cytochrome oxidase, which gives H_2O. However, it can also be incompletely reduced and transformed into $O_2^{\bullet -}$ which will eventually form H_2O_2. The e^- leak happens at the end of the pathway when e^- are transferred from complexes I and II to complexes III and IV. This phenomenon happens because of a mismatch of the activities

of complexes I and II and those of complexes III and IV (Zhao et al., 2019). It is estimated that 1-2% of the total O_2 used is turned into ROS, making this pathway a substantial source of ROS under physiological conditions (Thomas et al., 2008).

Xanthine oxidase. XO is an enzyme that comes from the oxidation of xanthine dehydrogenase, which, in physiological condition, catalyzes the degradation reaction of purines, more specifically it oxidizes hypoxanthine to xanthine and subsequently xanthine to uric acid (Cantu-Medellin and Kelley, 2013). However, under certain conditions of stress (e.g., hypoxemia) xanthine dehydrogenase is oxidized into XO, and when conditions return to normal, the XO that accumulated under the stressed system metabolizes hypoxanthine into uric acid, using O_2, thus generating $O_2^{\bullet -}$ and in turn H_2O_2 (Kelley, 2015; Xu et al., 2019).

NADPH oxidase. NOX is a family of enzymes that has the particularity to purposedly generate ROS, contrary to other previously described ROS generating pathways that either produce them as a byproduct of their regular function or because of dysfunction. The NOX enzymes are predominantly found on the surface of phagocytes and are responsible for the “respiratory burst” that facilitates the elimination of pathogens. However, these enzymes are also present in vascular smooth muscle and endothelial cells of the vasculature and are responsible for the non-cytotoxic production of ROS necessary for normal redox signaling. In some pathological situations (e.g.: hypertension, diabetes, hyperlipidemia) they can be overactivated, resulting in an overproduction of ROS (Drummond and Sobey, 2014). To date, seven isoforms have been discovered, that are expressed in different tissues and cells: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2 (Thomas et al., 2008). Four of the seven isoforms are found in endothelial cells (Nox1, Nox2, Nox4 and Nox5) and the most important one in the context of vascular dysfunction is Nox2. Nox2 requires the recruitment of 5 regulatory subunits in order to be functional, $p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac1 (Panday et al., 2015). Once assembled, the complex becomes active and generates $O_2^{\bullet -}$ catalyzing the transfer of e^- from NADPH in the cytosol to O_2 in the luminal or extracellular space: $NADPH + 2\ O_2 \rightarrow NADP^+ + 2\ O_2^{\bullet -} + 2\ H^+$ (Bedard and Krause, 2007; Jones et al., 2000).

When the concentration of $O_2^{\bullet-}$ in the vascular wall is increased, it can react with NO to produce the potent oxidant peroxynitrite $ONOO^-$: $O_2^{\bullet-} + {}^{\bullet}NO \rightarrow ONOO^-$. NO could stabilize by autoxidation, and $O_2^{\bullet-}$ could spontaneously dismutate, but the rate constant of $ONOO^-$ formation is higher than those of NO and $O_2^{\bullet-}$ reactions. In addition, the coupling of NO with $O_2^{\bullet-}$ occurs faster than the non-spontaneous transformations of NO and $O_2^{\bullet-}$. That makes the formation of $ONOO^-$ kinetically favored over other NO reactions and tending to occur whenever NO and $O_2^{\bullet-}$ are present (Radi, 1996). One deleterious effect of $ONOO^-$ is its incapacity to activate sGC, greatly decreasing the bioactivity of NO in the vascular wall, eventually causing endothelial dysfunction (Thomas et al., 2008).

Only a fraction of the generated ROS enters the oxidation reactions and causes damage to the tissues. The organism can neutralize these ROS using various mechanisms either locally or at the tissue level. To counter the oxidant action of free radicals, the organism has two families of antioxidants, i.e., the active enzymatic systems and the passive non-enzymatic systems.

Enzymatic antioxidant systems

Superoxide Dismutase (SOD). This enzyme increases the speed of $O_2^{\bullet-}$ dismutation into H_2O_2 and O_2 by 10'000 times (Forman and Boveris, 1982). By oxidizing $O_2^{\bullet-}$, SOD cuts the chain reaction at the beginning, preventing $O_2^{\bullet-}$ from reacting with H_2O_2 and creating hydroxyl radical ($OH^{\bullet}$; Powers and Lennon, 1999). In mammals, the SOD family comprises three metallic forms, the copper and zinc SOD (Cu-ZnSOD also known as SOD1) located in the cytosol, the manganese SOD (Mn-SOD also known as SOD2) located in the mitochondria, and the extracellular Cu-ZnSOD (EC-SOD also known as SOD3) located in the extracellular liquids (Ighodaro and Akinloye, 2018).

Glutathione peroxidase (GPx) and glutathione reductase. The role of GPx is to inhibit the formation of $OH^{\bullet}$ by reducing H_2O_2. Two molecules of glutathione (GSH) give two protons (H^+)

to H_2O_2, which in turn is reduced into two molecules of H_2O: $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$. The remaining GSH that have been oxidated into GSSG is recycled back into GSH by glutathione reductase using the NADPH cofactor in the process, preventing its oxidation by NOX: $GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$ (Richard et al., 1997).

Catalase. The rigid and stable structure of catalases makes them more resistant to pH, thermal denaturation, and proteolysis than most other enzymes. Their main role is to control the concentration of H_2O_2 (Goyal and Basak, 2010). To achieve that, they increase the speed of transformation of H_2O_2 into H_2O and O_2: $2H_2O_2 \rightarrow 2H_2O + O_2$ (Chelikani et al., 2004). This reaction prevents H_2O_2 from being used to produce $OH^\bullet$ during the Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow OH^\bullet + OH^- + Fe^{3+}$ (Fenton, 1894).

Non-enzymatic antioxidant systems

These substances are capable of neutralizing a single free radical at a time and act at high concentrations. They can be grouped according to their affinity for lipids or water.

Acid uric. This water-soluble molecule plays an important role as a reducer in plasma, erythrocytes and leucocytes. It reduces $OH^\bullet$ and its oxidation product is allantoin (Grootveld and Halliwell, 1987). Its concentration is increased in particular during an intense physical activity via activation of XO (Green and Fraser, 1988).

Glutathione. As mentioned above, GSH exists in its reduced and oxidized forms (GSSG). Its main antioxidant mechanism resides in its ability to reduce H_2O_2, which is a reaction catalyzed by GPx. It also plays an important role in the antioxidant network by recycling the oxidized forms of vitamin C, reinstating its antioxidant capacity (Linster and Schaftingen, 2007; Meister, 1994).

Ascorbic acid (vitamin C). Ascorbic acid exists under three oxidoreduction states, the reduced form, also called ascorbic acid (A-HOOH), the semi-reduced form, also called semi-dehydroascorbic acid, and the oxidized form, also called dehydroascorbic acid (A-OO). It is a

hydrogen donor that reacts with $OH^{\bullet}$ and $O_2^{\bullet -}$ to transform them into H_2O: $2OH^{\bullet}$ + A-HOOH $\rightarrow$ A-OO + $2H_2O$ and $O_2^{\bullet -}$ + 2A-HOOH $\rightarrow$ A-OO + $2H_2O$. Ascorbic acid also recycles vitamin E, restoring its antioxidative properties (Linster and Schaftingen, 2007).

Vitamin E (tocopherol). Vitamin E is the main antioxidant present in membranes. It is incorporated into membranes via a hydrophobic bond with phospholipids polyunsaturated fatty acids. α-tocopherol is an important antioxidant of lipid structures. Its reactivity lies in the capacity of its core to trap free radicals formed during lipid peroxidation (Miyazawa et al., 2019).

Carotenoids. Natural liposoluble pigments, exclusively synthesized by plants and specific microorganisms, carotenoids are derived from common precursors called lycopene and β-carotene. They are mostly found in fruits and vegetables and about ten of them are vitamin A precursors. Some carotenoids, such as β-carotene, lycopene or lutein, have antioxidative effects and reduce ROS (Han et al., 2012).

Coenzyme Q10. Located in the internal membrane of the mitochondria, it transports e^- and H^+ inside the cell. In its reduced form it is the first liposoluble antioxidant, and it reduces the highly toxic perferryl radical (Fe^{3+}-$O_2^{\bullet -}$) into Fe^{3+} and H_2O_2. It also prevents lipid peroxidation (Björnstedt et al., 2004).

Flavonoids. These substances originating from plants have antioxidative properties more powerful than vitamin E. They are also recycled by vitamin C (Han et al., 2012).

3.1.2.2. Insufficient supply of substrate and NOS uncoupling

In healthy human adults, L-arginine is a non-essential amino acid that can be synthesized de novo from citrulline and therefore its supply is rarely a limiting factor for the production of NO under physiological conditions. But in certain situations, where the L-arginine transporter is defective in the membrane of endothelial cells, or when its breakdown by arginase is accelerated (increased endothelial arginase activity caused by aging, diabetes mellitus or

hypertension), L-arginine can become a limiting factor to the production of NO (El-Hattab et al., 2012).

In order to generate NO from L-arginine, eNOS needs a specific cofactor called tetrahydrobiopterin (BH_4). Initially, eNOS is synthesized as a monomer but it has to form dimers with BH_4 and L-arginine to be able to produce NO. In the absence of BH_4, a phenomenon called eNOS uncoupling occurs (Zou et al., 2002). The latter takes place when oxidative stress oxidizes BH_4. When BH_4 levels are defective, eNOS becomes unstable and uncoupled, and as a consequence, less NO is produced and more $O_2^{\bullet-}$ is generated. This same $O_2^{\bullet-}$ will interact with NO and lead to the formation of $ONOO^-$, which further oxidizes BH_4 (Kietadisorn et al., 2012). BH_4 has two synthetization pathways, the de novo and the salvation pathways, and it is the latter that plays a key role in eNOS uncoupling. In this pathway, BH_4 is generated from its oxidized form 7,8-dihydrobiopterin (BH_2), which is in direct competition with BH_4 to interact with eNOS, except it has no cofactor properties (Crabtree et al., 2008). Therefore, more than the absolute concentration of BH_4, it is the ratio between BH_4 and BH_2, along with the supply of eNOS, that dictates eNOS uncoupling (Crabtree et al., 2009). In the same line, if eNOS alone is over expressed, the cofactor imbalance will lead to eNOS uncoupling (Kietadisorn et al., 2012).

3.2. Prostaglandins

Despite NO being the maestro of vascular reactivity, another class of molecules is implicated in this phenomenon, i.e., prostanoids. Prostanoids are a subclass of eicosanoid compounds, formed from arachidonic acid. In cell membranes, cytosolic phospholipase A_2 is activated by sub-micromolar concentrations of Ca^{2+} and phosphorylation, and once activated, it forms arachidonic acid from membrane phospholipids. Arachidonic acid is then transformed by cyclooxygenase enzymes into prostaglandin H_2. Prostaglandin synthase then act on prostaglandin H_2 to form potent vasoactive prostanoids such as prostacyclin, prostaglandin E

and F, and TxA_2 (Félétou et al., 2011; Parkington et al., 2004). Some of these prostanoids are mostly present in vascular endothelial cells, however depending on the receptor they bind to, they can either have a vasodilating or a vasoconstricting effect. Prostacyclin and TxA_2 are the two main prostanoids acting in the endothelial cells-vascular smooth muscle cells system. Prostacyclin binds to its receptor IP on the surface of vascular smooth muscle cells, leading to the activation of adenylyl cyclase and increased production of cyclic adenosine monophosphate (cAMP). cAMP in turn activates protein kinase A that phosphorylates target protein to induce vasorelaxation either via Ca^{2+} dependent mechanisms, and/or via membrane hyperpolarization. The NO-cGMP-PKG pathway being very similar, evidence show that both system can cross-react, with cGMP and cAMP being able to activate each other's kinase, although with a lower affinity (Parkington et al., 2004).

As briefly mentioned in chapter *2.2. Vasoconstriction*, TxA_2 is secreted by platelets and does not only act as a platelet aggregation factor, but it is also involved in vasoconstriction. The mechanism induced by TxA_2 leading to vasoconstriction has been detailed in chapter *2.2. Vasoconstriction*. In endothelial cells, the activation of TxA_2 receptors inhibits the production of NO, and although TxA_2 is the preferred ligand for these receptors, other potent prostanoids can also bind to it (Cohen et al., 2010). Cyclooxygenase activation has also been shown to contribute to oxidative stress by generating $O_2^{\bullet-}$ in the presence of NADH or NADPH (Kukreja R C et al., 1986).

3.3. Hydrogen sulfide

Similar to NO, H_2S is a gasotransmitter with important roles in the vessel wall, including vasoreactivity. The H_2S and NO pathways interact with one another, meaning that they have positive or negative impact on the properties and concentrations of one another (Hosoki et al., 1997). Three enzymes are responsible for the generation of H_2S, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate (3MST), all three expressed in

vascular cells (Olas, 2015). It has been shown that ROS and shear stress stimulate the expression of CSE and 3MST respectively, and that increased Ca^{2+} concentration stimulates CSE in vascular smooth muscle cells. H_2S has a vasodilator function because it acts as an EDHF, by activating potassium-ATP channels on vascular smooth muscle cells. There are also evidence showing that H_2S modulates other channels such as Ca^{2+} sensitive ones (Olas, 2015). It has also been shown that H_2S is involved in NOS activation, as well as cyclooxygenase in human micro-vessels (Kutz et al., 2015). Several studies have demonstrated that the involvement of H_2S in vascular tone regulation is dependent on its concentration. It would appear that lower concentrations of the molecule elicit vasoconstriction whereas higher concentrations would elicit vasodilation (Kanagy et al., 2017).

NO and H_2S share multiple biological, signaling and chemical attributes. They are not always independent from one another, since various interactions, both direct and indirect, between the two compounds or the pathways they regulate have been reported (Kuschman et al., 2021). It has been shown that H_2S and NO can both stimulate and attenuate each other's biological synthesis. Through Ca^{2+} uptake stimulation, Akt-mediated phosphorylation of eNOS and stimulation of eNOS mRNA synthesis, H_2S can activate eNOS and induce NO release (Cardounel et al., 2011; Kida et al., 2013; Szabo, 2017). On the other hand, NO can also influence the production of H_2S, via the stimulation of CSE activity, as it has been shown in a study conducted in rat vascular tissues, and it has been proposed that this stimulation may be due to NO-mediated cGMP induction of protein kinases (Zhao et al., 2001). H_2S and NO also work in synergy to maintain and increase cGMP. When NO activates sGC for cGMP production, H_2S defers its degradation, indicating that both compounds work together to coordinate angiogenesis and vascular reactivity (Kuschman et al., 2021).

However, the volatility of H_2S makes it a difficult component to investigate and further studies need to be conducted in order to fully comprehend its role in vascular tone regulation.

II. Exercise training and cardiovascular health

1. Vascular responses of the body to exercise training

O_2 consumption at rest varies between individuals, but one thing that is common to everyone is that it increases during exercise. The maximum value of O_2 consumption is called $\dot{V}O_{2max}$. At rest, ~80% of total cardiac output (total is about 5 $L.min^{-1}$, so about 4 $L.min^{-1}$) is directed towards the brain, heart, kidneys and liver. However, during an effort, a redistribution of blood is required to match the new demands of tissues. Therefore, while the brain still needs the same amount of blood, skeletal muscles require much more blood than they do at rest due to their increased metabolic demand and high ATP turnover. In order to match this increased need, cardiac output rises (from 5 $L.min^{-1}$ at rest to 20 $L.min^{-1}$ in non-athletes) and vascular adaptations are triggered (Joyner and Casey, 2015). This augmentation of blood flow to the contracting muscles is called exercise hyperemia and its ultimate role is to match O_2 delivery to demand. A complex interaction between neural, mechanical and chemical factors is necessary in order to raise and maintain blood flow overtime during exercise (Clifford, 2007). Researchers have been trying to elucidate specifically which of these pathways is the one that would entirely orchestrate this rapid vascular response to exercise. However, studied individually none of them can induce a sufficient or fast enough increase in blood flow that would explain the observed exercise hyperemia (Clifford, 2007; Dinenno and Joyner, 2003; Joyner and Casey, 2015; Joyner and Wilkins, 2007). Therefore, a theory of synergistic interaction is most likely.

Neural. The nervous system contributes to exercise hyperemia in two ways. First sympathetic withdrawal, also named “functional sympatholysis” (Remensnyder et al., 1962) which is a blunt of the sympathetic nervous system vasoconstriction. Second, an ACh spillover from active motoneurons, where the ACh released in the neuromuscular junction spills over onto muscarinic receptors in the skeletal muscle vasculature, contributing to vasodilation (Clifford, 2007; Welsh and Segal, 1997).

Mechanical. The muscle pump is defined by an increase in blood pressure gradient in arteries and veins because of skeletal muscle contraction. When muscles contract, veins are emptied, and venous pressure is decreased. In order to compensate for this decreased venous pressure, blood from arteries is rushed into those affected venous portions, thus increasing arterial flow (Tschakovsky and Sheriff, 2004). Another mechanical inducer of hyperemia is the sensitivity of endothelial cells and vascular smooth muscle cells changes in intravascular pressure and intraluminal shear stress (Clifford, 2007).

Chemical. A few substances directly released by contracting muscles have been studied and play a role in exercise hyperemia. Adenine nucleotides adenosine, ATP and adenosine diphosphate are known muscle-released substances, as well as NO and prostanoids (Joyner and Wilkins, 2007). The underlying mechanisms behind chemically induced vasodilation has been described in chapter *2.1. Vasodilation*. Once again NO has been investigated and it was found that it can bind to hemoglobin and be released by erythrocytes when they unload O_2 (Stamler et al., 1997). Together with NO, ATP can also be discharged by erythrocytes when O_2 is being released from hemoglobin (Ellsworth, 2004).

2. Exercise and cardiovascular disease prevention

General physical activity, including ExT, is a major element of therapy for most CVD. In a society where a sedentary lifestyle prevails, and the prevalence of obesity is rising, promoting physical activity and ExT is essential (Pelliccia et al., 2020). The efficiency of ExT is widely recognized, to the point that major cardiovascular societies included ExT in their CVD prevention guidelines, as a class I recommendation (“evidence and/or general agreement that a given treatment or procedure is beneficial, useful, effective”) (Haskell et al., 2007; Pelliccia et al., 2020; Piepoli et al., 2016). There is a dose-effect relationship between ExT and cardiovascular as well as all-risk mortality, with studies reporting between 20 and 30%

reduction in physically active individuals compared to sedentary (Lear et al., 2017; Wahid Ahad et al.).

2.1. Exercise training modalities

The characteristics of ExT can be modulated, from the frequency of the training, to the duration, type and intensity. All these characteristics induce different cardiovascular adaptations, however for the treatment and prevention of CVD, international guidelines recommend to exercise 30 min/day, 5 days/week at moderate-intensity, amounting to about 150 min/week (Pelliccia et al., 2020; Piepoli et al., 2016).

However, the optimal ExT protocol that would improve cardiovascular risk factors and endothelial function remains unclear. Meta-analyses of human studies show how disparate ExT protocols are in literature, with intensities evaluated in different ways (percent of maximal heart rate HR, percent of $\dot{V}O_{2max}$, metabolic equivalent for task, energy expenditure etc.), duration of sessions varying from 20 to 60 min, duration of intervention going from 4 to 52 weeks, frequency going from 1 to 7 times per week, making it essentially impossible to compare ExT modalities and their effects on CVD (Ashor et al., 2015). One study is currently being conducted in the United States aiming to compare benefits of resistance, aerobic and combined ExT with harmonized parameters (Brellenthin et al., 2019). Nevertheless, this study will only be the first step in answering the commonly asked question of "*What type and combination of exercise are most effective for cardiovascular benefits?*" (Brellenthin et al., 2019).

2.2. Impact of moderate-intensity continuous training on endothelial function

It is assumed that the beneficial effects of ExT on cardiovascular health was mediated by improved lipid profile, carbohydrate metabolism, neuro-hormonal release and blood pressure (Clarkson et al., 1999), until 1996 when Burkhard et al. demonstrated that four weeks of daily handgrip training restored flow mediated dilation (FMD) in patients with chronic heart failure, most likely via increased release of NO from the endothelium. However, the observed improvement was limited to the exercising limb. From there, Clarkson et al. (1999) extended these findings by proving that in healthy young men, 10 weeks of general ExT improved systemic flow-mediated endothelium-dependent relaxation. Since then, numerous studies have supported these findings. A study conducted on patients diagnosed with coronary artery disease showed that 8 weeks of combined aerobic and resistance training, improved endothelium-dependent conduit vessel dilation (Walsh et al., 2003a). Another study conducted on male patients with chronic heart failure showed that a four-week bicycle ergometer protocol corrected the endothelial dysfunction observed in the upper extremity of untrained patients, indicating a systemic effect of ExT (Linke et al., 2001). In hypercholesterolemic subjects, 8 weeks of combined aerobic, resistance training and lipid-lowering medication improved endothelium-dependent vessel function of conduit and resistance vessels (Walsh et al., 2003b). In patients with lower extremity peripheral artery disease (PAD) and intermittent claudication, a study has showed that 12 weeks of supervised treadmill ExT increased FMD and maximum walking distance, indicating a generalized effect of ExT on endothelial function (Januszek et al., 2014). Aerobic ExT for 12 weeks has also been proven efficient to improve vascular function in hypertensive patients (Higashi Yukihito et al., 1999). These results were also observed in animal models. In old mice fed a western diet, 10-14 weeks of voluntary wheel running reduced arterial stiffness and improved endothelial function via increased NO bioavailability, thanks to a lower scavenging by $O_2^{\bullet -}$ (Lesniewski et al., 2013). In our lab, we showed that 9 weeks of swimming ExT improved endothelial function in atherosclerotic apolipoprotein E deficient ($ApoE^{-/-}$) mice via increased NO bioactivity (Pellegrin et al., 2007).

More recently, another study showed that 8 weeks of moderate aerobic ExT improved endothelium-dependent vasodilation in thoracic aorta of obese mice, via decreased perivascular adipose tissue oxidative stress (Sousa et al., 2019).

Mechanistically, analyses of conduit arteries from patients suffering from coronary artery disease showed a doubled eNOS expression, accompanied with a four times higher phosphorylation of eNOS Ser^{1177} after four weeks of moderate aerobic ExT (Hambrecht et al., 2003a). As mentioned in chapter *3.1.1. NO production*, the main initiator of eNOS Ser^{1177} phosphorylation is shear stress, via the Akt pathway. It is not clear how exactly ExT improves vascular function, however studies agree on the fact that it is mediated by the NO pathway, either by direct production of NO through eNOS activation, or increased NO bioavailability through a shift in the pro-/antioxidant balance, or both. Tanaka et al. (2015) submitted healthy Wistar rats to acute aerobic exercise and showed that endothelial function improved in exercised versus control rats, concomitantly with an upregulation of phospho-eNOS (p-eNOS), whereas total eNOS expression remained unchanged. They also show that H_2O_2 production is increased in the aorta, despite no differences in total lipid hydroperoxide concentration and reduced to oxidized glutathione ratio (oxidative stress markers in the aorta), indicating a positive role of H_2O_2 on vascular reactivity regulation. The positive role of H_2O_2 on the NO pathway has been documented, with studies demonstrating that it promotes Ca^{2+} dependent eNOS activity (Thomas et al., 2002), oxidizes PKG (see pathway in *3.1.1. NO production*, (Burgoyne et al., 2007) and induces eNOS expression (Cai Hua et al., 2001). A study conducted in C57BL/6 and $eNOS^{-/-}$ mice found that three weeks of moderate-intensity ExT increased eNOS and extracellular SOD expression in wild type mice, whereas they did not change in trained $eNOS^{-/-}$ mice, suggesting that this effect of ExT is modulated by endothelium derived NO (Fukai et al., 2000). A recent study showed that lifelong voluntary aerobic ExT protected C57BL/6 mice from developing age related endothelium dysfunction, even when fed a high-fat diet, through an alleviated vascular oxidative stress and inflammation (Gioscia-Ryan et al., 2021).

2.3. Impact of high-intensity exercise training on endothelial function

So far, the optimal ExT characteristics needed to improve endothelial function remain unclear. However vascular reactivity seems to be sensitive to ExT intensity in humans, with high-intensity ExT improving endothelial function more efficiently than lower intensity protocols, due to an increased shear stress and subsequent stimulation of endothelial cells. Jo et al. (2020) conducted a study in hypertensive metabolic syndrome patients, which showed that 8 weeks of treadmill high-intensity interval training (HIIT; 5 bouts of 3 min at 80% of heart rate reserve (HRR) interspersed with active recovery of 3 min at 40% of HRR) increased FMD more than moderate-intensity ExT (35 min at 60% of HRR). Another study investigated the effects of 12 weeks of HIIT (~15 min of 100 m sprints running) combined with regular physical activity (30 min, 3x/week) on endothelial function in normal-weight and overweight-obese adolescents. Not only did they show that FMD increased in normal-weight and overweight-obese subjects, but also that there was no difference between normal-weight and obese participants anymore (da Silva et al., 2020). In healthy older adults, acute cycling based high-intensity exercise (30 min at 75-80% of their age-predicted maximal HR) caused an increased FMD compared to low-intensity (30 min at 50-55% of their age-predicted maximal HR), and returned to baseline levels after 1 h (Iwamoto et al., 2018). In a recent study, Hasegawa et al. (2018) conducted in parallel two studies on Sprague-Dawley rats and healthy young men, to investigate the effects aerobic training and HIIT on arterial stiffness and the NO pathway. Rats were submitted to 8 weeks of either aerobic treadmill ExT (60 min at 30 m/min, 5 days a week) or 6 weeks of swimming HIIT (14 bouts of 20 s swimming, interspersed with 10 s rest, 4 days a week), and human subjects to 8 weeks of aerobic cycling ExT (45 min at 60-70% of $\dot{V}O_{2max}$, 3 days/week) or 6 weeks of cycling HIIT (six to seven sets of 20 s cycling at about 170% of $\dot{V}O_{2max}$ interspersed with 10 s rest, 4 days/week). Pulse wave velocity was measured as an index of arterial stiffness (aortic for rats and carotid-femoral for humans). Both HIIT and aerobic treadmill ExT improved pulse wave velocity, associated with an increase in p-eNOS and p-Akt expression and increased plasma nitrate and NO_2 (NOx) levels in rats from both ExT groups.

The same results were observed in human subjects, with both ExT protocols improving pulse wave velocity, alongside an increase in plasma NOx levels. Another study investigated the effects of 12 weeks of high-intensity ExT (four 4 min intervals at 90-95% of peak HR, interspersed with 3 min active recovery, consisting in cycling at 50-70% of peak HR) vs. aerobic continuous training (37 min of cycling at 70-75% of peak HR) in patients with coronary artery disease. Once again, high-intensity ExT and aerobic continuous training induced the same improvement of FMD (Van Craenenbroeck et al., 2015). Overall, studies conducted in humans show that high-intensity ExT is a training option that needs to be considered over lower intensity, more tedious ExT because it induces at least the same benefits on endothelial function, probably more according to some studies, while being less time consuming and more enjoyable (Bond et al., 2015).

In rodent studies, the benefits of high-intensity ExT in relation to endothelial function are uncertain. Several studies have reported higher aortic endothelium-dependent vasorelaxation following high-intensity ExT, such as presented in a paper from Haram et al. (2009) that subjected rats with metabolic syndrome to 8 weeks of continuous moderate-intensity ExT (5 days/week from 1.5 to 2 h at 70% of $\dot{V}O_{2max}$, with a slope of 25°) or high-intensity ExT (4 min bouts at 85-90% of $\dot{V}O_{2max}$ interspersed with 3 min active recovery periods at 70% of $\dot{V}O_{2max}$, for a total of 60 min, 5 days/week, with a slope of 25°). They found that high-intensity ExT increased endothelial function more than moderate-intensity ExT, which was associated with an increased eNOS protein expression. Another study on type 1 diabetes rats examined the effects of 6 weeks moderate (5 days/week, for 60 min at 15 m/min, representing about 50-60% of $\dot{V}O_{2max}$, with a slope of 6°) and high-intensity (5 days/week, for 60 min at 27 m/min, representing about 70-80% of $\dot{V}O_{2max}$, with a slope of 6°) endurance training, and showed that high-intensity ExT was more effective at improving endothelial function, via an increased eNOS protein content in iliac and femoral arteries (Murias et al., 2013). More recently, Batacan et al. (2018) found that 12 weeks of high-intensity ExT (10 m/min at the beginning with a progressive increase up to 50 m/min, for 10-15 min/day divided into four 2.5 min ExT bouts interspersed

with 3 min rest periods, with no slope at first, and a 10% inclination towards the end, 5 days/week) on adult rats fed a high-fat high carbohydrate diet increased endothelium-dependent vasorelaxation.

On the other hand, other animal studies reported no benefits of high-intensity ExT on endothelial function, such as Battault et al. (2016) who found that 6 weeks of high-intensity aerobic ExT (60 min at 80% of maximal aerobic velocity, 5 days/week,) on spontaneous hypertensive rats did not improve endothelium-dependent vasorelaxation. Kemi et al. (2005) compared the effects of 10 weeks of high-intensity (60 min, 5 days/week, with a 25° slope, five intervals alternating between 8 min at 85-90% of $\dot{V}O_{2max}$ and 2 min at 50-60% of $\dot{V}O_{2max}$) and moderate-intensity interval ExT (60 min, 5 days/week, with a 25° slope, five intervals alternating between 8 min at 65-70% of $\dot{V}O_{2max}$ and 2 min at 50-60% of $\dot{V}O_{2max}$) on endothelial function in female adult Sprague-Dawley rats, and observed no difference in endothelial function improvement between the two training intensities. More recently, another study found that C57BL/6J mice trained four weeks at high-intensity ExT (6 bouts of running at 85% of maximal speed for 8 min, interspersed with 2 min active rest at about 50% of maximal speed, 5 days/week, with a 10° slope) did not improve their endothelium-dependent vasorelaxation compared to moderate-intensity continuous ExT mice (about 70 min at 65% of maximal speed, 5 days/week, with a 10° slope) and even sedentary (Kim et al., 2020).

Some studies even show a deleterious effect of high-intensity ExT on endothelial function compared to lower training intensities, such as presented in a paper by Ye et al. (2019) who trained spontaneous hypertensive rats at high (26-28 m/min, which is about 75-85% of maximal aerobic velocity, 60 min, 5 days/week) and moderate-intensity (18-20 m/min, which is about 55-65% of maximal aerobic velocity, 60 min, 5 days/week) for 8 weeks. High intensity ExT rats had a decreased endothelium-dependent relaxation, as well as higher oxidative stress and ROS contribution, and lower NO contribution.

Thus, there is still controversy regarding the potential beneficial effect of high-intensity ExT on endothelial function in animal models.

III. Hypoxic exercise training

1. Definition

Hypoxia can be defined as a condition where the combination of barometric pressure and inspired fraction of O_2 (F_iO_2) results in an inspired pressure of O_2 (P_iO_2) lower than a normoxic value of 150 mmHg (Conkin and Wessel, 2008). Hypoxia can therefore be achieved by lowering barometric pressure (i.e., hypobaric hypoxia) or by lowering F_iO_2 (i.e., normobaric hypoxia). Apart from natural/terrestrial altitude, which is hypobaric hypoxia, there are essentially two ways to obtain normobaric hypoxia, either by diluting O_2 concentration with additional nitrogen or by O_2 filtration, reducing its molecular concentration as it fills up the chamber. In both cases, the low O_2 gas mixtures can be delivered through a mask or a chamber (Wilber, 2007). When P_iO_2 decreases, the alveolar O_2 pressure decreases as well, along with distribution of O_2 to the tissues (Luks et al., 2016), triggering rapid physiological responses. If respiratory, hematological and muscular responses are beyond the scope of this work, molecular and cardiovascular responses are of high importance.

At the molecular level, the master regulator of O_2 homeostasis is the transcription factor hypoxia inducible factor 1 (HIF-1) which controls both O_2 delivery and utilization. HIF-1 is a heterodimer composed of an O_2-monitored subunit, HIF-1α, and a constitutively expressed subunit, HIF-1β (Semenza, 2014). Under normoxic conditions, HIF-1α is repeatedly generated and degraded. O_2-dependent degradation of HIF-1α is induced by hydroxylation of the proline residues 402 and/or 564 by the prolyl hydroxylase domain protein 2, allowing the von Hippel-Lindau protein to bind to HIF-1α. This leads to the recruitment of an E3 ubiquitin-protein ligase complex that ubiquitinates HIF-1α, making it a target for degradation by the 26S proteasome (Figure 2; Jaakkola et al., 2001; Kamura et al., 2000; Maxwell et al., 1999). Under hypoxic conditions, the activity of prolyl hydroxylase domain protein 2 is reduced, thus HIF-1α degradation is inhibited, leading to a build-up of the protein, which dimerizes with HIF-1β and

in turn regulates transcription of target genes through recruitment of coactivator proteins (Figure 2; Semenza, 2007).

Numerous genes are known to be activated at the transcriptional level by HIF-1α, however, at least 2% of all human genes are regulated by HIF-1 in endothelial cells, showing the key role hypoxia plays in vascular reactivity regulation (Manalo et al., 2005).

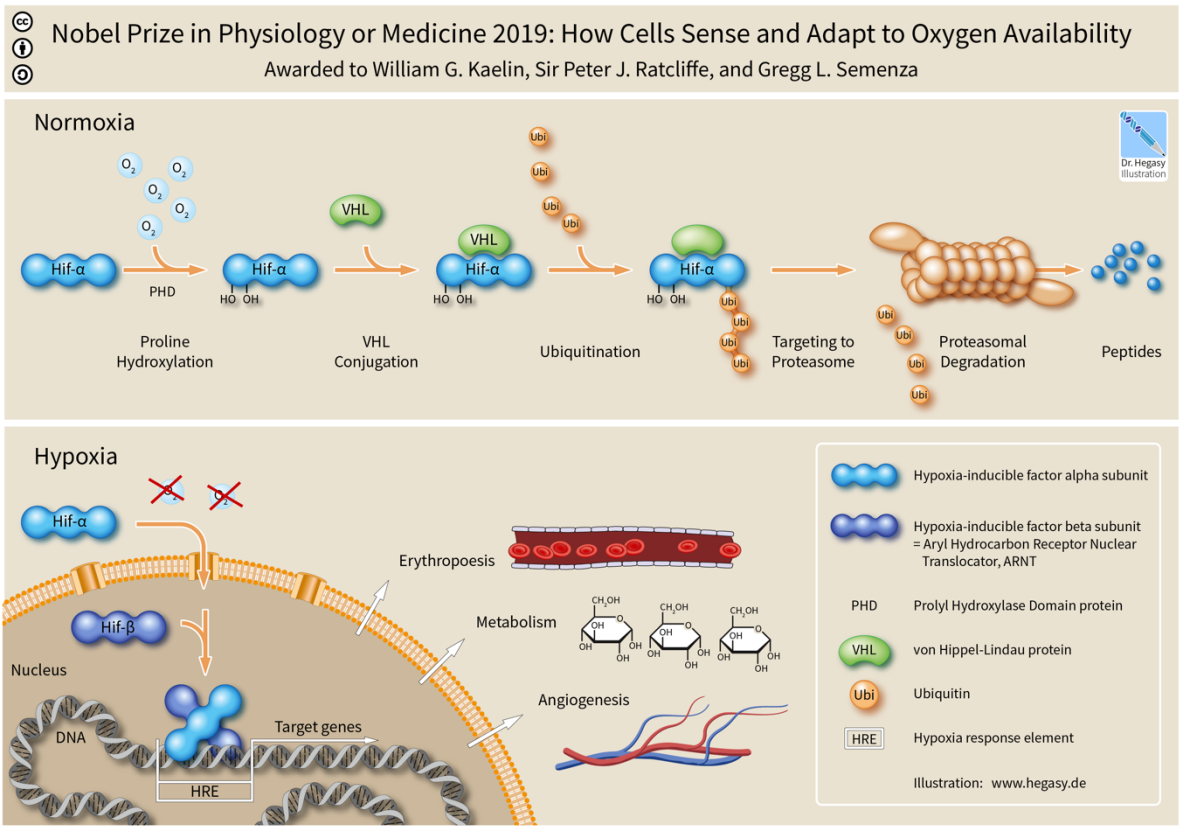

Figure 2 : Nobel Prize in Physiology or Medicine 2019. How Cells Sense and Adapt to Oxygen Availability. Courtesy of Dr. Guido Hegasy (2019).

In a number of vessels, hypoxia has been shown to cause acute contraction of vascular smooth muscle cells that is more distinct when the preparations are pre-constricted, hence its name, “hypoxic augmentation” of vasoconstriction (Vanhoutte et al., 2017). Most of this hypoxic augmentation is endothelium-dependent (Mey and Vanhoutte, 1983) and requires the presence of NO as well as the activation of sGC (Chan et al., 2011). Pulmonary hypertension caused by chronic hypoxia is concomitant with reduced endothelium-dependent relaxations in

pulmonary arteries, due to an overproduction of ROS, reducing eNOS activity and thus NO bioavailability (Fresquet et al., 2006). In a pathology such as sleep apnea, the induced intermittent hypoxia reduces endothelium-dependent vascular reactivity through a reduction in NO production, mediated by ROS and endothelin-1 production (Budhiraja et al., 2007). It has been shown that *in vitro*, hypoxia decreases eNOS expression in endothelial cells (Fish et al., 2010). *In vivo*, studying the impact of hypoxia on endothelial function is not as straightforward because parameters other than the availability of O_2 have to be considered (Man et al., 2014). Regardless, it has been shown that both acute and chronic hypoxia decreased endothelial NO production in rat pulmonary arteries (Shaul et al., 1993). Even without a decreasing in eNOS protein levels, it can be impacted, through a reduction of the phosphorylation of Ser^{1177} or increased phosphorylation of Thr^{495}, which are both associated with diminished eNOS function in hypoxia. eNOS can also co-localize with arginase 2, which competes with eNOS for L-arginine as a substrate (Murata et al., 2002; Prieto et al., 2011).

If hypoxia has long been associated with alterations of vascular reactivity, it now appears to be dose-dependent and can have therapeutic benefits, especially when combined with exercise (Millet et al., 2016).

2. Combination of exercise training and hypoxia

Altitude training is an important research topic in the field of sport sciences, starting with the discovery of the symptoms of acute mountain sickness (Bert, 1878), moving towards physical preparation in view of a competition occurring at high altitude, such as the Olympic Games of 1968 in Mexico, where the disciplines were to be held at an altitude of 2300 m, and continuing today with the use of altitude training to improve sea-level performances (Figure 3) (Faiss et al., 2015; Kasai et al., 2015; Wilber et al., 2007). In a review analyzing the responses of human muscle tissue to hypoxia, Lundby et al. (2009) concluded that chronic exposure to hypoxia alone was not sufficient to induce notable adaptations in the skeletal muscle, whereas the

combination of ExT and hypoxia might generate structural as well as functional modifications. In line with these results, the combination of an innovative form of training – i.e., repeated sprint training in hypoxia (Faiss et al., 2013) – with “traditional” live high-train low method was proven efficient in increasing the ability to repeat sprints of team sport athletes (Brocherie et al., 2015). Repeated sprint training in hypoxia alone has shown to improve the ability to repeat sprints in moderately trained cyclists, concomitantly with muscular adaptations (Faiss et al., 2013b). Using near infrared spectrometry, Faiss et al. (2013b) also showed that these results were accompanied by improved muscle oxygenation, with changes in total hemoglobin concentration. However, that does not reflect the impact of this training on endothelial function.

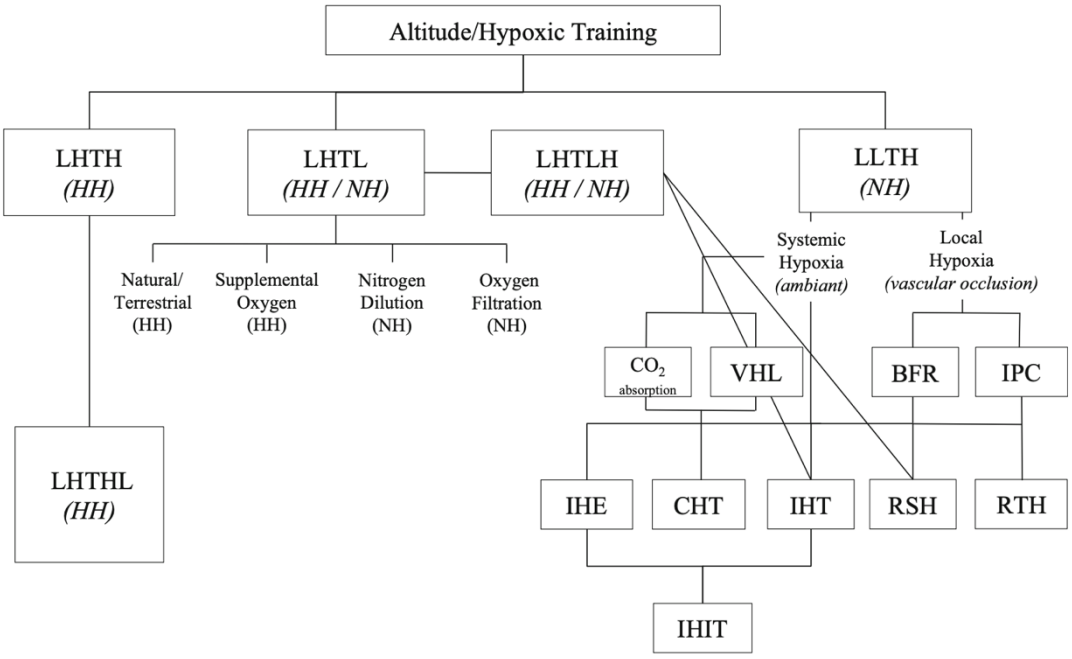

Figure 3. Updated panorama of the different hypoxic/altitude training methods used for a range of athletes. Adapted from Girard et al. (2017). BFR blood flow restriction, CHT continuous hypoxic training, CO_2 absorption rebreathing with a mask, HH hypobaric hypoxia, IHE intermittent hypoxic exposure, IHIT IHE during interval-training, IHT interval hypoxic training, IPC ischemic pre-conditioning, LHTH live high-train high, LHTL live high-train low, LLTH live low-train high, LHTHL live high-train high and low, LHTLH live high-train low and high, NH normobaric hypoxia, RSH repeated sprint training in hypoxia, RTH resistance training in hypoxia, VHL voluntary hypoventilation at low lung volume.

There are clear evidence that submaximal exercise in hypoxia triggers specific responses that are not observed with the same training in normoxia (Lundby et al., 2009). It has been shown that during a hypoxic exercise, muscle blood flow is increased compared to the same level of exercise in normoxia (Calbet et al., 2003; Katayama et al., 2013; Wilkins et al., 2006). This "compensatory" vasodilation aims at preserving tissue O_2 delivery, and ensuring it is matched to demand by triggering additive effects on vascular dilator function (Casey et al., 2010; Montero and Lundby, 2016). However, the compensatory vasodilation exceeds what is predicted by a simple sum of the individual dilator responses to hypoxia alone and normoxic exercise (Casey and Joyner, 2012). Interestingly, during hypoxic exercise there is an increased sympathetic vasoconstrictor activity directed towards skeletal muscles (Hanada et al., 2003). That means that the signals concomitant to the compensatory vasodilation are antagonistic to the vasoconstrictor activity (Casey and Joyner, 2012; Wilkins et al., 2006). In order to understand this interplay, Wilkins et al. (2008) investigated the effects of an α-adrenergic blockade on the compensatory vasodilation during hypoxic forearm exercise, with the hypothesis that β-adrenergic receptor-mediated vasodilation might be partly responsible for the increased hypoxic exercise hyperemia. This study revealed that there was a greater vasodilation following hypoxic exercise in subjects injected with the α-adrenergic blockade compared to the control group (i.e., saline injection), and confirmed a major contribution of β-adrenergic mechanisms in the observed vasodilation. However, despite raised sympathetic vasoconstrictor activity during hypoxic exercise, considerable compensatory vasodilation prevails (Casey and Joyner, 2012). A possible explanation could have been an increased functional sympatholysis, i.e., the blunting of the sympathetic vasoconstriction that naturally occurs under normoxic condition in the vascular beds of contracting muscles. However, Wilkins et al. (2006) demonstrated that the observed compensatory vasodilation during hypoxic exercise is due to an augmented vasodilator signal rather than a blunted vasoconstriction.

Studies have investigated some of the reported metabolic vasodilating substances as possible mediators of compensatory vasodilation during hypoxic exercise. At rest, at least part of the β-

adrenergic receptor activation is mediated through the NO pathway (Weisbrod et al., 2001), and as previously mentioned, it was found that β-adrenergic mechanisms play an important role in the compensatory vasodilation (Wilkins et al., 2008). These findings alongside those of Casey et al. (2010) indicate that the observed compensatory vasodilation during hypoxic exercise is at least partly induced by the β-adrenergic receptor mediated NO release (Figure 4). However, this β-adrenergic contribution decreases as exercise intensity increases, despite NO still playing a central role, indicating that another pathway must be involved in the release of NO to mediate the compensatory vasodilation (Casey et al., 2011; Wilkins et al., 2008). Several candidates have been considered. There could be a direct release of NO from the endothelium on account of shear stress (Ando and Yamamoto, 2013), NO release from erythrocytes in the form on S-nitrosohemoglobin (Stamler et al., 1997) and/or NO release from ATP and prostaglandins (Parkington et al., 2004; Yamamoto et al., 2007). According to a study from Leuenberger et al. (2008), in humans, hypoxia increases plasma but not skeletal muscle NO levels, suggesting an endovascular or endothelial source. Adenosine has also been considered as a putative candidate to compensatory vasodilation. A study showed that after NOS inhibition, adenosine does not contribute to compensatory vasodilation, meaning that its possible implication does not rely on an NO-independent pathway (Casey et al., 2010). However studies have been unable to confirm that it holds a central role (Casey et al., 2009). As mentioned in chapter II.1. *Vascular responses of the body to exercise training: matching oxygen supply and demand*, ATP can be discharged from erythrocytes, contributing to the regulation of blood flow and its distribution (Ellsworth, 2004). Acute exposure to hypoxia at rest as well as exercise in normoxia have been shown to induce increases in venous plasma ATP levels (González-Alonso, 2012). However, due to the lack of specific pharmacological antagonist for ATP P_2 receptors available for use in humans, the study of the contribution of ATP to compensatory vasodilation is limited (Casey and Joyner, 2012). Thus, this lead remains elusive.

It is clear that exercise in hypoxia elicits specific vascular adaptations with a compensatory vasodilation that matches the O_2 demand and supply to tissues throughout the body. The cornerstone of these vascular adaptations is NO, and at low-intensity, its release is mostly stimulated by activation of β-adrenergic receptors. However, there is evidence towards intensity being a key characteristic of exercise capable of inducing different vascular adaptations, with a decreased β-adrenergic contribution as intensity increases. Studies have also showed that hypoxic high-intensity ExT induced beneficial hematological and cardiorespiratory changes mediated by significant increase in serum concentration of proangiogenic factors and NO compared to the same training in normoxia (Żebrowska et al., 2019). However, the pathways initiating NO release during high-intensity ExT in hypoxia have yet to be identified.

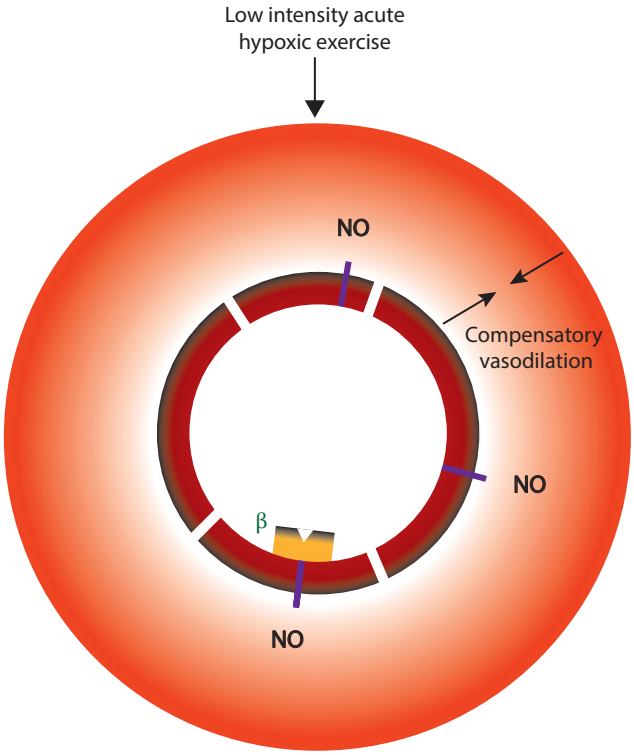

Figure 4. The observed compensatory vasodilation during hypoxic exercise is at least partly induced by the β-adrenergic receptor mediated NO release.

Adapted from Casey and Joyner (2012).

IV. Atherosclerosis

1. Physiopathology

Atherosclerosis is the primary cause of CVD. It is the most frequent cause of coronary artery disease, carotid artery disease and PAD. Endothelial cells, vascular smooth muscle cells and leukocytes are the main actors of the development of this disease (Falk, 2006). In injury-prone areas, atherosclerotic lesions begin to develop under a seemingly intact, yet dysfunctional endothelium. The physiopathology of atherosclerosis has been extensively reviewed (Falk, 2006; Libby et al., 2019; Ross, 1999), however the basic pathophysiological steps of atherosclerotic plaque formation can be summarized as follows: first, low-density lipoprotein cholesterol in excess from the blood infiltrates the dysfunctional endothelial cells and enters the artery wall where it oxidizes (Figure 5a). Then, activated endothelial cells express leukocyte adhesion molecules such as vascular cell adhesion molecules (e.g., VCAM-1), E-selectin and P-selectin that help capture blood monocytes, which then migrate to the arterial wall. Third, the recruited monocytes differentiate into macrophages and absorb the oxidized low-density lipoproteins to become foam cells, which, histologically, appear as a fatty streak. Those are the earliest lesions of atherosclerosis (Figure 5b). When the foam cells die, they release their lipid content, creating a lipid core that is one of the two parts of the atherosclerotic plaque. Fourth, several endogenous molecules (oxidized lipoproteins or their component oxidized lipids; Hovland et al., 2015) can link to toll-like receptors on the foam cells, inducing the release of inflammatory cytokines (i.e., interleukin-1β, interleukin-6, tumor necrosis factor-α and interleukin-12p35). Fifth, vascular smooth muscle cells proliferate and form a fibrous cap (the second characteristic part of the atherosclerotic plaque) overlying the lipid core (Figure 5c and 5d). Finally, the plaque will protrude into the lumen, decreasing the blood flow (Libby et al., 2011; Muller et al., 2013; Toutouzas et al., 2015; Vancraeynest et al., 2011). Atherosclerotic plaques can evolve into an unstable/vulnerable plaque, which can rupture,

leading to dramatic thrombosis and acute cardiovascular events (e.g., unstable angina, acute myocardial infarction, stroke) (Pellegrin et al., 2009a).

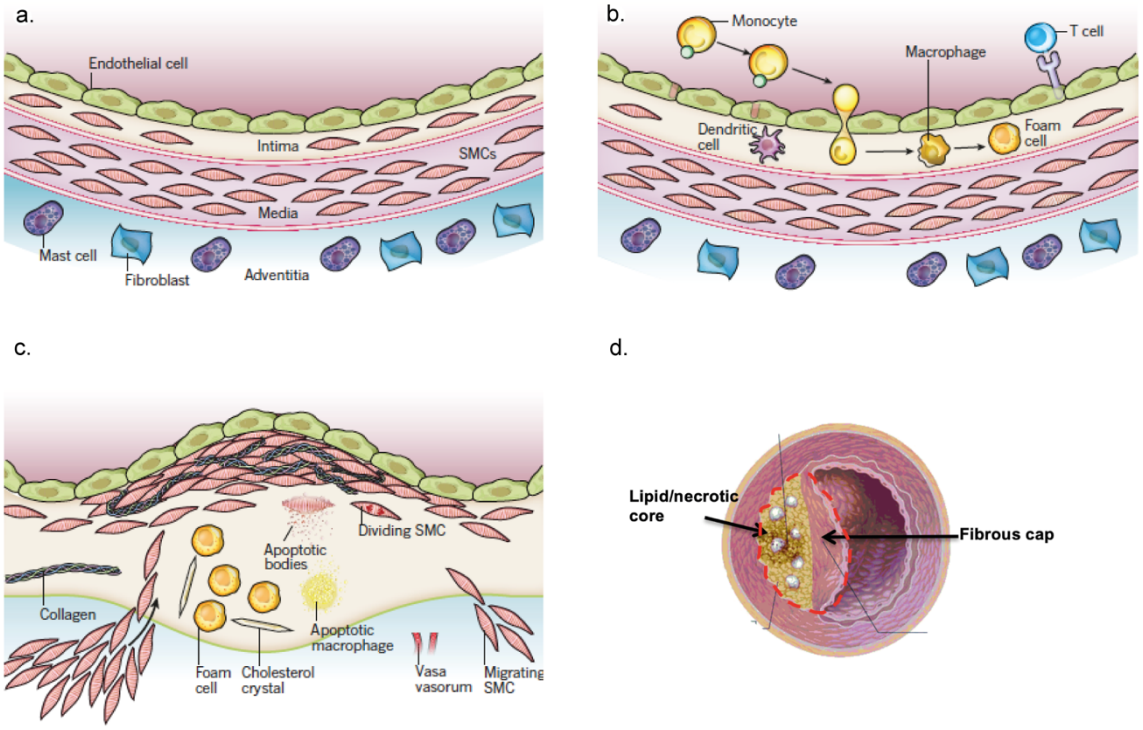

Figure 5. Major pathogenic steps of atherosclerosis.

Adapted from (Libby et al., 2011). (a) Normal artery with healthy layers. (b) The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes into macrophages, and their uptake of lipid, yielding foam cells. (c) Lesion progression involves the migration of smooth muscle cells from the media to the intima, the proliferation of resident intimal smooth muscle cells and media-derived smooth muscle cells, and the heightened synthesis of extracellular matrix macromolecules such as collagen and elastin. Plaque macrophages and smooth muscle cells can die in advancing lesions, some by apoptosis. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid core. Advancing plaques also contain cholesterol crystals and micro-vessels. (d) Atherosclerotic plaque characterized by a lipid core and a fibrous cap covering the lipid core.

2. Exercise as preventive tool against atherosclerosis development

As previously mentioned, exercise is the gold standard for reducing cardiovascular risks in patients, partly through its direct beneficial effect on the development and progression of

atherosclerosis (Green et al., 2017; Shimada et al., 2011). In patients, exercise programs are divided into two categories, supervised and unsupervised programs (Novakovic et al., 2017). Previous studies comparing both types of trainings have shown that supervised ExT provides better outcome due to a higher adherence to programs (Aboyans et al., 2018; Hageman et al., 2018).

Chronic inflammation is a substantial characteristic of atherosclerosis, with reported increase in pro-inflammatory cytokines both in atherosclerotic lesions and in the circulation (Geovanini and Libby, 2018; Taleb, 2016). In an experimental mouse model of atherosclerosis, Pellegrin et al. (2009) showed that 24 weeks of swimming ExT, for 50 min, 5 days per week, “stabilized” atherosclerotic plaques (i.e., less prone to rupture) in $ApoE^{-/-}$ mice, with a decrease in macrophage and an increase in smooth muscle cells content in the plaque. They also showed that 4 weeks of voluntary wheel running significantly hampered the progression and helped the stabilization of pre-existing angiotensin II-mediated atherosclerotic lesions in a 2-kidney, 1-clip renovascular hypertension mouse model. This result was accompanied by a shift towards a systemic anti-inflammatory profile (Pellegrin et al., 2015). Another study conducted in $ApoE^{-/-}$ mice fed a high-fat diet showed that 10 weeks of voluntary wheel running led to a decrease in pro-inflammatory mediators when compared to sedentary high-fat fed mice (Fukao et al., 2010).

Oxidative stress is another major characteristic of atherosclerosis. Excess ROS generation and low density lipoprotein (LDL) oxidation are linked to atherosclerosis risk factors (Kattoor et al., 2017). A study showed that 30 to 45 min of swimming, three times a week for 8 weeks in $ApoE^{-/-}$ mice fed a high-fat diet boosted the antioxidant system via the NO pathway, restraining the progress of experimental atherosclerosis (Okabe et al., 2007).

As explained before, macrophages are recognized as key agents in widespread diseases processes associated with chronic inflammation, including atherosclerosis and they become foam cells by taking up oxidized LDL, which appear histologically as a fatty streak, the earliest

lesions of atherosclerosis (Libby et al., 2011). A study showed that 30 min of treadmill running (15 m/min five times per week for six weeks) improved the cholesterol trafficking from macrophages to the liver in wild type mice (Pinto et al., 2015).

As previously mentioned, it is recognized that the underlying reason for atherosclerosis-induced dramatic clinical events is plaque destabilization and rupture. In a mouse model of renovascular hypertension, Pellegrin et al. (2009b) reported that 50 min of swimming, five days a week, for five weeks prevented the development of vulnerable atherosclerotic plaques.

The efficiency of ExT to improve atherosclerosis has therefore been established. However, no study has investigated the effects of high-intensity interval training alone or combined to hypoxia on the development and the progression of atherosclerotic plaques.

V. Aims and hypothesis

Based on this review of the literature, we believe that, in patients with CVD, the combination of high-intensity ExT and hypoxia could induce physiological benefits that would be above those obtained with the ExT protocols currently recommended (i.e., low to moderate-intensity). Therefore, the general aim of this thesis was to set the foundations to investigate the effects of three ExT intensities (i.e., low, maximal and supramaximal) combined with hypoxia on vascular reactivity and NO pathways in mouse models of CVD, more specifically atherosclerotic mice. To do so, we first developed and tested the feasibility of supramaximal-intensity training in healthy mice and determined a tolerable and adequate protocol (intensity, run duration, recovery, etc.). This part of the study is presented in Study 1, Article 1.

Then we compared the effects of the three different ExT intensities in normoxia and in hypoxia on vascular reactivity in healthy C57BL/6 mice. We hypothesized that the vascular adaptations would depend on the combination of both ExT intensity and O_2 availability, and therefore that (i) vascular reactivity (i.e., vasoconstriction and vasodilation) would improve to a greater extent as ExT intensity increases either in normoxia or hypoxia; and (ii) hypoxic ExT would improve vascular reactivity to a greater extent than the same intensity in normoxia. This is presented in Study 1, Article 2.

Finally, in collaboration with the laboratory of Professor Ying Zhang from the Beijing Sport University we conducted a study to investigate the therapeutic and preventive effects of low-intensity and high-intensity ExT in hypoxia on hypercholesterolemic ApoE$^{-/-}$ mice. We hypothesized (i) that high-intensity ExT in hypoxia would be more efficient in preventing plaque development (i.e., preventive) and reverse pre-existing plaques (i.e., therapeutic) than low-intensity ExT, both in normoxia and hypoxia; and (ii) that hypoxic ExT would prevent plaque development and reverse pre-existing plaques more than the same intensity in normoxia. This is presented in Study 2.

Chapter 2

Summary of experimental results

Summary of Experimental Results

In this section, only the main results of the second article of study 1 are being presented (i.e., Chapter 6 – Article 2). Due to the originality of this research, we first had to set up the hypoxic training facilities and set up the exercise protocol. Therefore, the first article of the first study (i.e., Chapter 5 – Article 1) was a description of the method, intended for testing the feasibility of the novel supramaximal intensity repeated-sprint training protocol in mice.

I. Effects of exercise training intensity and hypoxia on endothelial function

In the aorta, the endothelium-dependent vasorelaxation to ACh was significantly improved in LowH compared to LowN (+10.0%, $P < 0.001$), as well as in MaxH compared to MaxN (+14.8%, $P < 0.0001$) and SupraH compared to SupraN (+20.0%, $P < 0.0001$). Acetylcholine-induced relaxation was 9.2% greater in MaxH and 8.2% greater in SupraH than in LowH ($P < 0.001$ and $P < 0.01$, respectively). There was no significant difference in ACh-induced relaxation between LowN, MaxN and SupraN (Figure 6A, B).

Endothelium-independent relaxation to the NO donor DEA/NO was significantly increased in LowH compared to LowN (+4.7%, $P < 0.01$). It was also significantly greater in MaxN compared to LowN (+4.3%, $P < 0.05$) and in MaxH compared to SupraH (+3.2%, $P < 0.05$) (Figure 6C, D).

The vascular constriction to phenylephrine (Phe) was significantly improved in LowH, MaxH and SupraH compared to LowN, MaxN and SupraN respectively (+42.1%, $P < 0.05$; +48.3%, $P < 0.01$ and +119.9%, $P < 0.0001$). Vasoconstriction was greater in SupraH compared to LowH (+25.3%, $P < 0.05$) (Figure 7A, B).

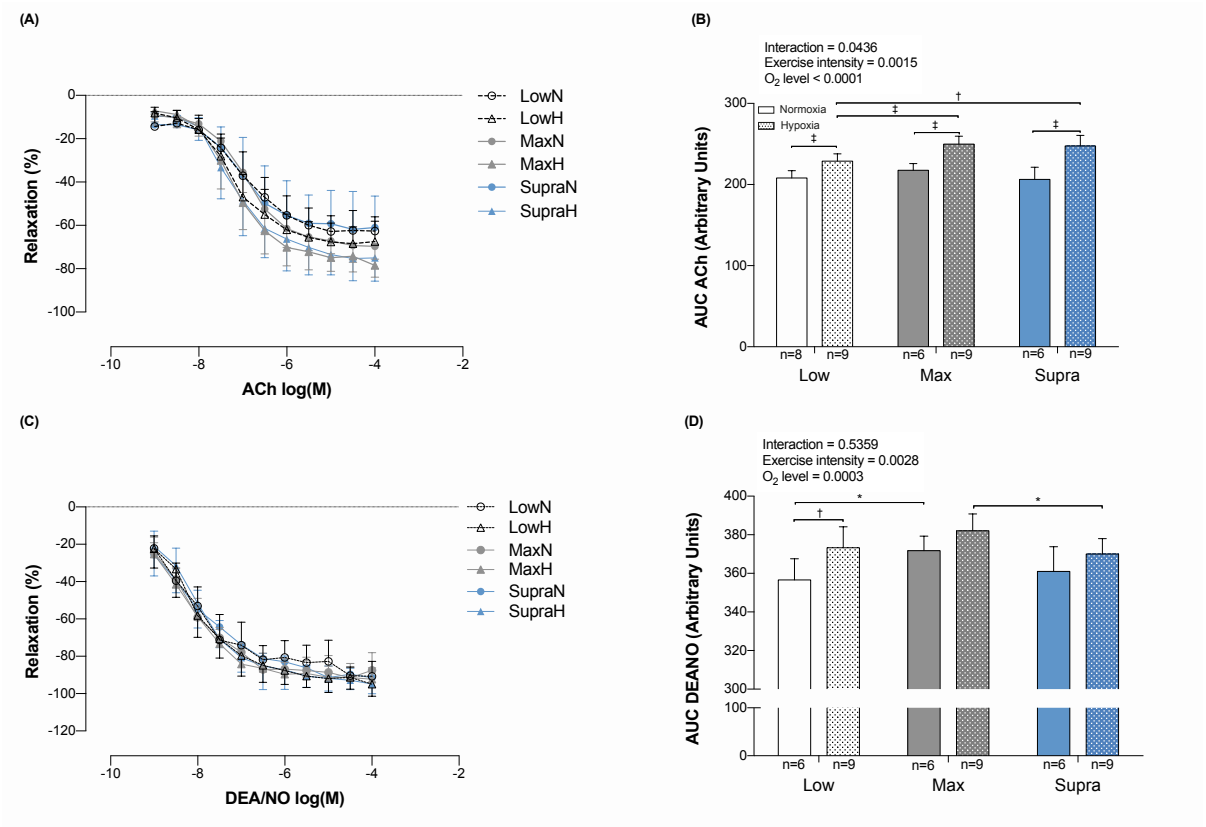

Figure 6. Effect of hypoxic exercise training at different intensities on endothelium-dependent and independent vasorelaxation.

Dose-response curves to acetylcholine (ACh, **A**) and to DEA/NO (**C**) of isolated abdominal aorta pre-constricted with phenylephrine in LowN, LowH, MaxN, MaxH, SupraN and SupraH mice. Bar graphs show the area under the curve of the vascular responses (**B**, **D**), calculated from the relaxation curves shown in a and c. Data are expressed as mean ± SD (n = 6 to 9 mice per group) of the percent of change in tension induced by the vasodilator. Two-way ANOVA with Sidak post-hoc test: * $p<0.05$; † $p<0.01$; ‡ $p<0.001$. Groups: low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN) and supramaximal intensity training group in hypoxia (SupraH).

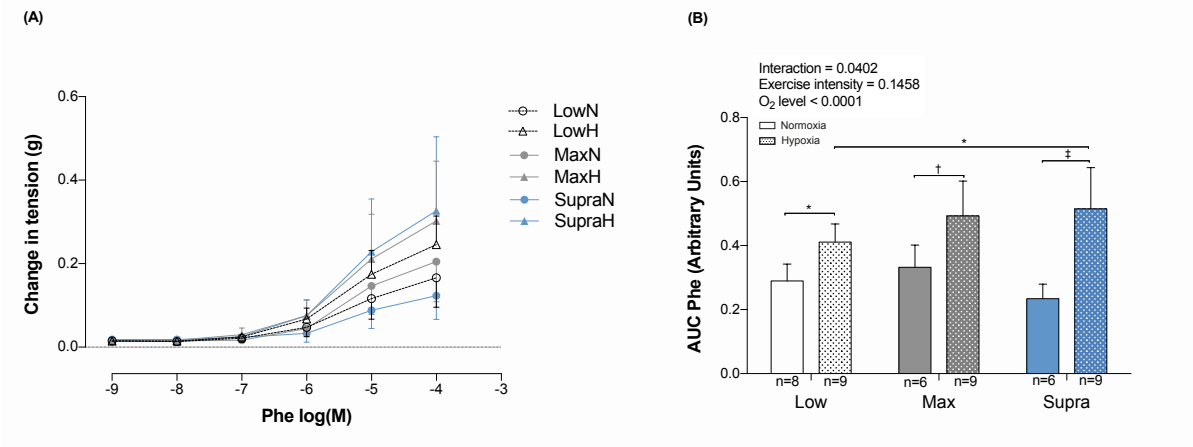

Figure 7. Effect of hypoxic exercise training at different intensities on vasoconstriction.

Dose-response curves to α1-adrenergic receptor agonist Phenylephrine (Phe, **A**) of isolated aorta of LowN, LowH, MaxN, MaxH, SupraN and SupraH mice. Bar graph shows the area under the curve of the vascular responses (**B**), calculated from the contraction curves shown in a. Data are expressed as mean ± SD (n = 6 to 9 mice per group). Two-way ANOVA with Sidak post-hoc test: * p<0.05; † p<0.01; ‡ p<0.001. Groups: low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN) and supramaximal intensity training group in hypoxia (SupraH).

II. Effects of exercise training intensity and hypoxia on aortic nitric oxide production and bioavailability

To understand the vasoreactivity results, we investigated the aortic mRNA and protein expression of markers involved in NO production and bioavailability. We found that mRNA level of eNOS was higher in SupraH compared to SupraN ($P < 0.05$), while no significant differences were observed in LowH and MaxH compared to their respective normoxic groups (Figure 8A). mRNA level of eNOS was significantly higher in LowH and SupraH compared to MaxH ($P < 0.05$; Figure 8A). No significant difference was observed between LowN, MaxN, and SupraN (Figure 8A). Neither protein expressions of eNOS nor p-eNOS showed significant differences between the six training groups (Figure 8B). The ratio of p-eNOS to eNOS expression revealed no significant difference between any of the groups either (Figure 8C).

As shown in Figure 9A, there were no significant differences in mRNA level of antioxidant SOD3 as well as in the protein expression of antioxidant SOD1, SOD2 and SOD3 among any of the groups. Neither the mRNA level nor the protein expression of pro-oxidant p47phox significantly differed between groups (Figure 9B).

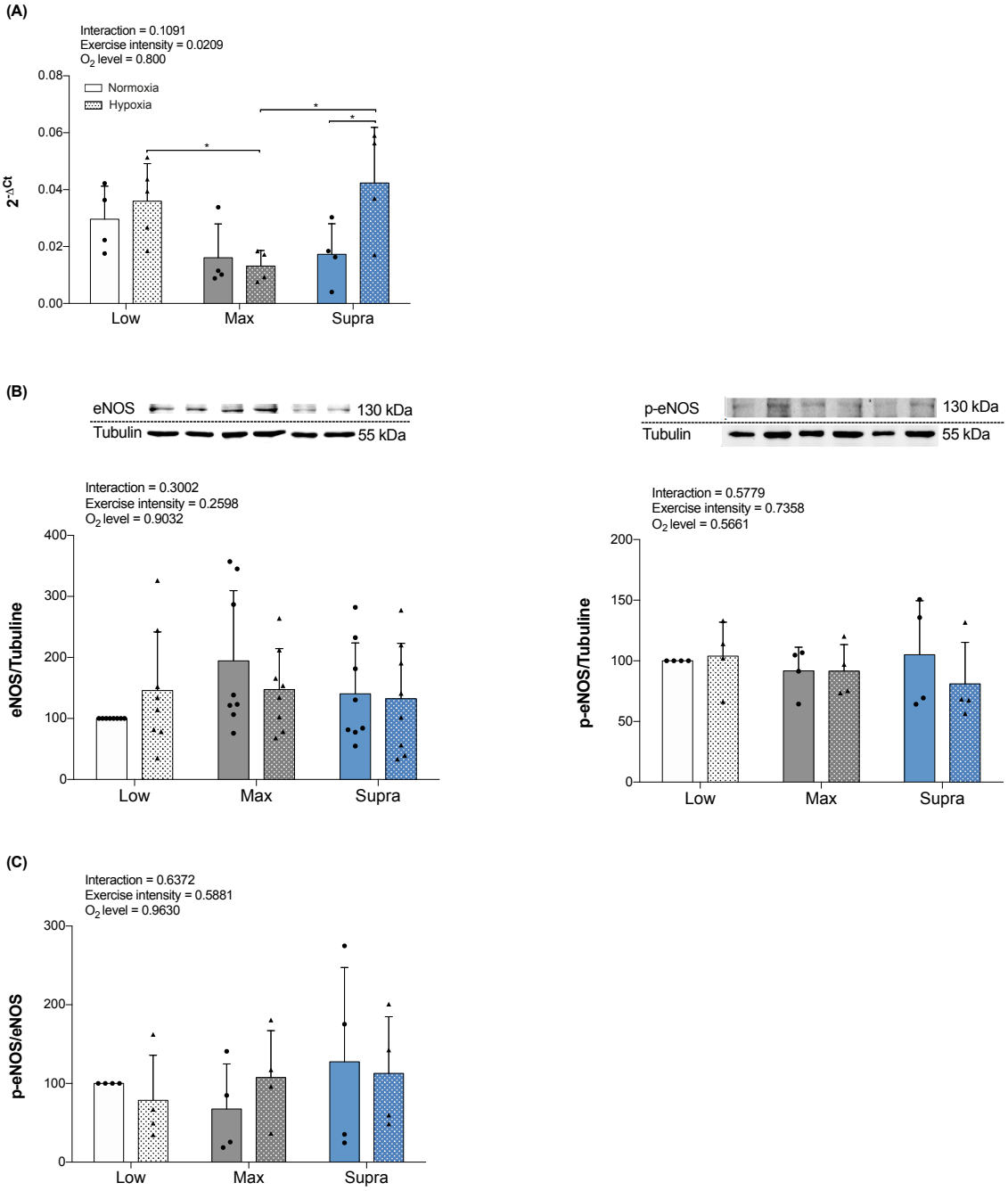

Figure 8. Effect of hypoxic training at different intensities on mRNA and protein expression of markers involved in NO production in aorta.

(**A**) Gene expression analysis of eNOS. Results are expressed as $2^{-\Delta Ct}$ using 36B4 as housekeeping gene. (**B**) Western blot analysis of eNOS and phospho-eNOS protein expression. Protein expressions were normalized to tubulin content in each sample and expressed as the percentage of the protein expression values obtained in the LowN group. Top panel: representative WB images; bottom panel: quantitative analysis. (**C**) Ratio of phospho-eNOS to eNOS. Data are presented as mean ± SD (n = 4 to 8 mice per group). Two-way ANOVA with Sidak post-hoc test: * p<0.05. Groups: low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN), and supramaximal intensity training group in hypoxia (SupraH).

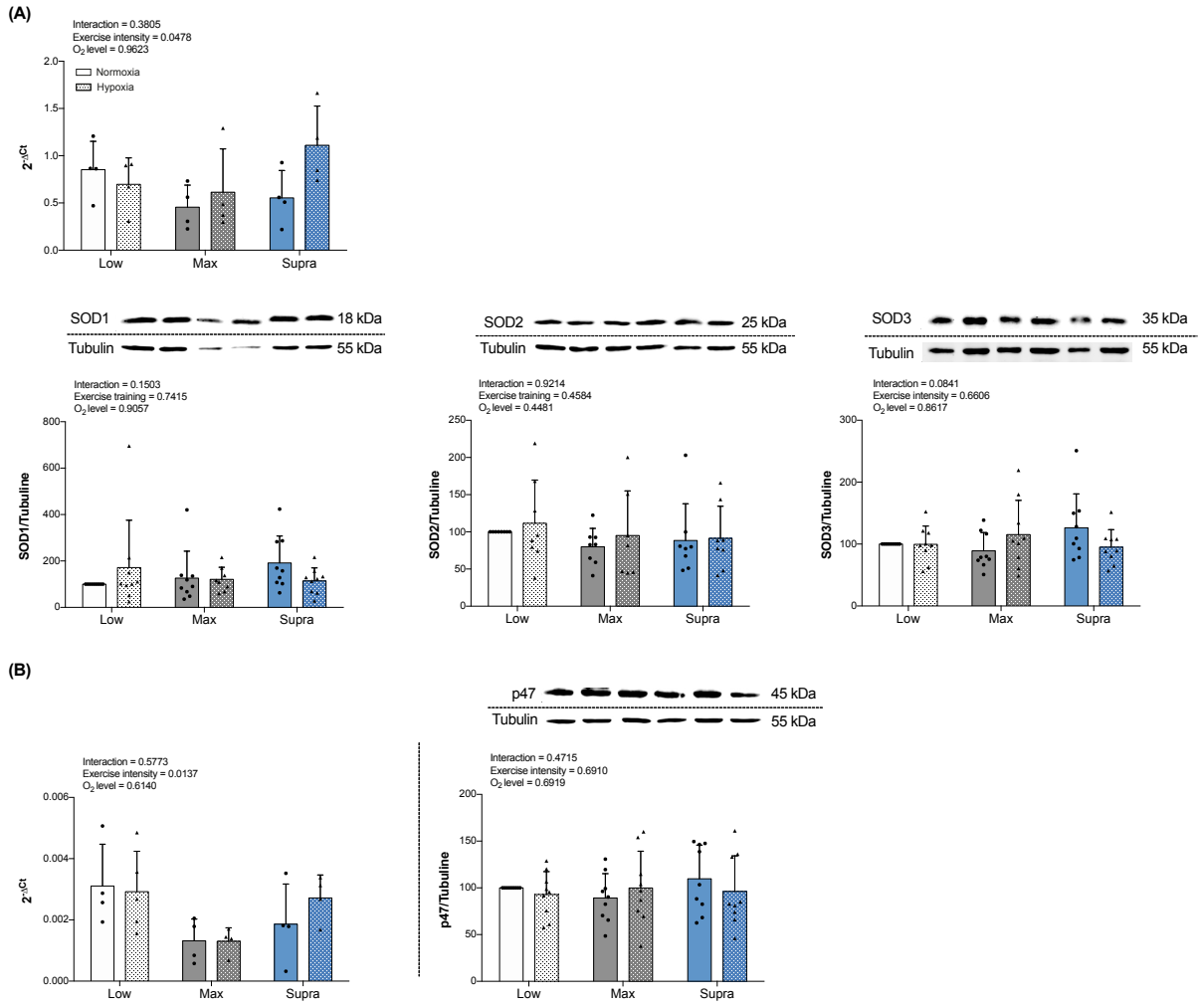

Figure 9. Effect of hypoxic training at different intensities on mRNA and protein expression of markers involved in NO bioavailability in aorta.

(**A**) Gene expression analysis of antioxidant SOD3 (**top**) and Western blot analysis of SOD1, SOD2 and SOD3 protein expression (**low**). (**B**) Gene expression analysis (**left**) and Western blot analysis (**right**) of pro-oxidant

p47phox. For gene expression analysis, results are expressed as $2^{-\Delta Ct}$ using 36B4 as housekeeping gene. For western blot analysis, protein expressions were normalized to tubulin content in each sample and expressed as the percentage of the protein expression values obtained in the LowN group. Top panel: representative WB images, and bottom panel: quantitative analysis. Data are presented as mean ± SD (n = 4 to 9 mice per group). Two-way ANOVA with Sidak post-hoc test. Groups: low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN), and supramaximal intensity training group in hypoxia (SupraH).

III. Effects of exercise training intensity and hypoxia on circulating nitric oxide metabolites, pro- and antioxidant markers

We then investigated NO bioavailability and oxidative stress in the circulation. No significant changes between any of the six training groups were observed in the concentration of NOx (Figure 10A). Concentration of NO_2 alone was significantly higher in SupraN compared to MaxN (4.5 ± 1.9 $\mu mol.L^{-1}$ vs 2.9 ± 0.9 $\mu mol.L^{-1}$, $P < 0.05$; Figure 10B). No other significant differences were observed between the other groups.

Regarding oxidative stress markers, no significant changes between any of the six training groups were observed in advanced oxidation protein products (AOPP; Figure 10C) and in XO (Figure 10D). As shown in Figure 10E, nitrotyrosine (3-NT) concentration was significantly decreased in MaxH and SupraH compared to the same intensities in normoxia ($P < 0.05$ and $P < 0.001$, respectively), and in SupraH compared to LowH ($P < 0.01$).

No significant differences in plasma SOD (Figure 10F) and catalase activities (Figure 10G) were observed between LowH and LowN, MaxH and MaxN, or SupraH and SupraN. SupraH mice exhibited higher plasma SOD ($P < 0.05$) and catalase ($P < 0.01$) activities than LowH (Figure 10F and 10G).

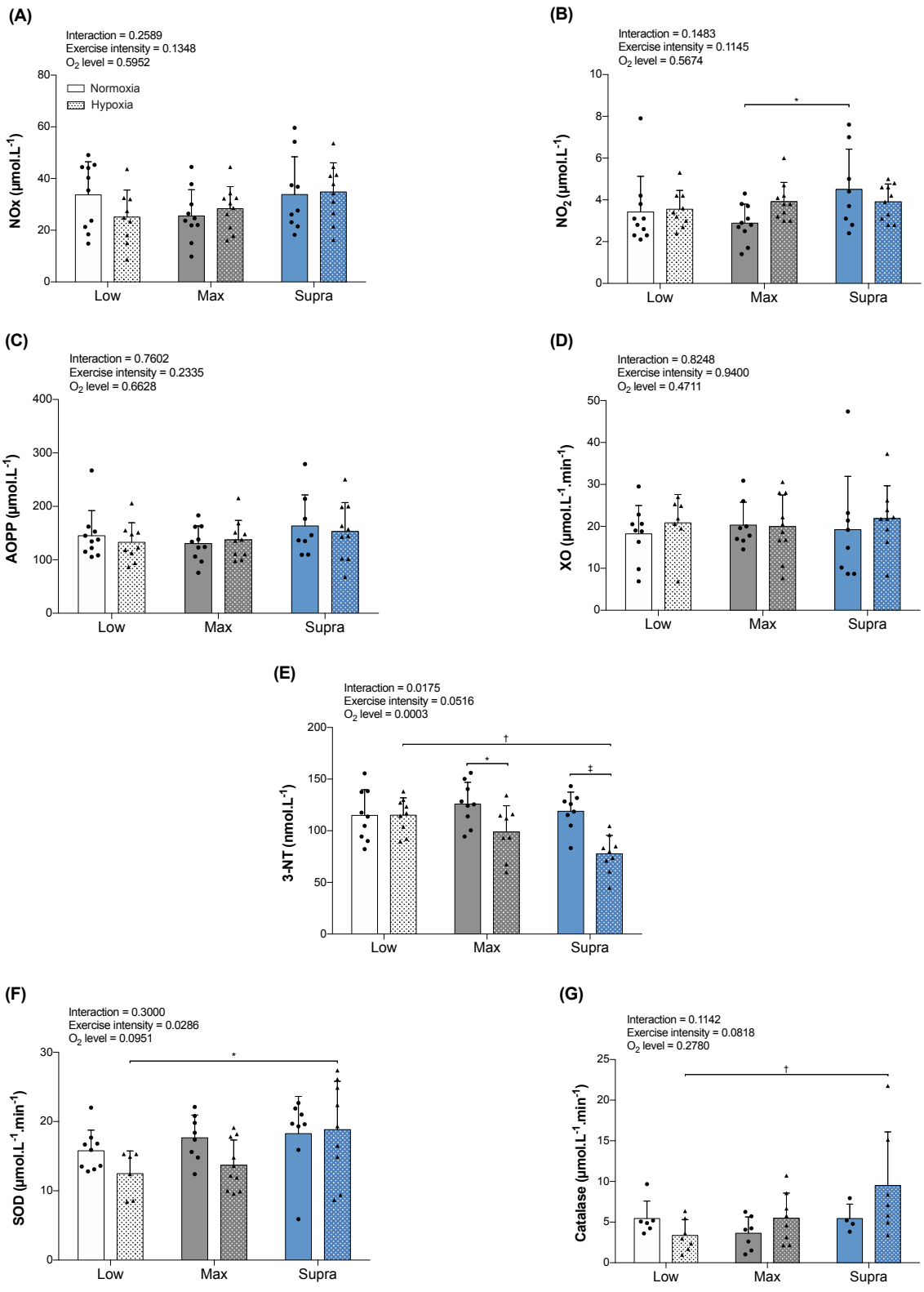

Figure 10. Effect of hypoxic training at different intensities on circulating NO metabolites, oxidative stress markers and antioxidant markers.

Plasma concentrations of NO metabolism end-product nitrite and nitrate (NOx, A), nitrite alone (NO_2, B), advanced oxidation protein products (AOPP; C), xanthine oxidase (XO; D) and nitrotyrosine (3-NT, E), and plasma superoxide

dismutase (SOD; F) and catalase (G) activities in low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN) and supramaximal intensity training group in hypoxia (SupraH). Data are presented as mean ± SD. Two-way ANOVA with Sidak post-hoc test: * $p<0.05$; † $p<0.01$; ‡ $p<0.001$.

Chapter 3

Discussion and perspectives

Discussion

Impaired endothelium function is the first step in the development of atherosclerosis and is associated with CVD occurrence. With 523 million people worldwide in 2019 affected by CVD, preventing and improving endothelial dysfunction is a major public health challenge (Roth et al., 2020). ExT is a cornerstone in primary and secondary prevention of CVD, with evidence leaning towards a positive effect of high-intensity ExT on endothelial function in patients (Chidnok et al., 2020). Combining hypoxia with low/moderate continuous ExT has shown to potentiate the effects of exercise on vascular reactivity, with a compensatory vasodilation working to increase blood flow to maintain tissue O_2 delivery (Casey and Joyner, 2012; Montero and Lundby, 2016). To date, however, there has been a lack of research on the effects of combining high-intensity ExT and hypoxia on endothelial function.

Our work focused on the effects of low, maximal and supramaximal intensity ExT in normoxia and hypoxia on vascular reactivity in healthy C57BL/6 mice. We showed that ExT performed in hypoxia significantly improved vasodilation compared to normoxia, independently of exercise intensity. Moreover, for the first time to date, we reported a larger effect of higher ExT intensities in hypoxia on endothelium-dependent relaxation. The improved vasorelaxation was not associated with an increased vascular eNOS activation and/or NO production but rather with a limited NO inhibition at the systemic level, as indicated by the observed decrease in 3-NT in higher intensity groups training in hypoxia compared to the same trainings in normoxia. Therefore, it appears that benefits of high-intensity ExT in hypoxia on endothelium-dependent vasorelaxation are not mediated by an increased NO production, but rather by increased NO bioavailability secondary to an increased antioxidant status (Figure 11). If the functional results of this work are innovative, the understanding of the underlying mechanisms requires further investigation.

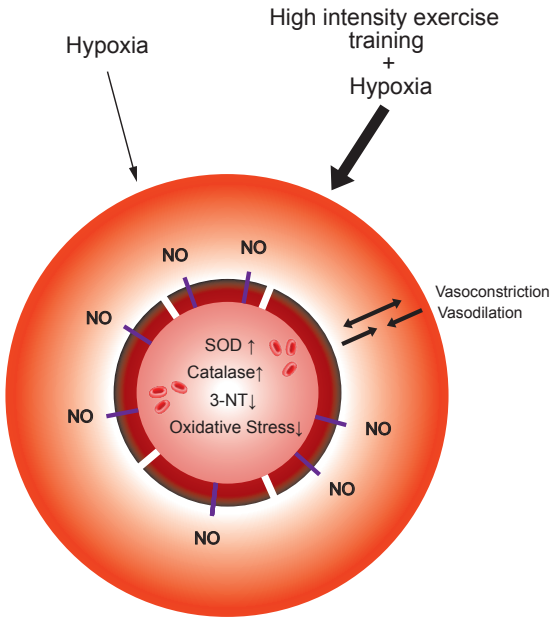

Figure 11. The benefits of high-intensity ExT in hypoxia on endothelium-dependent vasoreactivity are mediated by increased NO bioavailability secondary to an increased antioxidant status in the circulation.

Adapted from (Casey and Joyner, 2012)

ExT increases blood flow and thus shear stress which is a substantial stimulus that plays a role in the stimulation of NO production in the endothelium (Noris et al., 1995). It is known to upregulate eNOS mRNA and protein expression (Hambrecht et al., 2003a; Woodman et al., 1997). It has been shown that long-term ExT induces vascular structural adaptation that induces a chronic increase in vessel diameter, partially perhaps through NO-mediated remodeling (Prior et al., 2003). In turn, shear stress is structurally normalized and NO bioavailability returns to basal levels (Di Francescomarino et al., 2009). Thanks to animal studies, it has been possible to look into the time course of the effects of ExT on NO-dependent vascular reactivity (Green et al., 2004), however there is no consensus on the exact timing (i.e., duration of ExT and rest time between the last training and the sacrifice) required to observe both an effect of ExT on vascular reactivity and on eNOS, pro-/antioxidant gene and/or protein expressions. A study conducted in healthy rats showed that up to four weeks of high-intensity continuous training was sufficient to induce increased endothelial-dependent vasorelaxation, accompanied by increased eNOS protein levels in the aorta compared to

sedentary rats (timing of sacrifice not specified) (Delp and Laughlin, 1997). Another study conducted in mice showed that four weeks of moderate-intensity aerobic ExT did not increase vasodilation in trained wild type mice compared to sedentary mice, and no differences in total eNOS, p-eNOS/eNOS and catalase protein expressions were observed (sacrifice 48 h after the last training session) (Guizoni et al., 2016). The same study also investigated the effects of four weeks of moderate-intensity aerobic ExT on low-density lipoprotein receptor knockout mice and found that vasodilation was significantly improved compared to sedentary mice of the same strain. This improvement was not concomitant with an increased eNOS and catalase protein expression, but with an increase in the p-eNOS/eNOS ratio (Guizoni et al., 2016). In a study on C57BL/6 mice, Kim et al. (2020) found that four weeks of moderate-intensity continuous training (MICT) and HIIT did not improve vasodilation compared to sedentary mice, and NOS gene expression was not modulated (sacrifice 48 h after the last training session). Another study conducted in wild type mice showed that six weeks of low-intensity continuous training improved aortic vasodilation in trained mice compared to sedentary mice, which was concomitant with increased eNOS, p-eNOS, catalase and SOD2 protein expression (sacrifice 12 h after the last training session) (Chen et al., 2016). Several studies, however, used longer periods of ExT in their protocols. In wild type mice trained at moderate-intensity for 8 weeks, Silva et al. (2016) found that thoracic aorta vasodilation was unchanged compared to sedentary mice. Surprisingly, total eNOS protein expression was significantly higher in the trained group compared to the sedentary, however the p-eNOS/eNOS ratio and SOD2 protein expressions were unchanged (timing of sacrifice not specified). Another study found that perivascular adipose tissue deprived aortas of C57BL6/J mice, training at moderate intensity for 8 weeks, had a more potent vasorelaxation response to ACh compared to sedentary mice. However, protein expression of eNOS, SOD1, SOD2 and SOD3 remained unchanged (sacrifice 48 h after the last training session) (Sousa et al., 2019). Araujo et al. (2015) found that rats trained at moderate intensity for 8 weeks did not increase neither aortic vasodilation nor eNOS protein expression compared to sedentary rats (sacrifice 48 h after the last training session). Taking these studies into consideration, one cannot exclude that we might have

missed the time frame allowing us to link our vascular reactivity results to mRNA and protein expression of marker involved in NO production, as well as NO bioavailability.

If the phosphorylation of eNOS is known to be a determinant of its action (Corson et al., 1996; Hambrecht et al., 2003b), its location is critical for the regulation of its activity (Prabhakar et al., 1998; Sánchez et al., 2006). Although RT-qPCr and western blotting give a general view of the molecular mechanisms, they are not precise enough to visualize this aspect of eNOS functioning. It has been shown that ACh stimulates the favored movement of cell membrane-bound eNOS to specific intracellular locations, suggesting a functional meaning for the molecular movement of eNOS (Sánchez et al., 2006). A study showed both in vivo and in vitro, that eNOS translocated from the cell membrane to the Golgi within 30 s, indicating that translocation cues for the onset of vasodilation (Sánchez et al., 2006). For that they used regular and confocal fluorescence microscopy, which would have been an interesting technique to use in our work, had we not been limited in samples. Using an optical microscope equipped with a fluorescein filter, it would also have been interesting to directly look at NO production by submitting aortic segments to an NO-sensitive fluorescent dye. However, this technique is limited in that it not only detects NO, but also intermediate forms such as NO_2 (Sousa et al., 2019). Vascular redox status could have been an interesting trail to investigate, using liquid chromatography and an electrochemical detector to quantify the GSH to GSSG (Tanaka et al., 2015). Unfortunately, as previously mentioned, we had very limited amounts of aortic tissue.

We also explored the possible involvement of H_2S in hypoxic high-intensity ExT-modulated vasoreactivity, since it has been reported to have a protective role on the endothelium through regulation of oxidative stress (Li et al., 2019). Our results were inconclusive, however only one study has looked into the involvement of the H_2S pathway in the impact of ExT on endothelial function (Gu et al., 2013), and none have ever investigated the effects of ExT in hypoxia on this specific pathway. Therefore, further research must be conducted before completely excluding this lead.

As previously mentioned, we found surprising that the results of gene and protein expression in the aorta did not support the functional results of vascular reactivity and thought that we missed the pathway behind it, since we did see an effect of our protocols in the circulation. However, rather than the pathway, one cannot rule out that we missed the tissue responsible for these vascular reactivity improvements.

The perivascular adipose tissue (PVAT) is well established as a functional component of the vasculature due to its paracrine and endocrine influence on vascular reactivity (da Rocha et al., 2017). PVAT is located around large conduit arteries, veins and resistance vessels among others (Xia and Li, 2017). In some vessels, PVAT is an intrinsic part of the vascular wall without any form of barrier (e.g., laminar structure) separating it from the adventitia (Gil-Ortega et al., 2015). Substances released by PVAT can either reach the inner layers of the vessels by direct diffusion or via the vasa vasorum (i.e., small vascular network supplying the wall of larger vessels) (Gil-Ortega et al., 2015). In 1991, Soltis and Cassis published the first study suggesting a possible effect of adipose tissue on the regulation of blood vessel vascular tone, as they observed a decreased norepinephrine-induced vasoconstriction in rat aorta due to PVAT. It has also been established that PVAT decreases the vasoconstriction generated by agonists such as endothelin, angiotensin II and Phe (Szasz and Webb, 2011). PVAT can regulate the vascular tone in both an endocrine and paracrine way by releasing bioactive molecules called adipocyte-derived relaxing factors (ADRF) because of their anticontractile properties (Xia and Li, 2017). Like EDRF and EDHF, it is most likely that there is not a unique ADRF but rather an interaction between several substances, depending on the type of stimulus received, the targeted vessel and the structure of the local PVAT (Xia and Li, 2017). Several molecules have been proposed as candidates for ADRF, with some being the same as EDRF. These include NO and H_2S, prostacyclin, angiotensin II, endothelin 1, ROS, but also adipokines (e.g.., leptin, adiponectin, resistin etc.) and cytokines (e.g., interleukin 6, tumor necrosis factor α, etc.) (Gollasch, 2012). In a study to investigate the effects of PVAT on endothelial dysfunction in obese C57BL/6 mice, it was shown that thoracic aortas without

PVAT did not present a lessen vasodilation to ACh when comparing the obese and lean groups, contrary to the aortas that were left with PVAT. Using an eNOS inhibitor, L-NG-Nitroarginine Methyl Ester (L-NAME), they found that this lack of vasodilation was NO dependent. Most importantly, they discovered that all the pathological changes associated with experimental obesity (i.e., eNOS dephosphorylation, arginase upregulation, L-arginine deficiency and eNOS uncoupling) were observed in PVAT but not in the aorta, indicating that the reduced vascular reactivity observed in obese mice was due to a dysfunction of eNOS in the PVAT rather than the endothelium (Xia and Li, 2017; Xia et al., 2016). A few studies have investigated the effects of ExT on PVAT and endothelial function. A study conducted in obese C57BL/6 mice showed that 8 weeks of aerobic ExT enhanced the expression of antioxidant proteins and decreased PVAT oxidative stress with a positive impact on vasodilation of the thoracic aorta (Sousa et al., 2019). Araujo et al. (2015) conducted a study in which they trained healthy Wistar rats for 8 weeks at moderate-intensity (60-80% of maximal aerobic speed). Interestingly, they did not find any difference in vasorelaxation between thoracic aortas with and without PVAT, and there was no difference in eNOS protein expression in the aorta between sedentary and trained rats. However, in the PVAT, eNOS protein expression was 50% higher in trained compared to sedentary rats. These studies indicate that both in physiological and pathological situations – where PVAT has become dysfunctional – there seems to be a clear effect of ExT on PVAT. There is close to no data on the impact of ExT modalities (i.e., intensity, volume) on the characteristics of PVAT (i.e., inflammation or redox status), and none on the combination of ExT and hypoxia, therefore further studies must be conducted in this direction.

The fact that we did not see any changes in gene and protein expression despite our vascular reactivity results might be due to molecular adaptations in the PVAT. The way our aortas were cleaned out before being set inside the organ chambers for the vascular reactivity tension studies most likely obliterated the surrounded PVAT. According to the aforementioned study (Xia et al., 2016), we should not have seen differences in vasorelaxation between the groups, which is actually what we observed in normoxia. However, hypoxia alone and its combination

with high-intensity ExT might have induced a strong enough stimulus that could have caused long-term adaptations, explaining why even without PVAT, we observed an improved vasodilation in our hypoxic groups, independently of exercise intensity, but also in our high-intensity exercise groups compared to the low intensity group in hypoxia. Future studies in our laboratory must take this into consideration for future projects, and make sure to investigate the role of PVAT.

Perspectives

Peripheral artery disease is one of the most well-known types of CVD, and a highly prevalent public health problem affecting 237 million people worldwide in 2015 (Song et al., 2019). Peripheral artery disease in the lower limbs is called lower extremity peripheral artery disease. It occurs when there is a significant narrowing of the arteries supplying the legs, most often due to atherosclerosis, resulting in a decreased circulation and a range of symptoms. The typical symptom is intermittent claudication. It manifests as ischemic muscular pain in the legs during physical activity, because of an arterial narrowing that impairs blood flow, which in turn cannot meet the metabolic needs of the active muscles (Figure 12). As a consequence, physical capacity of patients with PAD and intermittent claudication is significantly reduced, and their quality of life as well as their capacity to complete daily living activities requiring the use of their legs is altered (Askew et al., 2014).

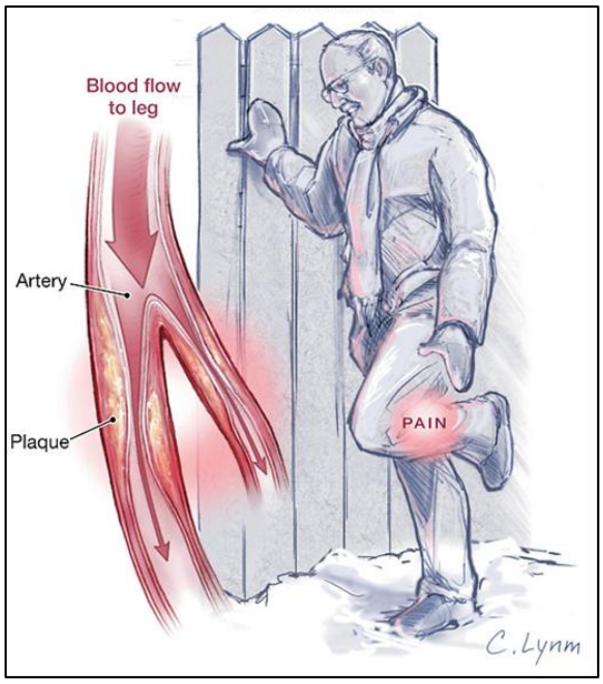

Figure 12. Intermittent claudication symptom.

During exercise, limited blood flow due to atherosclerotic plaques causes insufficient blood supply to the muscle (ischemia) causing muscular pain/cramps. This symptom, called intermittent claudication, forces the patient to stop walking.

For patients with symptomatic PAD, the efficiency of walking ExT as the cornerstone of treatments to improve walking capacity is well recognized and is included in current European guidelines as a class I recommendation ("evidence and/or general agreement that a given treatment or procedure is beneficial, useful, effective") (Aboyans et al., 2018). Patients are urged to undergo supervised ExT programs that include at least 3h per week of walking to the maximal or submaximal distance eliciting symptoms (Pelliccia et al., 2020). However, the characteristics of ExT that are the most effective to improve functional capacity of patients, remain largely unknown. In a previous study, we have investigated the effects of different ExT frequencies on walking capacity in a mouse model of PAD and intermittent claudication (see Annex 2). We found that 3 times per week and 5 times per week ExT have the same benefits on walking capacity improvement.

HIIT has emerged as an alternative ExT to low-intensity ExT because of its similar or superior efficacy on exercise capacity and cardiovascular parameters, in patients with coronary artery disease (Gomes-Neto et al., 2017). This form of exercise has the additional benefits of being time-efficient and more enjoyable, leading to greater adherence of patients to training programs compared to longer, more tedious lower intensity exercise programs. Because of the advantages of HIIT, there is growing clinical interest in using this exercise modality for PAD, even more so with recent evidence showing that it could be beneficial for improving walking capacity in patients (Iso et al., 2018; Pymer et al., 2019).

Recent clinical evidence has demonstrated that the combination of HIIT with hypoxia increased serum concentration of proangiogenic factors and NO more than the same training in normoxia in healthy individuals (Żebrowska et al., 2019) as well as in patients with type 1 diabetes (Hall et al., 2018). In line with the findings of the present work (Lavier et al., 2021), preliminary data from an ongoing research in our group have revealed that low-intensity ExT in hypoxia improves the brachial artery flow-mediated dilation by 50% in PAD patients, indicating a systemic vascular function improvement (Lanzi et al. unpublished data). Collectively, these data strongly suggest that hypoxic training might be highly efficient for improving lower

extremity arterial function in PAD. Restoring endothelial vasomotor function of lower extremities, leading in turn to an increased blood flow and O_2 delivery to the muscles, is a crucial goal of walking capacity rehabilitation therapy for PAD patients. As far as we know, no studies have investigated the specific effect of training in hypoxia and in particular the effect of HIIT in hypoxia on lower extremity arterial function in the setting of PAD.

Therefore, the next step of this project will be to investigate the effects of low-intensity ExT and HIIT combined with hypoxia on lower extremity arterial endothelial function and walking capacity in a mouse model of PAD and intermittent claudication (Pellegrin et al., 2020) and to explore the underlying molecular mechanisms.

Experimental design

Animals

A total of 60 C57BL/6 ApoE$^{-/-}$ 8-week-old male mice will be used in this study. They will be obtained from our mouse breeding colony in the UNIL animal facility in Epalinges. The original breeding pairs were purchased from the Jackson Laboratory (Bar Harbor, USA). Animals will be transferred to our local animal facility at least one week before the beginning of the experiments. Animals will be housed under conventional housing conditions in ventilated cages (Mouse IVC Library System, Techniplast, Italy) under a 12-h light/dark cycle in a temperature-controlled environment (22±2°C). All mice will have free access to a standard laboratory chow (Kliba Nafag, Switzerland) and water throughout the study. The protocol has already been approved by an internal animal experimentation committee as well as the Veterinary Office of the Canton de Vaud

Mouse model of peripheral arterial disease and intermittent claudication

Mice will be subjected to right iliac artery ligation to induce PAD, as previously described in detail (Pellegrin et al., 2014, 2020). Mice will be anesthetized by isoflurane inhalation (4-4.5% in O_2 for induction and 1- 2% in O_2 for maintenance) and placed in a supine position on a

heating pad in order to maintain body temperature at 37±1°C. Then, hindlimbs will be depilated with a depilatory cream (SurgexTM, Cooper Cosmetics SA, Switzerland). Through a small abdominal incision, the right common iliac artery will be exposed and ligated with 7-0 silk suture (Deknatel®) with sterilized surgical tools, just above the internal-external iliac artery bifurcation (Figure 13). Iliac vein and nerve will be preserved. Abdominal incision will then be sutured with a resorbable 5-0 silk suture (Vicryl).

For post-operative analgesia, mice will be treated with Dafalgan® (200 mg/kg in drinking water) for a week. In addition, Tamgesic® (0.1 mg/kg) will be administrated subcutaneously just before surgery, and once a day for 3 days. Contralateral non- ligated hindlimb will served as an internal control for each mouse.

Mice will be allowed to recover from the surgery for a week before any manipulations will be performed.

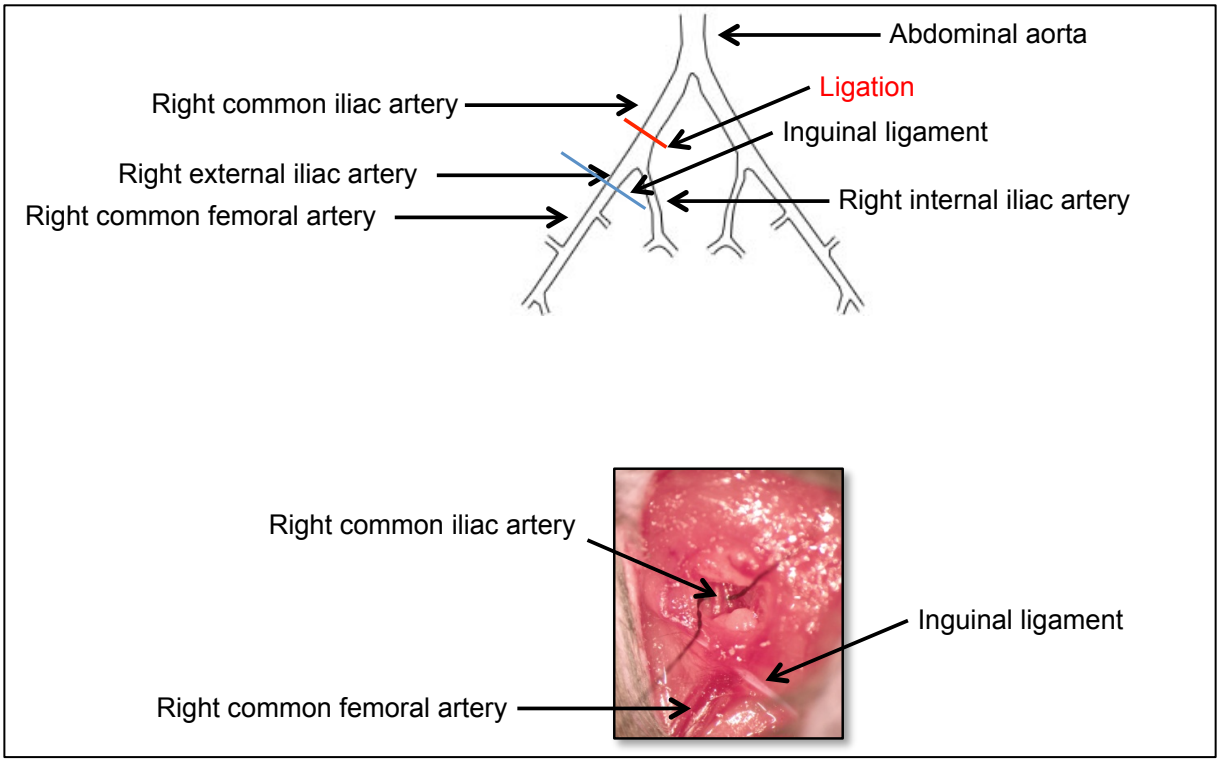

Figure 13. Common right iliac artery ligation.

Through a small abdominal incision, right common iliac artery will be exposed and will be ligated with 7-0 silk suture just above the internal-external iliac artery bifurcation.

Study design and aerobic exercise training protocol

One week after ligation, ApoE$^{-/-}$ mice will be randomly divided into six groups: 1. Sedentary (control) in normoxia (n=10); 2. Sedentary (control) in hypoxia (n=10); 3. MICT in normoxia (n=10); 4. MICT in hypoxia (n=10); 5. HIIT in normoxia (n=10); 6. HIIT in hypoxia (n=10) (Figure 14).

One week after surgery, mice will be submitted to an incremental test to exhaustion in order to assess their walking capacity (maximal waking distance, time and speed). ExT will consist in treadmill running three times a week for six weeks. MICT mice will run continuously for 40 min at 40-50% of their maximal aerobic speed. HIIT mice will run 8 bouts of 1.5 min at 90% of their maximal aerobic speed, with 1.5 min of active recovery running at 40% of their maximal aerobic speed between each bout. For the hypoxic regimens, the treadmill will be placed in a home-made chamber with an F_iO_2 of 0.13.

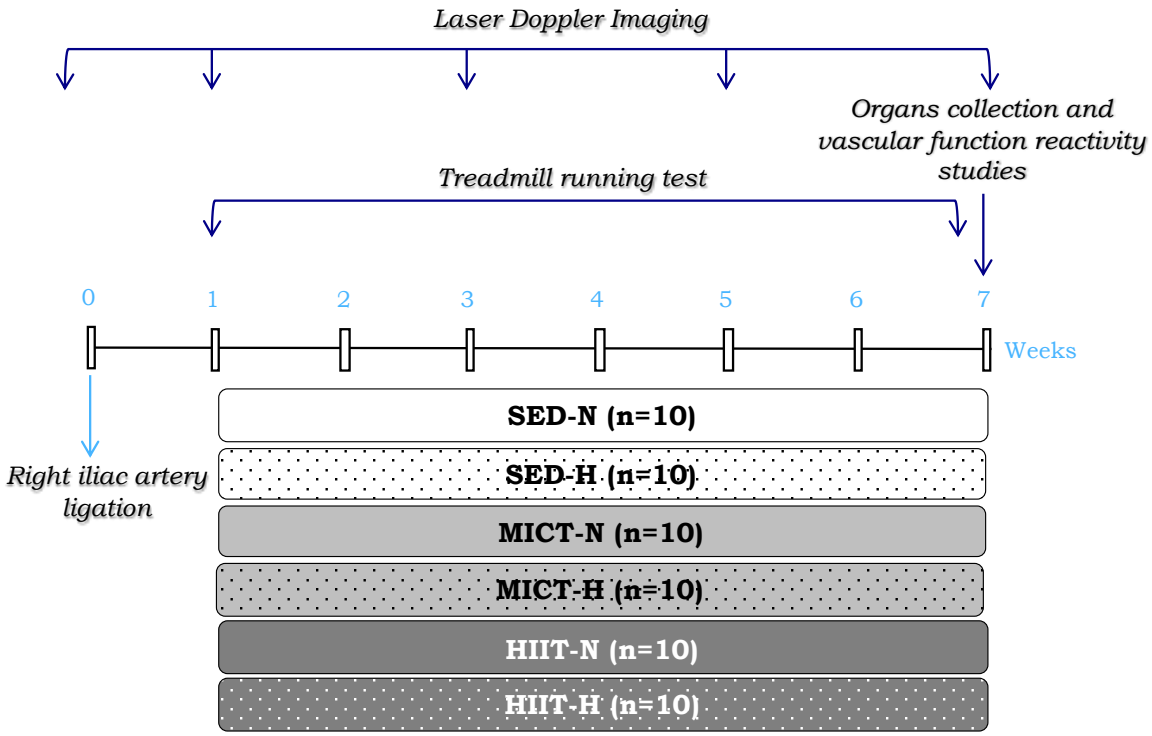

Figure 14 : Study Design.

Laser doppler imaging for hindlimb perfusion assessment

Hindlimb perfusion will be assessed using laser doppler imaging before artery ligation, 1-week post-ischemia and every two weeks until euthanasia (Figure 14). Mice will be anesthetized and placed in a prone position on a heating pad to maintain a constant temperature. For each mouse, six consecutive plantar foot images of both ligated and contralateral non-ligated hindlimbs will be scanned at 30 seconds interval and averaged. Moor LDI Image Review Software will be used to calculate perfusion within the ischemic and the contralateral non-ischemic hindlimbs, on the basis of colored histogram pixels (Figure 15). To minimize biases due to environmental variables such as ambient light and temperature variations, tissue perfusion in ligated/ischemic hindlimb will be expressed as a percentage of that measured in contralateral non-ligated/non-ischemic hindlimb.

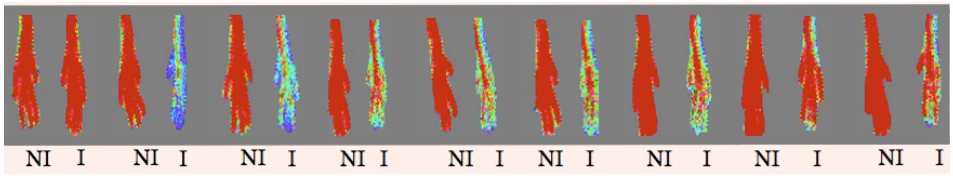

Figure 15. Example of representative laser doppler images.

From left to right, non-ischemic (NI) and ischemic (I) hindlimb at week 1 before arterial ligation and weeks 1, 2, 3, 4, 5, 6, 7 and 8 after arterial ligation. Blue color indicates low perfusion while red color indicates high perfusion. *Personal data.*

Tissue collection and further analyzes

At the end of the protocol, blood will be collected to assess plasma cholesterol profile and oxidant status. Thoracic aortas, iliac arteries as well as PVAT will be used for western blot and RT-PCr analysis (pro-/anti-inflammatory status, eNOS pathway, pro-/antioxidant status). Ex-vivo vasoconstriction and vasodilation studies will be performed using isolated vessel tension experiments on the iliac artery and the aorta. Overall, the protocol will be the same as used in the first part of this work (Lavier et al., 2021), however we would like to examine the dose

response to the NOS inhibitor L-NAME in order to assess the extent to which NO contributed to the anticipated results.

Expected Results

The first expected outcome of this study is for hypoxic training to be more effective than normoxic training in improving vascular reactivity and walking capacity, independently of intensity. The second expected result is that HIIT in hypoxia would increase vascular reactivity and walking capacity compared to MICT in hypoxia. Finally, we expect the improved vasomotor function induced by hypoxic training to be mediated by the NO pathway, not necessarily directly in the aorta and iliac artery, but possibly in the PVAT, possibly accompanied with an improved hindlimb perfusion.

This study could help with the prescription of more effective ExT regimens for PAD patients in order to ameliorate their walking capacity, thus improving their quality of life. Eventually, it could allow the identification of potential new drug targets.

General Conclusion

The aim of the present PhD work was to investigate the effects of ExT at high intensity on vascular function in healthy mice, in order to then explore these ExT modalities in mouse models of atherosclerosis and PAD. Since supramaximal-intensity repeated-sprint training is a training protocol that had never been published in the mouse, we first needed to design it and test its feasibility in mice (Study 1, Article 1). Once the optimal running speeds and number of sprints had been established, we submitted healthy mice to a four-week training protocol comparing low, maximal and supramaximal-intensity ExT in hypoxia on vascular reactivity (Study 1, Article 2). After finding that hypoxia and in particular higher intensities ExT combined with hypoxia increased vasodilation and vasoconstriction more than lower intensity ExT via increased NO bioavailability, we continued with a mouse model of atherosclerosis (Study 2). We carried out two protocols, a preventive and a therapeutic one to investigate plaque development and progression. The ultimate goal would be to investigate the effects of high-intensity interval ExT in hypoxia on a mouse model of PAD and intermittent claudication (perspectives).

This project sets the groundwork for using high-intensity interval training in hypoxia as a tool for primary and secondary prevention of many pathologies for which endothelial dysfunction is a risk factor, may it be cardiovascular or metabolic diseases.

Chapter 4

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Chapter 5

Study 1 Article 1 - Supramaximal Intensity Hypoxic Exercise and Vascular Function Assessment in Mice

Study 1 Article 1 – Supramaximal intensity hypoxic exercise and vascular function assessment in mice

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Abstract

Exercise training is an important strategy for maintaining health and preventing many chronic diseases. It is the first line of treatment recommended by international guidelines for patients suffering from cardiovascular diseases, more specifically, lower extremity artery diseases, where the patients' walking capacity is considerably altered, affecting their quality of life.

Traditionally, both low continuous exercise and interval training have been used. Recently, supramaximal training has also been shown to improve athletes' performances via vascular adaptations, amongst other mechanisms. The combination of this type of training with hypoxia could bring an additional and/or synergic effect, which could be of interest for certain pathologies. Here, we describe how to perform supramaximal intensity training sessions in hypoxia on healthy mice at 150% of their maximal speed, using a motorized treadmill and a hypoxic box. We also show how to dissect the mouse in order to retrieve organs of interest, particularly the pulmonary artery, the abdominal aorta, and the iliac artery. Finally, we show how to perform ex vivo vascular function assessment on the retrieved vessels, using isometric tension studies.

Video Link

The video component of this article can be found at https://www.jove.com/video/58708/

Introduction

In hypoxia, the decreased inspired fraction of oxygen (O_2) leads to hypoxemia (lowered arterial pressure in hypoxia) and an altered O_2 transport capacity[1]. Acute hypoxia induces an increased sympathetic vasoconstrictor activity directed toward skeletal muscle[2] and an opposed 'compensatory' vasodilatation.

At submaximal intensity in hypoxia, this 'compensatory' vasodilatation, relative to the same level of exercise under normoxic conditions, is well established[3]. This vasodilation is essential to ensure an augmented blood flow and maintenance (or limit the alteration) of oxygen delivery to the active muscles. Adenosine was shown to not have an independent role in this response, while nitric oxide (NO) seems the primary endothelial source since significant blunting of the augmented vasodilatation was reported with nitric oxide synthase (NOS) inhibition during hypoxic exercise[4]. Several other vasoactive substances are likely playing a role in the compensatory vasodilatation during a hypoxic exercise.

This enhanced hypoxic exercise hyperemia is proportional to the hypoxia-induced fall in arterial O_2 content and is larger as the exercise intensity increases, for example during intense incremental exercise in hypoxia.

The NO-mediated component of the compensatory vasodilatation is regulated through different pathways with increasing exercise intensity[3]: if β-adrenergic receptor-stimulated NO component appears paramount during low-intensity hypoxic exercise, the source of NO contributing to compensatory dilatation seems less dependent on β-adrenergic mechanisms as the exercise intensity increases. There are other candidates for stimulating NO release during higher-intensity hypoxic exercise, such as ATP released from erythrocytes and/or endothelial-derived prostaglandins.

Supramaximal exercise in hypoxia (named repeated sprint training in hypoxia [RSH] in the exercise physiology literature) is a recent training method[5] providing performance enhancement in team- or racket-sport players. This method differs from interval training in hypoxia performed at or near maximal speed[6] (V_{max}) since RSH performed at maximal intensity leads to a greater muscle perfusion and oxygenation[7] and specific muscle transcriptional responses[8]. Several mechanisms have been proposed to explain the effectiveness of RSH: during sprints in hypoxia, the compensatory vasodilation and associated higher blood flow would benefit the fast-twitch fibers more than the slow-twitch fibers. Consequently, RSH efficiency is likely to be fiber-type selective and intensity dependent. We speculate that the improved responsiveness of the vascular system is paramount in RSH.

Exercise training has been extensively studied in mice, both in healthy individuals and in pathological mouse models[9,10]. The most common way to train mice is using a rodent treadmill, and the traditionally used regimen is low-intensity training, at 40%–60% of V_{max} (determined using an incremental treadmill test[11]), for 30–60 min[12,13,14,15]. Maximal intensity interval training and its impact on pathologies have been widely studied in mice[16,17]; thus, interval training running protocols for mice have been developed. Those protocols usually consist of about 10 bouts of running at 80%–100% of Vmax on a rodent motorized treadmill, for 1–4 min, interspersed with active or passive rest[16,18].

The interest in mice exercising at supramaximal intensity (i.e., above the V_{max}) in hypoxia comes from previous results that the microvascular vasodilatory compensation and the intermittent exercise performance are both more increased at supramaximal than at maximal or moderate intensities. However, to our knowledge, there is no previous report of a supramaximal training protocol in mice, either in normoxia or in hypoxia.

The first aim of the present study was to test the feasibility of supramaximal intensity training in mice and the determination of a tolerable and adequate protocol (intensity, sprint duration, recovery, etc.). The second aim was to assess the effects of different training regimen in

normoxia and hypoxia on the vascular function. Therefore, we test the hypotheses that (1) mice tolerate well supramaximal exercise in hypoxia, and (2) that this protocol induces a larger improvement in vascular function than exercise in normoxia but also than exercise in hypoxia at lower intensities.

Protocol

The local state's animal care committee (Service de la Consommation et des Affaires Vétérnaires [SCAV], Lausanne, Switzerland) approved all experiments (authorization VD3224; 01.06.2017) and all experiments were carried out in accordance with the relevant guidelines and regulations.

1. Animal housing and Preparation

1. House 6 to 8 week-old C57BL/6J male mice in the animal facility for at least 1 week prior to the beginning of the experiments in order for the mice to get used to their new housing conditions. For practical reasons, mice of the same experimental group are usually housed together.

2. Keep the mice in a temperature-controlled room (22 ± 1 °C) with a 12 h light/dark cycle with ad libitum access to food and water.

2. Determination of the Maximal Speed and Standard Assessment of Performance Improvement by Treadmill Incremental Test

NOTE: The following steps are critical to completing the training protocols.

1. Use a motorized treadmill for mice where mice can be on multiple lanes alongside each other, with a 0° inclination and mounted with an electric grid set to 0.2 mA at the back of the lane, in order to encourage the mice to run.

2. Prior to the first test, submit the mice to 4 days of acclimatization to the treadmill, according to the following protocol.

    1. On day 1, have mice run for 10 min at 4.8 m/min.
    2. On day 2, have the mice run for 10 min at 6 m/min.
    3. On day 3, have the mice run for 10 min at 7.2 m/min.

4. On day 4, have the mice run for 10 min at 8.4 m/min.

3. On day 5, submit the mice to an incremental test to exhaustion, according to the following protocol.

1. Let the mice warm up for 5 min at 4.8 m/min (at a 0° inclination).

2. Increase the speed by 1.2 m/min every 3 min (e.g., 5 min at 4.8 m/min, then 3 min at 6 m/min, 3 min at 7.2 m/min, 3 min at 8.4 m/min, etc.) until exhaustion, which is reached when the mouse either spends 3 consecutive seconds on the electric grid or receives 100 shocks (displayed by the apparatus).

3. Write down the achieved speed (considered as the V_{max}), duration, distance, number of shocks, and total time spent on the grid.

NOTE: Typically, V_{max} was 28.8 ± 3.7 m/min.

4. Mid-training, resubmit the mice to this test in order to readjust the speeds of training to the updated V_{max} of the mice (e.g., if the training protocol lasts 8 weeks, then perform a mid-training incremental test at 4 weeks. In that case, replace one of the scheduled trainings by the test), and do so again at the end of the study in order to assess performance improvements.

5. Implement a 48 h rest period before and after this test.

NOTE: All the incremental tests were performed in the morning.

3. Hypoxic Environment

1. For the training sessions in hypoxia, place the treadmill in the hypoxic box (**Figure 1**) linked to a gas mixer. Use a calibrated oximeter to regularly control the ambient fraction of oxygen (F_iO_2 [i.e., the level of hypoxia]) in the box.

2. Set the gas mixer on 100% of nitrogen (N_2) and use the oximeter to verify the level of hypoxia. Once F_iO_2 = 0.13, change the parameter of the gas mixer from 100% N_2 to 13% O_2.

3. In order to avoid prolonged passive exposure to hypoxia, place the mice in a temporary smaller cage with litter and enrichment, and quickly place it in the box once F_iO_2 = 0.13 has been reached. Verify that the environment is still at 13% O_2 after putting the cage in; if not, readjust it.

4. Regularly verify the level of O_2 over the course of a training session to make sure that it remains at F_iO_2 = 0.13 ± 0.002.

4. Normoxic Environment

1. For the training sessions in normoxia, keep the treadmill in the hypoxic box, but remove the gloves so that there is ambient air (F_iO_2 = 0.21). The aim is to recreate the same training environment as the mice in hypoxia.

5. Supramaximal Intensity Training

1. Place the mice on individual lanes in the treadmill (at a 0° inclination) and submit them to the following protocol.
   1. Have the mice warm up for 5 min at 4.8 m/min, followed by 5 min at 9 m/min.
   2. Set the speed of the sprints to 150% of the previously determined V_{max}.

      NOTE: Typically, the sprint velocity was 42.1 ± 5.5 m/min.
   3. Train the mice for four sets of 5x 10 s sprints with 20 s of rest between each sprint. The interset rest is 5 min (**Figure 2**).

      NOTE: Add a cooldown period if the total workload of the training session needs to match that of another training group.

2. Perform this training 3x per week, with preferably 48 h between training sessions.
3. Use cotton swabs as a complementary method to electric shocks to encourage the mice to run. Place a cotton swab in a slit at the top of the lane, between the mouse and the electric grid, and gently nudge the mouse when it reaches the back of the treadmill. This will avoid the delivery of electric shocks and stimulate the mice to run in a softer way.

6. Low-intensity Training

1. Place the mice on individual lanes in the treadmill (at a 0° inclination) and submit them to the following protocol**.**
    1. Have the mice warm up for 5 min at 4.8 m/min, followed by 5 min at 7.2 m/min.
    2. Set the speed of the continuous running session to 40% of the previously determined V_{max}.

        NOTE: Typically, the continuous running velocity was 9.9 m/min.
    3. Train the mice for 40 min.
    4. Perform this training 3x per week with preferably 48 h between training sessions.
    5. Use cotton swabs as a complementary method to electric shocks to encourage the mice to run.

7. Mice Euthanasia and Organ Extraction

1. At the end of the training protocol and at least 24 h after the last incremental test, anesthetize the mouse in an induction chamber using isoflurane (4%–5% in O_2 to induce anesthesia, and 1%–2% in 100% O_2 to maintain anesthesia). Confirm proper anesthetization using the paw retraction reflex (firmly pinch the animal's paw; anesthesia is considered proper when the animal does not react to the stimulus).

2. Using a 25 G needle, perform a percutaneous cardiac puncture, to collect maximum blood volume as previously described[19].

3. Perform a cervical dislocation and remove the skin of the mouse by cutting through the first layer of skin on the abdomen with round-tip scissors and pulling on the two sides of the incision (toward the head and the tail).

4. Cut through the peritoneum under the ribcage on the left side of the mouse with thin-point-tip scissors to reach the spleen and extract it if needed.

    NOTE: Dissect out muscles if needed.

5. Dissect out the pulmonary artery.

    1. Using both small scissors and forceps, remove the thoracic cage and clear the heart-lung area.
    2. With "self-closing" tweezers, pinch the heart as close as possible to the apex and pull gently to stretch the base of the aortic arch and the pulmonary artery.
    3. Using the right hand, insert curved tweezers under the pulmonary artery and the aorta, and then move the tweezers back a little to hold only the pulmonary artery (**Figure 3**).
    4. Use the left hand to insert another pair of tweezers to replace the one held with the right hand.
    5. Using sharp straight microscissors in the right hand, dissect the pulmonary artery as close to the heart as possible on one side, and as far away as possible on the other side.

        NOTE: It does not matter which hand holds which instrument, although we have found it easier to cut with the right hand than with the left.

    6. Put it in a 2 mL tube with cold phosphate-buffered saline (PBS) buffer and keep on ice.

6. Perform a whole-body perfusion.

1. At the top of the right lower limb of the mouse, use tweezers to clear out the external-internal right iliac artery down to the right femoral artery (under the inguinal ligament). Using sharp straight microscissors, make a full cut in the femoral artery.

2. Insert a 5 mL 25 G syringe filled with cold PBS in the left ventricle of the heart and gently inject the cold PBS to remove the remaining blood from the vessels.

NOTE: Due to the extraction of the pulmonary artery, it is possible that PBS does not circulate all the way to the incision.

7. Using tweezers, remove the soft tissue surrounding the aorta from the left and right inguinal ligaments to the heart as thoroughly as possible.

NOTE: The heart can be extracted for further analysis if necessary.

8. Using both tweezers and microscissors, dissect out the heart up to the lowest point of the external iliac artery (in both left and right limbs) and place the entirely dissected-out section in a 10 cm-diameter dish with cold PBS.

9. Using tweezers and/or microscissors, finish cleaning the remaining fat around the aorta and arteries by gently pulling or cutting it away from the vessels.

10. Using microscissors, cut the left iliac artery at the left-right iliac artery bifurcation and store it for further analysis.

11. Using microscissors, cut the abdominal aorta under the left renal artery, and place the extracted vessel in cold PBS buffer on ice (**Figure 4**).

12. Keep the remaining cleaned vessel, from the aortic arch to right above the left renal artery, in storage for further analysis.

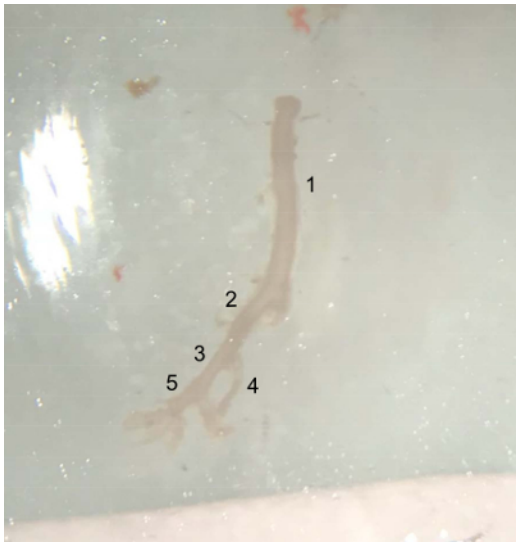

Figure 4: Picture of the dissected vessels. Extracted vessel from the top of the abdominal aorta (underneath the left renal artery) to the end of the right iliac artery, ready to be placed in cold PBS buffer on ice. (**1**) Abdominal aorta. (**2**) Right common iliac artery. (**3**) External iliac artery. (**4**) Internal iliac artery. (**5**) Femoral artery. Please click here to view a larger version of this figure.

8. Ex Vivo Vascular Function Assessment

NOTE: A wash corresponds to the emptying and refilling of the chambers with Krebs.

1. According to a previously described protocol[20], cut the isolated pulmonary artery, abdominal aorta, and right iliac artery segments into vascular rings of 1.0–2.0 mm long and mount each ring on two 0.1 mm-diameter stirrups passed through the lumen.
2. Suspend the vessel rings in vertical organ chambers filled with 10 mL of modified Krebs-Ringer bicarbonate solution (118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, and 11.1 mM glucose) maintained at 37 °C and aerated with 95% O_2-5% CO_2 (pH 7.4). One stirrup is anchored to the bottom of the organ chamber and the other one is connected to a strain gauge for the measurement of isometric force in grams.
3. Bring the vessels to their optimal resting tension: stretch the rings to 0.5 g for the pulmonary artery, 1.5 g for the iliac artery, and 2 g for the abdominal aorta, and wash

them after a 20 min period of equilibration. Repeat the stretch-equilibration-wash steps 1x.

4. To test the viability of the vessels, contract the rings with 235 µL of KCl (10^{-1} M) for 10 min, wash them for another 10 min, and contract again with 235 µL of KCl (10^{-1} M) for about 20 min until reaching a plateau.
5. Wash the vessels again for 10 min and add 58.4 µL of indomethacin (10^{-5} M) (an inhibitor of cyclooxygenase activity) for at least 20 min in order to avoid possible interference of endogenous prostanoids.
6. Add cumulative doses of phenylephrine (Phe) from 10^{-9} (10 µL) to 10^{-4} M (or 10^{-9} to 10^{-5} M for the pulmonary artery; 9 µL for all concentrations above 10^{-9} M) to contract the vessels.
7. After the last dose of Phe, wait for about 1 h until the vessels reach a relatively stable contraction state (plateau).
8. Add cumulative doses of the endothelium-dependent vasodilator acetylcholine (ACh), from 10^{-9} to 10^{-4} M (58.4 µL for 10^{-9} M, and alternately 12.6 µL and 40 µL for all concentrations above 10^{-9} M), to induce nitric oxide (NO)-mediated relaxation.
9. At the end of the relaxation curve, wash the vessels for 10 min, and add 58.4 µL of indomethacin (10^{-5} M), as well as 184 µL of NG-nitro-L-arginine (NLA, 10^{-4} M), which is an inhibitor of the NOS, for at least 20 min.
10. Contract the vessels again with a unique dose of 10 µL of Phe (10^{-5} and 10^{-4} M for the pulmonary artery and 10^{-4} M for the abdominal aorta and the iliac artery) for 1 h, to induce a relatively stable contraction.
11. Add a unique dose of 40 µL of ACh (10^{-4} M) until reaching a plateau.
12. Wash the vessels again for 10 min, before adding 58.4 µL of indomethacin (10^{-5} M) and 184 µL of NLA (10^{-4}) for 20 min.
13. Contract the vessels with 10 µL of Phe (10^{-5} and 10^{-4} M) for 1 h.

14. Add cumulative doses (10^{-9} [58.4 µM] to 10^{-4} M [40 µL for all concentrations above 10^{-9} M]) of the NO donor diethylamine (DEA)/NO, in order to assess the endothelium-independent NO-induced relaxation.

15. At the end of the experiment, store the vessels in liquid nitrogen for future analyses if needed.

Representative Results

To our knowledge, the present study is the first to describe a program of supramaximal intensity training in normoxia and in hypoxia for mice.

In this protocol, mice ran four sets of five 10 s sprints with a 20 s recovery in between each sprint. The sets were interspersed with 5 min of recovery periods. It was unknown whether the mice would be capable of sustaining such a protocol and complete it properly. However, according to **Figure 5**, the body weight gain of the mice undergoing the supramaximal intensity training was similar to that of the mice undergoing the low-intensity training, both in normoxia and in hypoxia.

The wellness of the animals was monitored twice a week, using score sheets, based on the following criteria: appearance, natural behavior, and body weight. Each of those criteria was graded up to a score of 3, and a mouse with a score of 3 in any of those criteria was considered in pain and/or distress due to the sustained protocol and had to be euthanized. No mouse ever reached a score of 3 over the course of any of the training regimens (**Table 1**).

As presented in the introduction, it has been hypothesized that supramaximal training, in particular when combined with hypoxia, would induce a compensatory vasodilation. This phenomenon aims at providing sufficient O_2 to the contracting muscles, thereby compensating for the imbalance between O_2 supply and demand that is enhanced by the combination of supramaximal intensity training and hypoxia. In order to investigate this hypothesis, we used the second technique presented here, the ex-vivo vascular function assessment, on the pulmonary artery, the abdominal aorta, and the right iliac artery. Figure 6 shows the dose-response curves obtained at the end of the protocol, on the abdominal aorta of a mouse from the group training at supramaximal intensity in hypoxia. This graph shows the whole process of contraction-relaxation observed after the addition of different pharmacological agents (KCL, Phe, ACh, NLA, and [DEA]/NO) in the organ baths.

Figure 7 shows the dose-response relaxation curve for the right iliac artery to increasing concentrations of ACh. The two represented groups are the supramaximal-intensity-in-normoxia group (SupraN) and the supramaximal-intensity-in-hypoxia group (SupraH). The preliminary results show that SupraH tended to improve ACh-induced relaxation compared to SupraN, with significant differences at 10^{-5} M and 10^{-4} M.

Figure 1: Hypoxic setup. The treadmill is placed inside the homemade glovebox, which is linked to a gas mixer. Please click here to view a larger version of this figure.

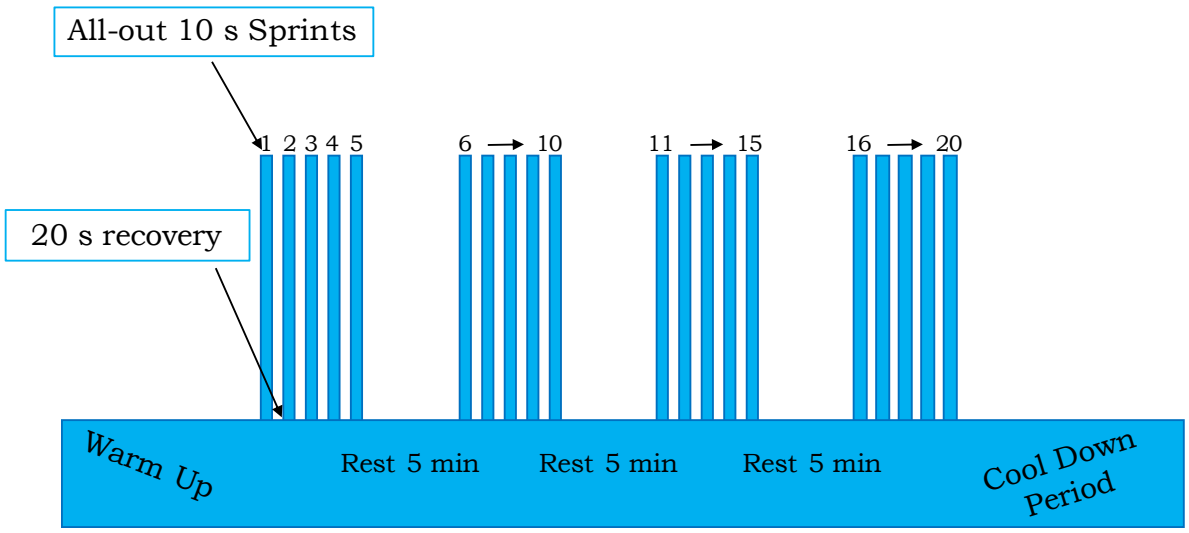

Figure 2: Description of a supramaximal intensity training session. The mice performed four sets of five 10 s sprints, interspersed with 20 s of rest. The interset rest was 5 min. This figure is adapted from Faiss et al.[21]. Please click here to view a larger version of this figure.

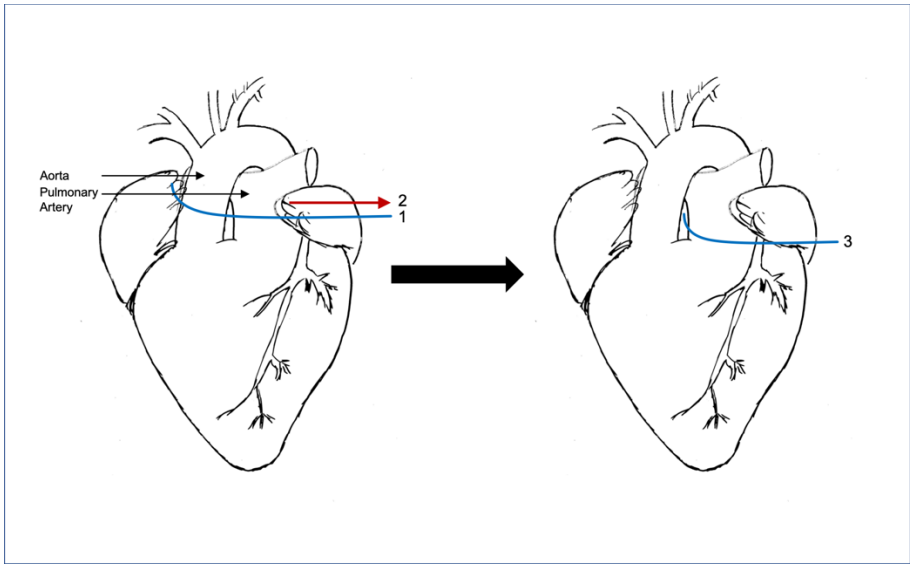

Figure 3: Schematic representation of the technique to retrieve the pulmonary aorta. (**1**) Place the tweezers under both the pulmonary artery and the aorta. (**2**) Pull back the tweezers in the direction of number 2 in order to keep the tweezers under the pulmonary aorta only. (**3**) Final position of the tweezers. Please click here to view a larger version of this figure.

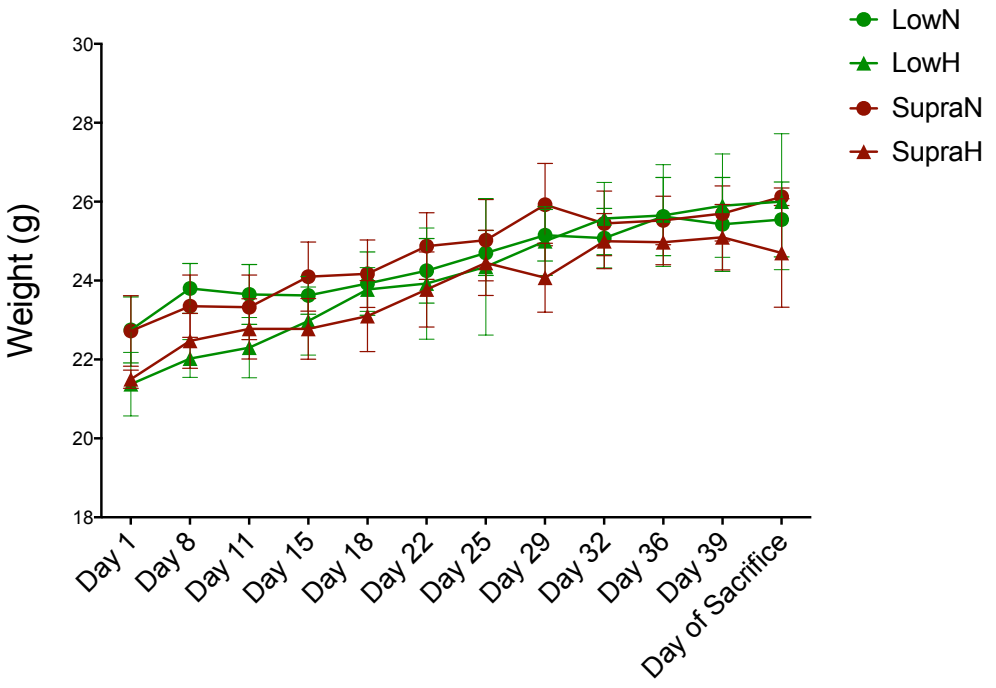

Figure 5: Body weight evolution over the course of the experiment. In green, the low-intensity training groups; in red, the supramaximal intensity training groups. There was no significant difference between any of the groups at any of the time points (*n* = 4 mice per group; the data are presented as mean ± SD). Statistical analysis was performed using a two-way repeated measure ANOVA). Please click here to view a larger version of this figure.

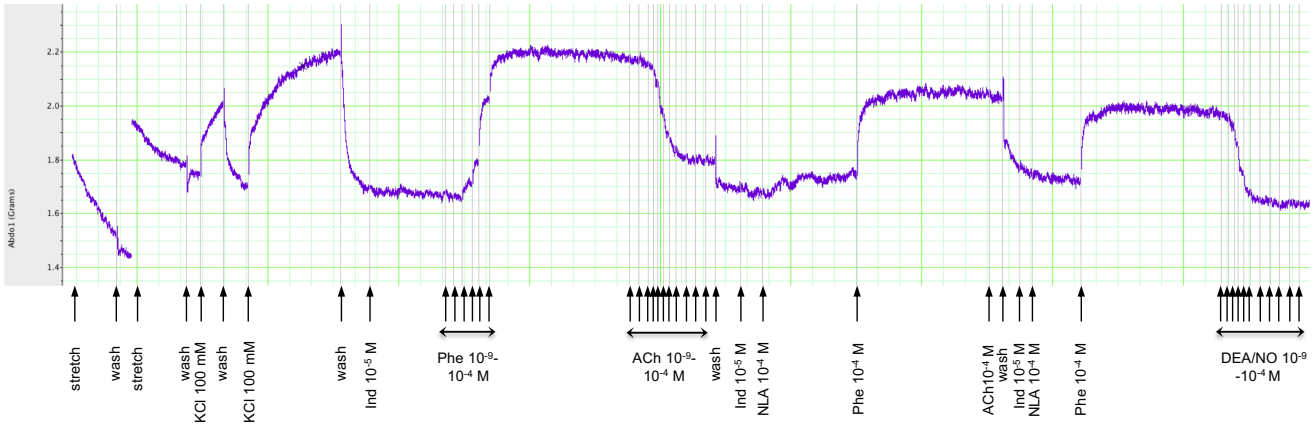

Figure 6: Vascular function assessment curves. Succession of contraction and relaxation phases induced throughout the entire protocol, expressed in grams. Representative recording of the variations in vessel tension in response to the applied substances, in a ring of the abdominal aorta isolated from a mouse trained at supramaximal intensity in hypoxia. Please click here to view a larger version of this figure.

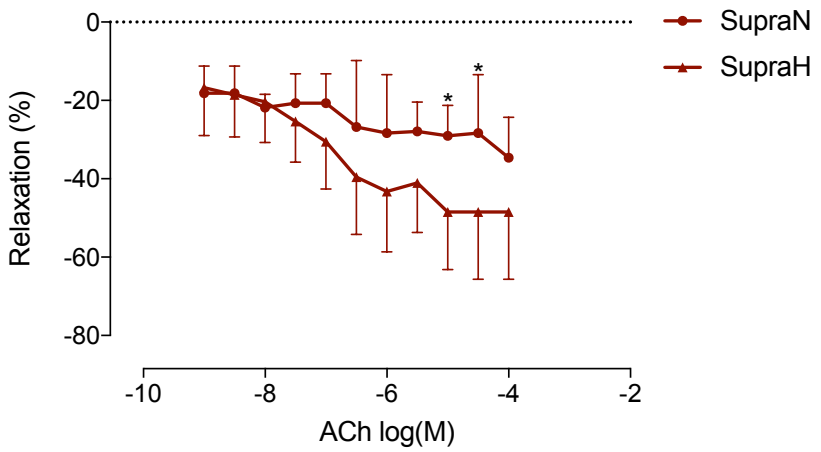

Figure 7: Pharmacological responses of an isolated iliac artery preconstructed with phenylephrine (Phe) to acetylcholine (ACh). Cumulative dose-response relaxation curve of the right iliac artery to increasing concentrations of ACh (10^{-9} to 10^{-4} M). The results are expressed as mean ± SD of the percentage of change in the tension induced by the vasodilator, with n = 3 in SupraN and n = 4 in SupraH. Statistical analysis was performed using a two-way ANOVA for repeated measures test. *p < 0.05 vs. SupraN. Please click here to view a larger version of this figure.

Animal Welfare Monitoring Sheet													
Authorization n°													
Experimentators:		Baseline						MidExp					End of Exp
	Date	24.07	31.07	03.08	07.08	10.08	14.08	17.08	21.08	24.08	28.08	31.08	04.09
Body weight baseline:	**Day**	01	08	12	15	19	22	25	29	32	36	39	43
ANIMAL ID:	**Score**	0	0	0	0	0	1	0	1	1	1	1	1
Types of interventions:		/	/	/	(1)	/	/	/	/	/	/	/	(3)
APPEARANCE													X
Normal	0	X	X	X	X	X	X	X	X	X	X	X	X
General lack of grooming	1												
Piloerection	2												
Hunched posture	3												
NATURAL BEHAVIOUR													
Normal	0	X	X	X	X	X	X	X	X	X	X	X	X
Less mobile and/or isolated	2												
Vocalization, self mutilation, restless or still	3												
BODY WEIGHT													
% of body weight change compared to mean body weight of the control group													
0-5% weight loss	0	X	X	X	X	X		X					
6-10% weight loss	1						X		X	X	X	X	X
11-15% weight loss	2												
>15% weight loss	3												
TOTAL SCORE		**0**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**1**
Signature (initials)													

CRITERIA FOR MICE EUTHANASIA													
Any mice with a total combined score ≥5					Type of interventions:				(1)	Tail vein blood collection			
Any mice with one or more individual maximum score of 3									(2)	Forced treadmill running test			
									(3)	Cardiac puncture blood collection			

Table 1: Typical score sheet of a mouse training at supramaximal intensity in hypoxia. We used score sheets to monitor the welfare of the mice. A score of 3 in any of the criteria indicated (appearance, natural behavior, and body weight) or a total score of 5 (by addition of the score of each category) meant the animal was suffering and had to be euthanized.

Discussion

The first objective of this study was to assess the feasibility of hypoxic high-intensity training in mice and to determine the adequate characteristics of the protocol that would be well tolerated by mice. Purposely, since there is no data using supramaximal (i.e., more than V_{max}) intensity training in mice, we had to perform trials based on previous protocols developed with athletes, which consisted of four to five sets of five all-out sprints (about 200% of V_{max}), interspersed with 20 s active recoveries, with an interset active recovery of 5 min[21,22]. Therefore, the initial protocol consisted of six sets of six 10 s sprints at 200% of V_{max}, interspersed with 20 s of passive recovery and with an interset passive recovery of 3 min, performed five times per week. After a few try-out runs at 200% of the V_{max}, considering the mice had trouble sustaining such a high intensity, we decided to lower the speed to 150% of the V_{max}. With that exercise intensity, we tried to run the mice over the length of a full protocol and adjusted both the number of sprints within each set and the number of sets per session. Finally, we increased the recovery time between sets and decreased the frequency of the training sessions. Following a trial-and-error method, we established a final optimal protocol that is very similar to the one used on athletes and made possible for mice to tolerate this supramaximal intensity test.

There is a slight possibility that the performance of the mice might be severely underestimated, as observed from large differences between previous studies utilizing animal exercise protocols[23,24]. However, in the present study, based on pre-experiment values, it would have

been impossible to impose a higher relative intensity on the animals considering the need to complete the entire repeated sprint session. Moreover, the Vmax values reported in this study (28.8 ± 3.7 m/min) seem to be in the range of values previously reported in the same C57BL/6J strain[25,26,27,28]. For example, Lightfoot et al.[25] reported values of ~28 m/min and Muller et al.[27] values of 28.3 m/min. Therefore, we are confident that the supramaximal intensity corresponds to sprint training intensity in these mice.

Although critical speed (CS) has been shown (1) to be a valuable mean for prescribing exercise intensity in healthy humans and patients[29] and (2) to be perfectly determined in mice[23,24,30], the exercise intensity prescription based on the determination of V_{max} remains relevant. It is known that, in mice, the determined VO_{2peak} and VO_{2max} depend on the protocol, and, as with humans, VO_{2max} can be determined with a ramp exercise protocol[11]. Since the aim of the present study was to determine the feasibility of supramaximal repeated sprint in mice, and despite the relevance of CS, we do not believe that using Vmax would be a flaw regarding the objectives of this study.

While observing mice behavior, it became clear that the electric grid at the rear of the treadmill admittedly encouraged mice to run; however, it also seemed to contribute to their fatigue. Indeed, the grid being slightly shifted from the running band, the mice had to generate an extra effort to get back on the lane. We decided to complement this stimulation with another, softer, one, namely the cotton swab stimulation, which decreased the number of shocks received by the animals and prevented them from having to get back from the grid to the lane. Despite the recommendation by Kregel et al.[31], it remains unclear whether stress is reduced using the air puff stimulation compared to the electric grid[32].

As far as we know, only one study has used "sprint interval training"[33]. However, since the highest intensity in that study corresponded to 75%– 80% of V_{max} and the sprint duration was 1.5 min, that protocol was very different from the present one (i.e., 150% of V_{max}; 10 s). It was unknown whether supramaximal intensity would be tolerated by the mice. In the present study, we provide results showing that the animals did perform very well in this supramaximal intensity training, both in hypoxia and normoxia. For instance, **Figure 5** shows an increase in body weight over the training period similar to that observed in the low-intensity groups. Similarly, **Table 1** reflects the level of welfare with a score lower than 3 in all groups. Altogether, those physiological parameters indicate that both hypoxia and supramaximal intensity training were very well tolerated by mice.

The second objective of the present study was to assess the vascular function of the pulmonary artery, the abdominal aorta, and the iliac artery, using isometric vessel tension studies[20]. This technique allows determining whether the intervention of interest impacted the ability of the vessels to contract and relax in response to pharmacological drugs. As shown in **Figure 7,** the iliac artery was relaxed using increasing concentrations of ACh. The observed curves reflect a progressive increase in the relaxation of the vessels, more marked for the SupraH group. If any of the observed curves had been completely flat and around 0% of relaxation, it could mean that the drug was not delivered to the organ chamber, or that the vessels had been damaged during the dissection or the mounting on the stirrups, or that one of the drugs was not administered at the optimal dose or for long enough.

The supramaximal intensity training in hypoxia is now transferred to mice and could potentially be used on pathological models in order to improve various parameters, including vascular function, which can be assessed using isometric vessel tension studies.

Disclosure

The authors have nothing to disclose.

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Chapter 6

Study 1 Article 2 - High-intensity exercise in hypoxia improves endothelial function via increased nitric oxide bioavailability in C57BL/6 mice

Study 1 Article 2 – High-intensity exercise in hypoxia improves endothelial function via increased nitric oxide bioavailability in C57BL/6 mice

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Abstract

Aim: The optimal exercise intensity to improve endothelial function remains unclear, as well as whether the addition of hypoxia could potentiate this function. Therefore, the aim of this study was to compare the effects of different exercise intensities in normoxia and hypoxia on vascular reactivity and nitric oxide (NO) bioavailability in mice.

Methods: C57BL/6 mice underwent treadmill running three times per week, for 4 weeks at either low, maximal or supramaximal intensity in normoxia or hypoxia (inspire oxygen fraction = 0.13). Vascular reactivity and expression of genes and proteins involved in NO production/bioavailability were assessed in aorta using isolated vessel tension experiments, RT-qPCR and western blot, respectively. Circulating NO metabolites and pro-/antioxidant markers were measured.

Results: Hypoxic exercise improved both acetylcholine-induced vasorelaxation and phenylephrine-induced vasoconstriction compared to normoxic exercise, independently of intensity. In hypoxia, a higher acetylcholine-induced vasorelaxation was observed with high intensities (supramaximal and maximal) compared to low intensity. Exercise protocols modulated endothelial nitric oxide synthase (eNOS) and α_1-adrenergic receptor (α_1-AR) mRNA level, but not superoxide dismutase 3 (SOD3) and p47phox. No significant differences were observed for protein expression of α_1-AR, total eNOS, phosphorylated eNOS, SOD isoforms and p47phox. However, plasma SOD and catalase activities were significantly increased in hypoxic supramaximal compared to hypoxic low intensity, while concentration of nitrotyrosine significantly decreased. The latter was also observed in hypoxic maximal and supramaximal compared to the same intensities in normoxia.

Conclusion: Hypoxic high-intensity exercise increases NO bioavailability and im- proves vascular function, opening promising clinical perspectives for cardiovascular disease prevention.

Introduction

Endothelial function lies in the ability of vessels to contract and relax under thorough control of endothelial cells and is directly linked to vascular health. Since the monolayer of endothelial cells is located between the blood flow and vessel wall components, it is a key regulator of different signaling pathways affecting vascular function and structure.[1] The most notorious one is the nitric oxide (NO) pathway. NO is synthesized in endothelial cells through the mobilization/activation of endothelial nitric oxide synthase (eNOS).[1] Impaired eNOS activity and/or reduction of NO bioavailability leads to endothelial dysfunction, an independent risk factor for cardiovascular diseases.[2] Thus, ameliorating endothelial function or preventing endothelial dysfunction through targeting eNOS and NO bioavailability is of clinical interest for the prevention of cardiovascular disorders.

Exercise training is a highly efficient non-pharmacological approach for maintaining cardiovascular health as well as for the primary and secondary prevention of cardiovascular diseases,[3] partly via its beneficial effect on endothelial function. There is extensive evidence showing that exercise training improved endothelial function in patients with cardiovascular risk factors or established cardiovascular diseases.[4–7] Such beneficial effects have also been reported in animal models.[8–10] Although the mechanisms behind the benefits of exercise training on endothelial function are not fully understood, it has been advocated that exercise training acts on NO bioavailability through shear stress-induced stimulation of endothelial cells, promoting the activation of eNOS. Increased NO bioavailability induced by exercise training can also be mediated by a reduction of oxidative stress and reactive oxygen species (ROS) production, through decreasing pro-oxidant agents such as NADPH oxidase subunits and/or increasing antioxidant defenses such as superoxide dismutase (SOD) isoforms and catalase.[10,11]

So far, the optimal characteristics of exercise training needed to improve endothelial function remain unclear. However, vascular reactivity seems to be sensitive to exercise training

intensity in humans, with high-intensity interval-training improving vascular reactivity more efficiently than lower intensity protocols.[12–14] Few studies in rodents have also reported higher aortic endothelium-dependent vasorelaxation following either high-intensity interval training or high-intensity endurance training compared to moderate intensity endurance training, which was associated with increased eNOS protein expression.[15,16] On the other hand, other animal studies reported no benefits with high-intensity continuous exercise training compared to moderate continuous exercise training, or with high intensity interval training compared to low intensity interval training.[17,18] Some studies even described a deleterious effect of high intensity continuous training compared to low and moderate intensity continuous training.[19,20] Thus, there is still controversy regarding the potential beneficial effect of high-intensity exercise training on endothelial function.

Hypoxia is a state that induces an imbalance between tissue oxygen (O_2) delivery and demand. Current evidence show that combining exercise training with hypoxia potentiates the vascular adaptations observed with the same level of exercise training in normoxia, including those related to the dilator function.[21] In fact, hypoxic training produces a “compensatory” vasodilatation and an augmented blood flow aiming at preserving tissue O_2 delivery, and ensuring it is matched to demand.[22,23] These hypoxic exercise training -induced compensatory vasodilatation and vascular responses have been shown to be mainly mediated by NO.[22,23]

To date, however, there has been a lack of research on the effect of combining high-intensity exercise training and hypoxia on endothelial function. More specifically, to our knowledge, no study has yet investigated the effects of supramaximal intensity training on endothelial function, in normoxia or in combination with hypoxia. In addition, mechanisms regulating the NO pathways responsible for the effect of hypoxic exercise training on vascular function remain incompletely understood.

Therefore, this study aimed at comparing the effects of different exercise training intensities (low, maximal and supramaximal) in normoxia and in hypoxia on vascular reactivity

in mice. We hypothesized that the vascular adaptations would depend on the combination of both exercise training intensity and O_2 availability, and therefore that (i) vascular reactivity (i.e., vasoconstriction and vasodilation) would improve to a greater extent as exercise training intensity increases either in normoxia or hypoxia; and (ii) hypoxic exercise training would improve vascular reactivity to a greater extent than the same intensity in normoxia.

Materials and Methods

Animals

A total of 58 8-week-old male C57BL/6J wild-type mice were used in this study. Mice were purchased from Charles River Laboratories (L'arbresle, France) and housed in ventilated cages under a 12-h light/dark cycle and in a temperature and humidity-controlled environment. Mice had free access to a standard chow (Kliba Nafag, Switzerland) and water throughout the study.

All experiments were conducted according to Swiss animal experimentation laws and guidelines and were approved by an internal animal experimentation committee as well as the Veterinary Office of the Canton de Vaud (authorization VD3224).

Study Design and Exercise Protocols

Mice were randomly divided into 6 groups: 1. Low-intensity continuous-training in normoxia (**LowN**, n = 10); 2. Low-intensity continuous-training in hypoxia (**LowH**, n = 9); 3. Maximal-intensity interval-training in normoxia (**MaxN**, n = 10); 4. Maximal-intensity interval-training in hypoxia (**MaxH**, n = 10); 5. Supramaximal-intensity repeated-sprints training in normoxia (**SupraN**, n = 9) and 6. Supramaximal-intensity repeated-sprints training in hypoxia (**SupraH**, n = 10).

Exercise training consisted in forced treadmill running 3 times per week for 4 weeks on a mouse treadmill (Panlab LE-8710, Bioseb, France). Low voltage stimuli (0.2 mA) and cotton

swabs were used to motivate mice to run throughout the training. Mice assigned to Low exercise training (groups 1 and 2) ran continuously for 40 min at 40% of their maximal aerobic speed (MAS). Max exercise training mice (groups 3 and 4) ran 8 bouts of 1 min at 90% of their MAS, with 1 min of passive recovery between each bout. Supra exercise training mice (groups 5 and 6) ran 4 sets of 5 x 10 s sprints at 150% of MAS, with 20 s of passive recovery between each sprint. The interset rest was of 5 min of passive recovery. The Low and Supra protocols were performed as previously published.[47] For all protocols, each training session began with a 5 min warm-up at 8 cm/s, followed by 5 min at 12 cm/s or 15 cm/s. Max and Supra mice were subjected to a cool-down period at the end of each exercise training in order to match the total workload of the Low groups. The treadmill was placed in a home-made chamber with a fraction of inspired O_2 (F_iO_2) of either 0.13 (hypoxic training) or 0.21 (normoxic training) as previously described.[47]

At the end of the 4 weeks of exercise training and 24 h after the last training session, blood was collected by cardiac puncture under isoflurane inhalation anesthesia (3-3.5% in O_2 for induction, 2.5% in O_2 for maintenance; Attane™ Isoflurane ad us. vet., Piramal Healthcare Limited, India) and the plasma was obtained by centrifugation for 10 min at 2500 rpm at +4°C. Plasma samples were then snap frozen and stored at -80°C until further analysis. Immediately after cardiac puncture, mice were euthanized by cervical dislocation, then aortas were isolated and cleaned of fat and connective tissue in cold phosphate-buffered saline. Tissues were either immediately used for ex-vivo vasoreactivity studies or immersed in RNA later (Ambion, Invitrogen, California, USA) at +4°C before storage at -80°C for further real-time reverse-transcriptase polymerase chain reaction and western blot analyzes.

Treadmill Incremental Test

Each mouse performed an incremental test to exhaustion to determine individual MAS as previously described.[47] After a 5 min warm-up at 8 cm/s, the running speed was increased by 2 cm/s every 3 min until exhaustion. Mice were considered exhausted when they failed to

maintain the running speed (i.e., when they stayed for 2 consecutive s on the electric grid at the rear of the treadmill or when they received a total of 100 electric shocks). Mice were acclimated to treadmill running for a week before the incremental test. Mean MAS was not different between the different groups: LowN: 41 ± 6 cm/s; LowH: 38 ± 4 cm/s; MaxN: 41 ± 5 cm/s; MaxH: 43 ± 4 cm/s; SupraN: 44 ± 4 cm/s and SupraH: 44 ± 5 cm/s.

Vascular Reactivity Tension Studies

Ex-vivo vasoconstriction and vasodilation studies were performed using isolated vessel tension experiments, as previously described in details.[47] The aorta was cut into vascular rings of 1.0-2.0 mm long and mounted on two 0.1 mm-diameter stirrups passing through the lumen. The rings were suspended in vertical organ chambers filled with 10 mL modified Krebs-Ringer bicarbonate (KRB) solution (118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, and 11.1 mM glucose) maintained at 37°C and aerated with 95% O_2-5% CO_2 (pH 7.4). Isometric tension was continuously recorded with a strain gauge system (PowerLab/8SP, ADInstruments). Afterwards, the vessel rings were progressively brought to their optimal resting tension by two steps of stretch (to 2 grams)-equilibration-wash. Potassium chloride (KCl, 10^{-1} M) was then added to the organ chambers to test the viability of the vessels.

Vasoconstriction responses of vessel rings were assessed by addition of cumulative doses of phenylephrine (Phe; 10^{-9} to 10^{-4} M). After washout, vasorelaxation responses were then assessed, after a pre-contraction with Phe (10^{-4}), by addition of cumulative doses of the endothelium-dependent vasodilator acetylcholine (ACh; 10^{-9} to 10^{-4} M). Endothelium-independent relaxation was also investigated using cumulating doses of the NO donor diethylamine DEA/NO (10^{-9} to 10^{-4} M), in the presence of the eNOS inhibitor N^G-nitro-L-arginine to exclude possible interference of endogenous NO. Vasorelaxation results were expressed as percentages of the initial contraction induced by Phe. Areas under the curve for ACh-, DEA/NO- and Phe-induced responses were calculated from the concentration-response plots

using GraphPad Prism version 6.05 (GraphPad Software, Inc., San Diego, CA, USA). All these experiments were performed in the presence of indomethacin (10^{-5} M), a cyclooxygenase activity inhibitor, to avoid possible interference of endogenous prostanoids with the vascular responses.

Real-time reverse-transcriptase polymerase chain reaction

Total RNA from aortic tissue was extracted using the RNeasy Micro Kit (Qiagen, Switzerland) according to the manufacturer's protocol. RNA was then reverse transcribed using PrimeScript™ RT Reagent Kit (TaKaRa Bio Inc., Japan). Quantitative real-time polymerase chain reaction was performed on a CFX96TM real-time system (Bio-Rad, Switzerland) with SYBR premix Ex Taq (TaKaRa Bio Inc., Japan), according to the manufacturer's protocols. mRNA expression of the following genes was detected: endothelial nitric oxide synthase (eNOS), superoxide dismutase 3 (SOD3), NADPH oxidase subunit p47phox, alpha-1 adrenergic receptor (α_1-AR), hypoxia inducible factor-1 alpha (HIF-1α), soluble guanylate cyclase subunit alpha 1 (sGCα1), cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), 3-mercaptopyruvate sulfurtransferase (3MST), and the housekeeping gene 36B4. Specific sequences of mouse primers are shown in Supplementary Table 1. For each sample, expression of target genes was normalized to the expression of the housekeeping gene 36B4 (ΔCt). Data were presented using the formula $2^{-\Delta Ct}$.

Gene	Forward sequence	Reverse sequence
36B4	5'-ATGGGTACAAGCGCGTCCTG-3'	5'-GCCTTGACCTTTTCAGTAAG-3'
eNOS	5'-GACCCTCACCGCTACAACAT-3'	5'-CTGGCC TTCCGCTCATTTTC-3'
SOD3	5'-TTCTACGGCTTGCTACTGGC-3'	5'-GCTAGGTCGAAGCTGGACTC-3'
p47phox	5'-AGGAGATGTTCCCCATTGAGG-3'	5'-CAGTCCCATGAGGCCGTTGAA-3'
α1-AR	5'-GCGGTGGACGTCTTATGCT-3'	5'-TCACACCAATGTATCGGTCGA-3'
HIF-1α	5'-TCAAGTCAGCAACGTGGAAG-3'	5'-TATCGAGGCTGTGTCGACTG-3'
sGCα1	5'-CCCCTGGTCAGGTTCCTAAG-3'	5'-GGAGACTCCCTTCTGCATTCT-3'
3-MST	5'-GGCCACCACTCTGTGTCATT-3'	5'-GGAGCTGATTGGCAGGTTCT-3'
CBS	5'-GGGACAAGGATCGAGTCTGGA-3'	5'-AGCACTGTGTGATAATGTGGG-3'
CSE	5'-TTGGATCGAAACACCCACAAA-3'	5'-AGCCGACTATTGAGGTCATCA-3'

Supplementary Table 1. List of primers sequences used for mRNA expression analyzes.

Western Blot Analyzes

Frozen aortas were grinded into powder using a ceramic mortar and pestle kept in liquid nitrogen. Total proteins were then extracted using a lysis buffer containing Nonidet P-40 0.5%, NaCl 150 mM, Na-orthovanadate 1 mM, NaF 10 mM, Tris-HCL pH=7.5 10 mM, PMSF 1 mM, EDTA pH=8.0 1 mM, aprotinin 10 $\mu g.mL^{-1}$, pepstatin 1 $\mu g. mL^{-1}$ and leupeptin 10 $\mu g\ mL^{-1}$, followed by sonication. Protein concentration was measured using a BCA protein assay kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Switzerland). 15 µg of these total proteins were separated using SDS-PAGE, then transferred to nitrocellulose membranes (Biorad) and blocked for 1 h at room temperature using Odyssey blocking buffer mixed with TBS buffer (1:1, LI-COR Biosciences, Bad Homburg, Germany). The membranes were probed overnight at 4°C with the following primary antibodies: mouse anti-eNOS/NOS Type III (1:500, 140 kDa, #610297, BD Transduction Laboratories™, USA), mouse anti-eNOS pS1177 (1:500, 140 kDa, #612392, BD Transduction Laboratories™, USA), rabbit anti-superoxide dismutase 1 (SOD1; 1:1'000, 18 kDa, ab13498, Abcam, UK), rabbit anti-superoxide dismutase 2 (SOD2; 1:5'000, 25 kDa, ab13533, Abcam, UK), rabbit anti-superoxide dismutase 3 (SOD3; 1:500, 35

kDa, ab83108, Abcam, UK), goat anti-NCF1/p47-phox (1:500, 45 kDa, ab795, Abcam, UK), rabbit anti-alpha 1 adrenergic receptor (α_1-AR; 1:500, 80 kDa, ab3462, Abcam, UK), and mouse anti-tubulin (1:10'000, 55 kDa, Sigma Aldrich, USA). Blots were incubated with the adequate secondary antibodies for 1 h at room temperature: donkey anti-mouse IRDye 800 (1:10'000, Rockland Immunochemicals, USA), goat anti-rabbit Alexa 680 (1:10'000, Molecular Probes, USA) or donkey anti-goat Alexa 594 (1:5'000, Invitrogen). The immunoblot signals were detected and quantified with the Odyssey infrared imaging system (LI-COR Biosciences, Bad Homburg, Germany). Individual values were normalized with the expression of tubulin, and then expressed as the percentage of the protein expression values obtained in the LowN group.

Circulating Nitric Oxide Metabolites and Pro- and Antioxidant Markers

As described previously,[48] NO metabolism was determined as the sum of nitrite and nitrate concentrations. After reduction of nitrates by nitrate reductase, the sum of nitrate and nitrite (NOx) fluorometric quantification was based on the reaction of nitrite with 2,3-diaminonaphtalene. The intra-assay coefficient of variation was 5.4%. The same technique was used to measure nitrites (NO_2), without the addition of nitrate reductase. Plasma advanced oxidation protein products (AOPP) were measured according to the semi-automated methods developed by Witko-Sarsat et al. (1996).[49] Using spectrophotometry, the AOPP plasma concentrations were determined and calibrated with a chloramine-T solution, which, given the presence of potassium iodide, absorbs at 340 nm. Absorbance was read at 340 nm and AOPP concentrations were expressed as $\mu mol.L^{-1}$ of chloramine equivalents. As described previously,[50] the intra-assay coefficient of variation was 5.4%. Xanthine oxidase (XO) activity was measured as described previously.[50] Briefly, the absorbance of the complex (formazan blue) formed by nitroblue tetrazolium and the superoxide produced by XO in the sample was read at 560 nm every 30 s during 5 min. The slope of the formation of formazan blue overtime corresponded to XO activity. The intra-assay coefficient of variation was 3.8%. Superoxide dismutase (SOD) activity was measured using the Oberley and Spitz method.[51] The degree of

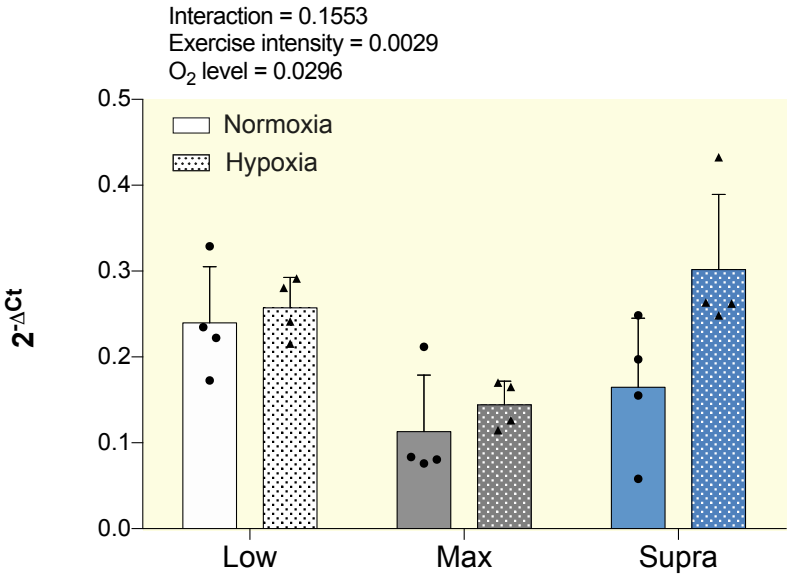
inhibition of the reaction between superoxide radicals, produced by a hypoxanthine - xanthine oxidase system, and nitroblue tetrazolium, determined SOD activity. The intra-assay coefficient of variation was 5.6%. The Johansson and Borg (1988)[52] method was used to determine catalase activity, with H_2O_2 as a substrate, and formaldehyde as a standard. Catalase activity was determined by the rate of formaldehyde formation, induced by the reaction of methanol and H_2O_2. The intra-assay coefficient of variation was 5.6%. Finally, concentrations of plasma nitrotyrosine (3-NT), as end product of protein nitration by peroxynitrite $ONOO^{\bullet-}$, were measured by ELISA as previously described.[53] The intra-assay coefficient of variation is 6.8%.

Statistical Analysis

All data are presented as mean ± standard deviation (SD). Data were analyzed using a two-way (O_2 level x exercise intensity) analysis of variance (ANOVA) followed by a post-hoc Sidak's multiple comparisons test. All statistical analyzes were performed using GraphPad Prism version 6.05 (GraphPad Software, Inc., San Diego, CA, USA), and a value of $p<0.05$ was considered to be statistically significant.

Results

Mice were submitted to a total of 12 training sessions and stayed in hypoxia for approximately 1 h each session. As expected, there was a significant main effect of hypoxia independently of exercise training intensity in mRNA expression of HIF-1α ($p<0.05$; Supplementary Figure 1).



Supplementary Figure 1. mRNA expression of hypoxia inducible factor 1α in aortas of low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN) and supramaximal intensity training group in hypoxia (SupraH). Results are expressed as $2^{-\Delta Ct}$ using 36B4 as housekeeping gene. Data are presented as mean ± SD (n = 4 mice per group). Two-way ANOVA with Sidak post-hoc test.

Body weight gain and mean arterial blood pressure

Body weight (BW) increased significantly in each group of mice between the beginning and end of the study ($p<0.05$, data not shown). BW gain was higher in LowH compared to LowN and in MaxH compared to MaxN ($p<0.001$ and $p<0.01$ respectively; Supplementary Table 2). No significant differences in BW gain were observed between SupraH and SupraN.

Mean arterial blood pressure (MBP) did not significantly change between the beginning and end of the study in any of the groups (Supplementary Table 2).

	LowN (n = 10)	LowH (n = 9)	MaxN (n = 10)	MaxH (n = 10)	SupraN (n = 9)	SupraH (n = 10)	P value		
							Exercise intensity	O_2 level	Interaction
Body weight gain (% of baseline)	11.1 ± 4.6	18.9 ± 3.0*	12.8 ± 2.8	19.1 ± 5.1†	11.9 ± 3.1	15.1 ± 4.6	0.1773	< 0.0001	0.2097
Mean blood pressure response (% of baseline)	1.9 ± 15.0	3.8 ± 18.9	1.4 ± 12.6	-6.5 ± 11.9	6.5 ± 8.3	-2.0 ± 11.1	0.4091	0.3870	0.1735

Supplementary Table 2. Effect of hypoxic training at different intensities on body weight and arterial blood pressure. Data are mean ± SD. The number of mice per group is in parentheses. Body weight gain and mean blood pressure response were expressed as percentage of change from baseline values (measured prior to the first ExT). Groups: low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN), and supramaximal intensity training group in hypoxia (SupraH). Two-way ANOVA with Sidak post-hoc test: * $p<0.001$ vs LowN; † $p<0.01$ vs MaxN.

Vascular reactivity tension studies

Vascular relaxation responses

As shown in Figures 1A and B, the endothelium-dependent vasorelaxation to ACh was significantly improved in LowH compared to LowN (+10.0%, $p<0.001$), as well as in MaxH compared to MaxN (+14.8%, $p<0.0001$) and SupraH compared to SupraN (+20.0%, $p<0.0001$). ACh-induced relaxation was 9.2% greater in MaxH and 8.2% greater in SupraH than in LowH ($p<0.001$ and $p<0.01$, respectively; Figures 1A and B). There was no significant difference in ACh-induced relaxation between LowN, MaxN, and SupraN (Figures 1A and B).

Endothelium-independent relaxation to the NO donor DEA/NO was significantly increased in LowH compared to LowN (+4.7%, $p<0.01$; Figures 1C and D). It was also significantly greater in MaxN compared to LowN (+4.3%, $p<0.05$) and in MaxH compared to SupraH (+3.2%, $p<0.05$) (Figures 1C and D).

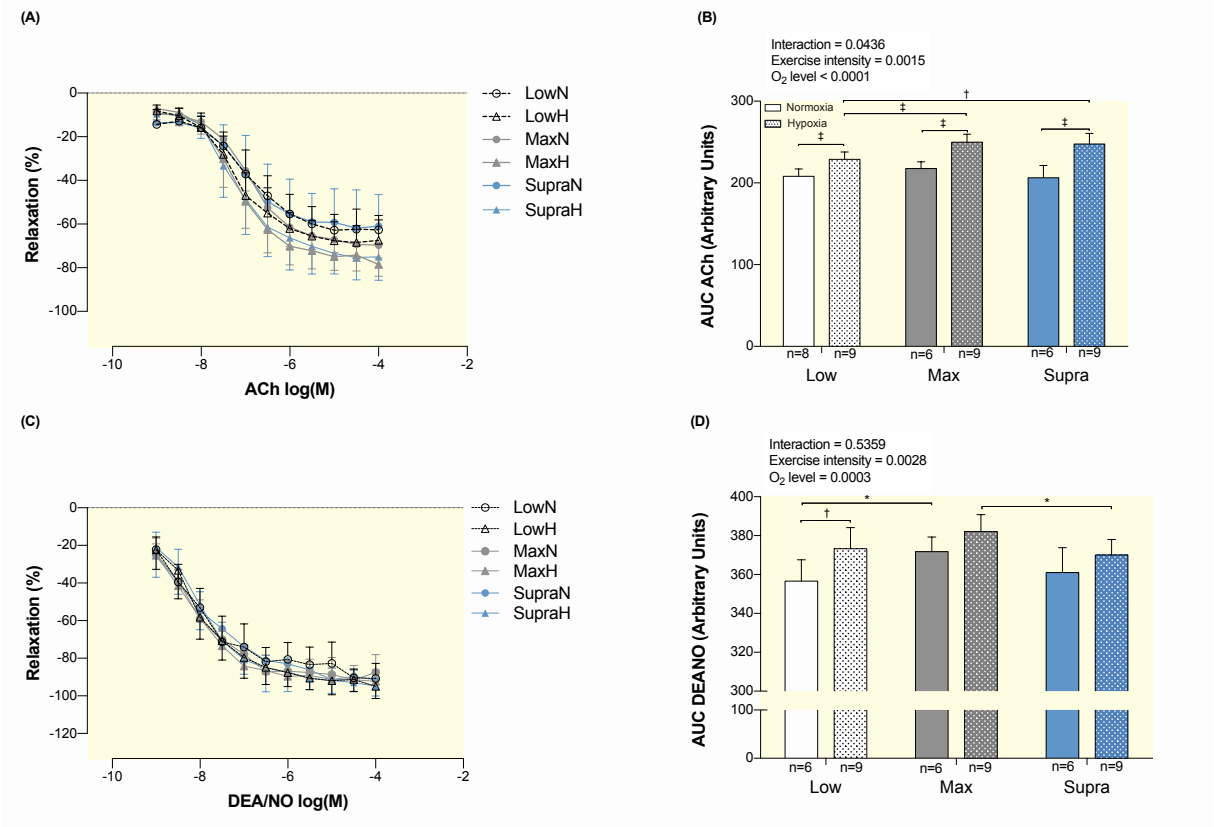


Figure 1. Effect of hypoxic exercise training at different intensities on endothelium-dependent and –independent vasorelaxation. Dose-response curves to acetylcholine (ACh, A) and to DEA/NO (C) of isolated abdominal aorta pre-constricted with phenylephrine in LowN, LowH, MaxN, MaxH, SupraN and SupraH mice. Bar graphs show the area under the curve of the vascular responses (B, D), calculated from the relaxation curves shown in a and c. Data are expressed as mean ± SD (n = 6 to 9 mice per group) of the percent of change in tension induced by the vasodilator. Two-way ANOVA with Sidak post-hoc test: * p<0.05; † p<0.01; ‡ p<0.001. Groups: low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN) and supramaximal intensity training group in hypoxia (SupraH).

Vascular constriction responses

The vascular constriction to Phe was significantly improved in LowH, MaxH and SupraH compared to LowN, MaxN and SupraN, respectively (+42.1%, p<0.05; +48.3%, p<0.01 and +119.9%, p<0.0001; Figures 2A and B). Vasoconstriction was greater in SupraH compared to LowH (+25.3%, p<0.05; Figures 2A and B).

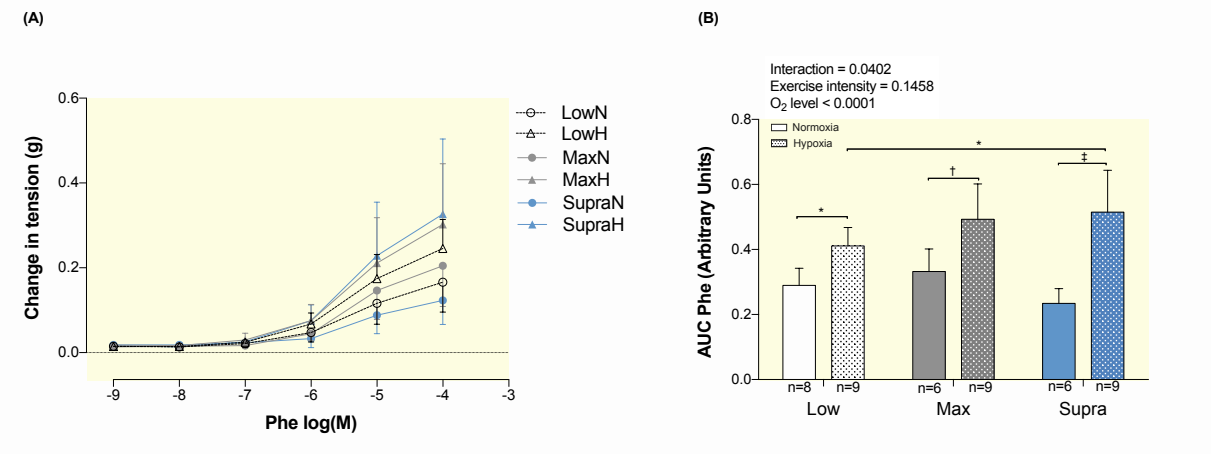


Figure 2. Effect of hypoxic exercise training at different intensities on vasoconstriction. Dose-response curves to α_1-adrenergic receptor agonist Phenylephrine (Phe, A) of isolated aorta of LowN, LowH, MaxN, MaxH, SupraN and SupraH mice. Bar graph shows the area under the curve of the vascular responses (B), calculated from the contraction curves shown in a. Data are expressed as mean ± SD (n = 6 to 9 mice per group). Two-way ANOVA with Sidak post-hoc test: * $p<0.05$; † $p<0.01$; ‡ $p<0.001$. Groups: low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN) and supramaximal intensity training group in hypoxia (SupraH).

Aortic mRNA and protein expression of markers involved in NO production

mRNA level of eNOS was higher in SupraH compared to SupraN ($p<0.05$), while no significant differences were observed in LowH and MaxH, compared to their respective normoxic groups (Figure 3A). mRNA level of eNOS was significantly higher in LowH and SupraH compared to MaxH ($p<0.05$; Figure 3A). No significant difference was observed between LowN, MaxN, and SupraN (Figure 3A). Neither protein expressions of eNOS nor phospho-eNOS (p-eNOS) showed significant differences between the six training groups (Figure 3B). The ratio of p-eNOS to eNOS expression revealed no significant difference between any of the groups either (Figure 3C).

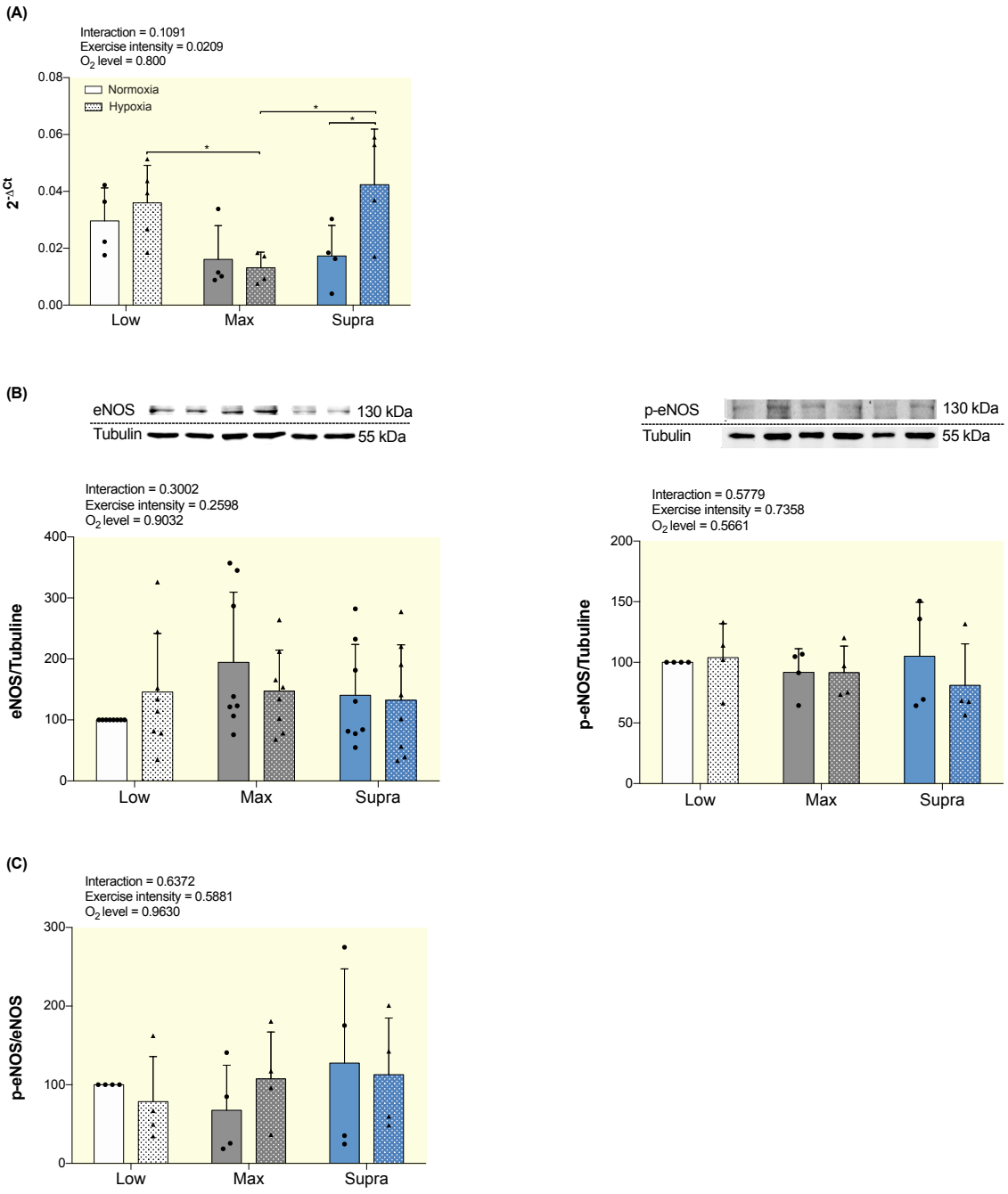


Figure 3. Effect of hypoxic training at different intensities on mRNA and protein expression of markers involved in NO production in aorta. (A) Gene expression analysis of eNOS. Results are expressed as $2^{-\Delta Ct}$ using 36B4 as housekeeping gene. (B) Western blot analysis of eNOS and phospho-eNOS protein expression. Protein expressions were normalized to tubulin content in each sample and expressed as the percentage of the protein expression values obtained in the LowN group. Top panel: representative WB images; bottom panel: quantitative analysis. (C) Ratio of phospho-eNOS to eNOS. Data are presented as mean ± SD (n = 4 to 8 mice per group). Two-way ANOVA with Sidak post-hoc test: * $p<0.05$. Groups: low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity

training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN), and supramaximal intensity training group in hypoxia (SupraH).

Aortic mRNA and protein expression of markers involved in NO bioavailability

As shown in Figure 4A, there were no significant differences in mRNA level of antioxidant SOD3 as well as in the protein expression of antioxidant SOD1, SOD2 and SOD3 among any of the groups. Neither the mRNA level nor the protein expression of pro-oxidant p47phox significantly differed between groups (Figure 4B).

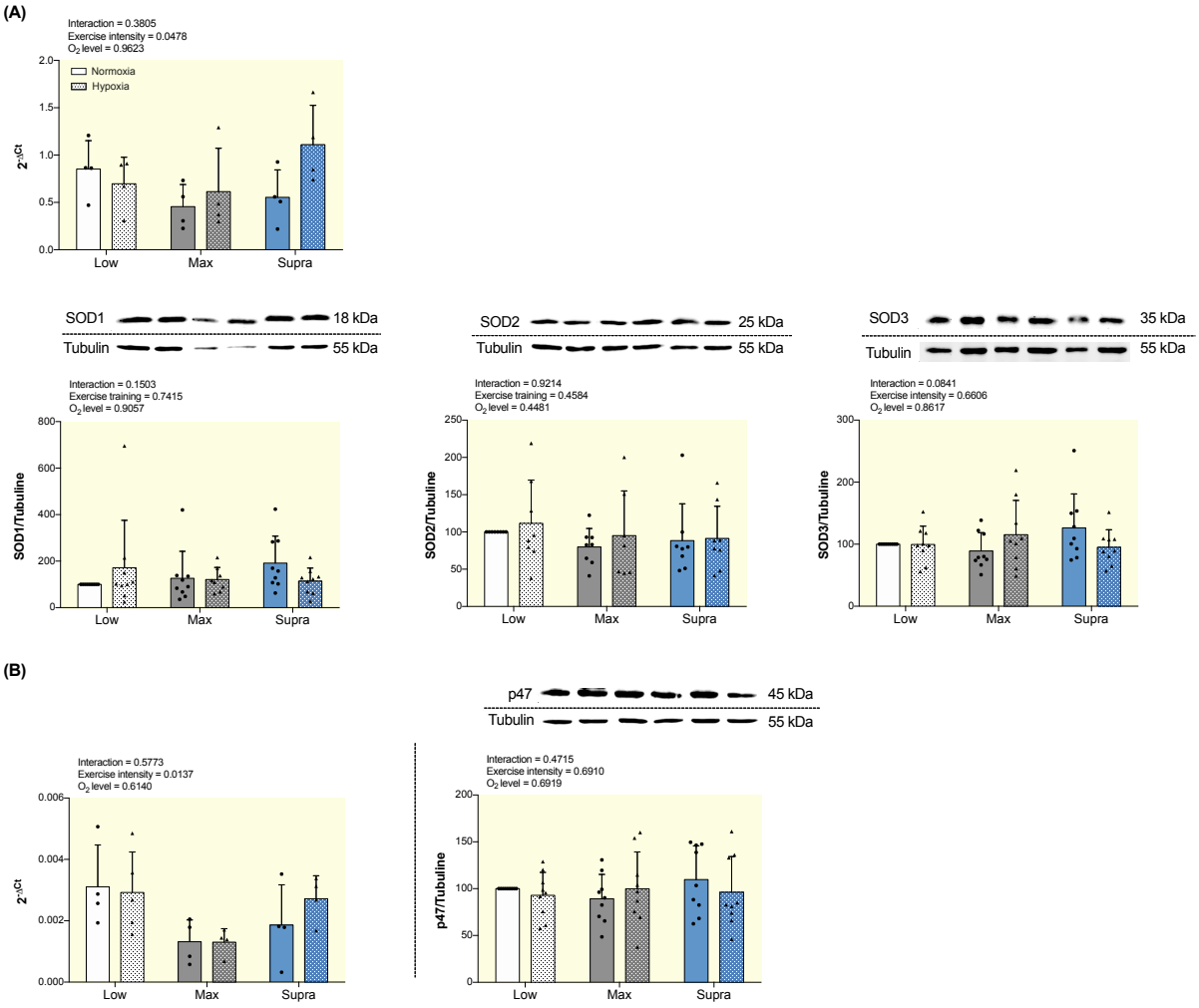
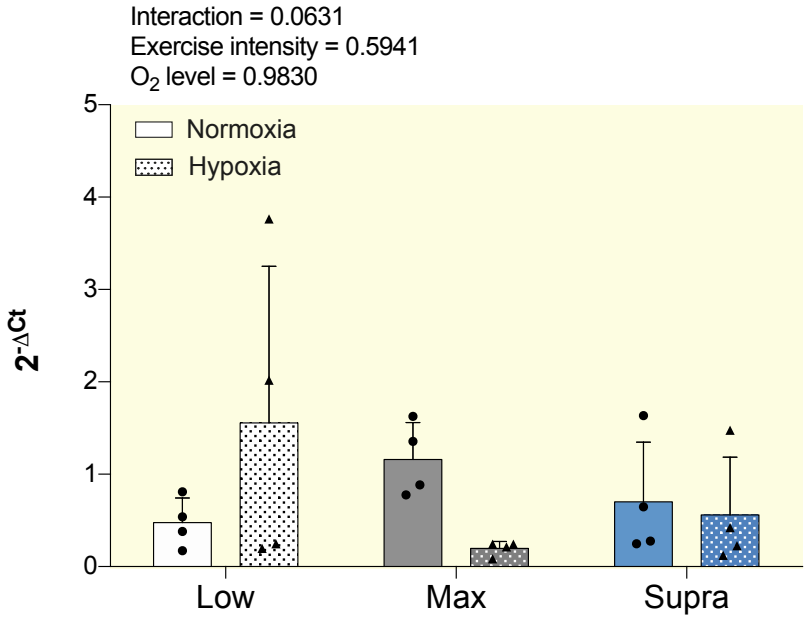


Figure 4. Effect of hypoxic training at different intensities on mRNA and protein expression of markers involved in NO bioavailability in aorta. (A) Gene expression analysis of antioxidant SOD3 (top) and Western blot

analysis of SOD1, SOD2 and SOD3 protein expression (low). (B) Gene expression analysis (left) and Western blot analysis (right) of pro-oxidant p47phox. For gene expression analysis, results are expressed as $2^{-\Delta Ct}$ using 36B4 as housekeeping gene. For western blot analysis, protein expressions were normalized to tubulin content in each sample and expressed as the percentage of the protein expression values obtained in the LowN group. Top panel: representative WB images, and bottom panel: quantitative analysis. Data are presented as mean ± SD (n = 4 to 9 mice per group). Two-way ANOVA with Sidak post-hoc test. Groups: low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN), and supramaximal intensity training group in hypoxia (SupraH).

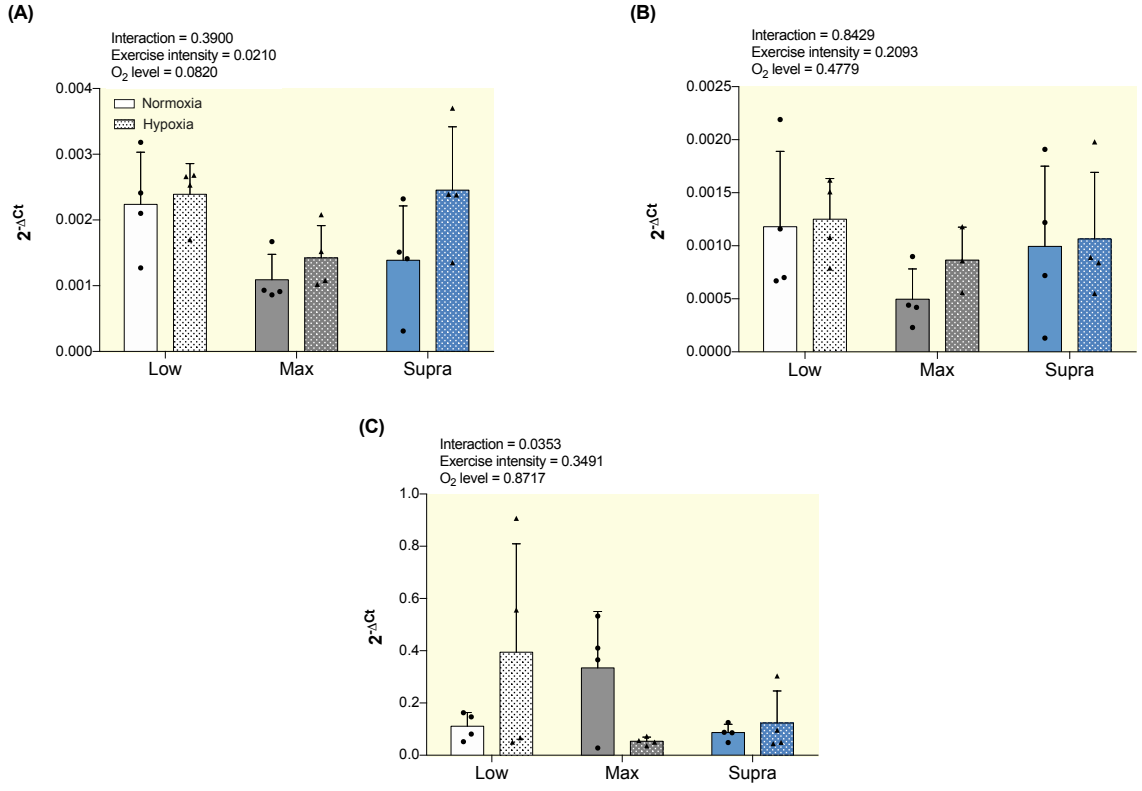
Aortic mRNA expression of soluble guanylate cyclase subunit alpha 1 and enzymes involved in hydrogen sulfide production

As shown in Supplementary Figure 2, there were no significant differences in mRNA level of soluble guanylate cyclase subunit alpha 1 (sGCα1) among any of the groups. Neither mRNA expression of CBS, CSE nor 3MST were significantly different between groups (Supplementary Figure 3).



Supplementary Figure 2. mRNA expression of soluble guanylate cyclase subunit alpha 1 in aortas of low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group

in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN), and supramaximal intensity training group in hypoxia (SupraH). Results are expressed as $2^{-\Delta Ct}$ using 36B4 as housekeeping gene. Data are presented as mean ± SD (n = 4 mice per group). Two-way ANOVA with Sidak post-hoc test.



Supplementary Figure 3. mRNA expression of cystathionine β-synthase (CBS, A), cystathionine γ-lyase (CSE, B) and 3-mercaptopyruvate (3MS, C) in aortas of low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN), and supramaximal intensity training group in hypoxia (SupraH). Results are expressed as $2^{-\Delta Ct}$ using 36B4 as housekeeping gene. Data are presented as mean ± SD (n = 3 to 4 mice per group). Two-way ANOVA with Sidak post-hoc test.

Aortic mRNA and protein expression of vasoconstriction marker α_1-adrenergic receptor

The mRNA expression of alpha 1 adrenergic receptor (α_1-AR) was significantly lower in MaxN and SupraN compared to LowN ($p<0.05$; Figure 5, left). It was also lower in MaxH and SupraH compared to LowH ($p<0.05$; Figure 5, left). There was no significant difference in the

expression of α_1-AR between normoxic and hypoxic groups for the same exercise training intensity (Figure 5, left). Protein expression of α_1-AR remained unchanged between the groups (Figure 5, right).

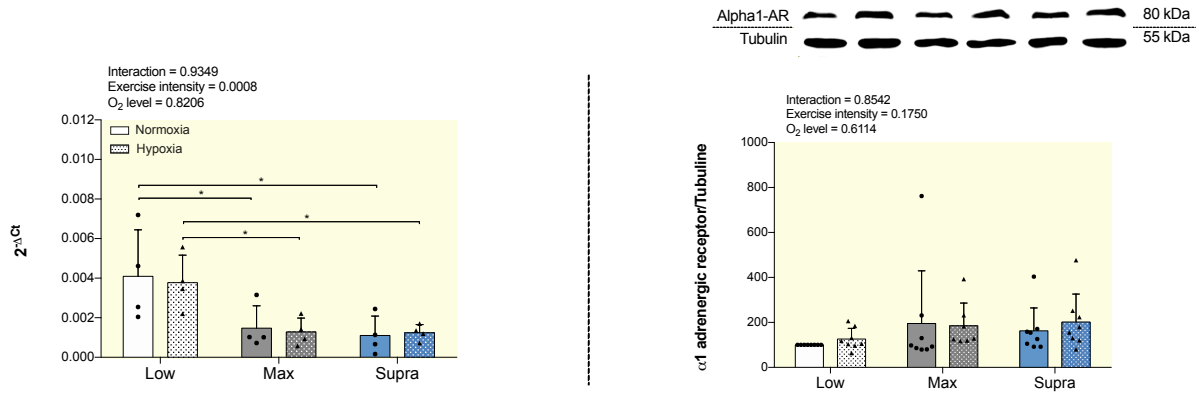


Figure 5. Effect of hypoxic training at different intensities on mRNA and protein expression of vasoconstriction marker α1-adrenergic receptor in aorta. Gene expression analysis (left) and western blot analysis (right) of α1-adrenergic receptor. For gene expression analysis, results are expressed as $2^{-\Delta Ct}$ using 36B4 as housekeeping gene. For western blot analysis, protein expressions were normalized to tubulin content in each sample and expressed as the percentage of the protein expression values obtained in the LowN group. Top panel: representative WB images, and bottom panel: quantitative analysis. Data are presented as mean ± SD (n = 4 to 8 mice per group). Two-way ANOVA with Sidak post-hoc test: * $p<0.05$. Groups: low intensity training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN) and supramaximal intensity training group in hypoxia (SupraH).

Circulating Nitric Oxide Metabolites, Pro- and Antioxidants Markers

No significant changes between any of the six training groups were observed in the concentration of the sum of nitrate and nitrite (NO_2; Figure 6A). Concentration of NO_2 alone was significantly higher in SupraN compared to MaxN (4.5 ± 1.9 µmol.L^{-1} vs 2.9 ± 0.9 µmol.L^{-1}, $p<0.05$; Figure 6B). No other significant differences were observed between the other groups.

Regarding oxidative stress markers, no significant changes between any of the six training groups were observed in AOPP (Figure 6C) and in XO (Figure 6D). As shown in Figure 6E, 3-

NT concentration was significantly decreased in MaxH and SupraH compared to the same intensities in normoxia ($p<0.05$ and $p<0.001$, respectively), and in SupraH compared to LowH ($p<0.01$).

No significant differences in plasma SOD (Figure 6F) and catalase activities (Figure 6G) were observed between LowH and LowN, MaxH and MaxN, or SupraH and SupraN. SupraH mice exhibited higher plasma SOD ($p<0.05$) and catalase ($p<0.01$) activities than LowH (Figure 6F and 6G).

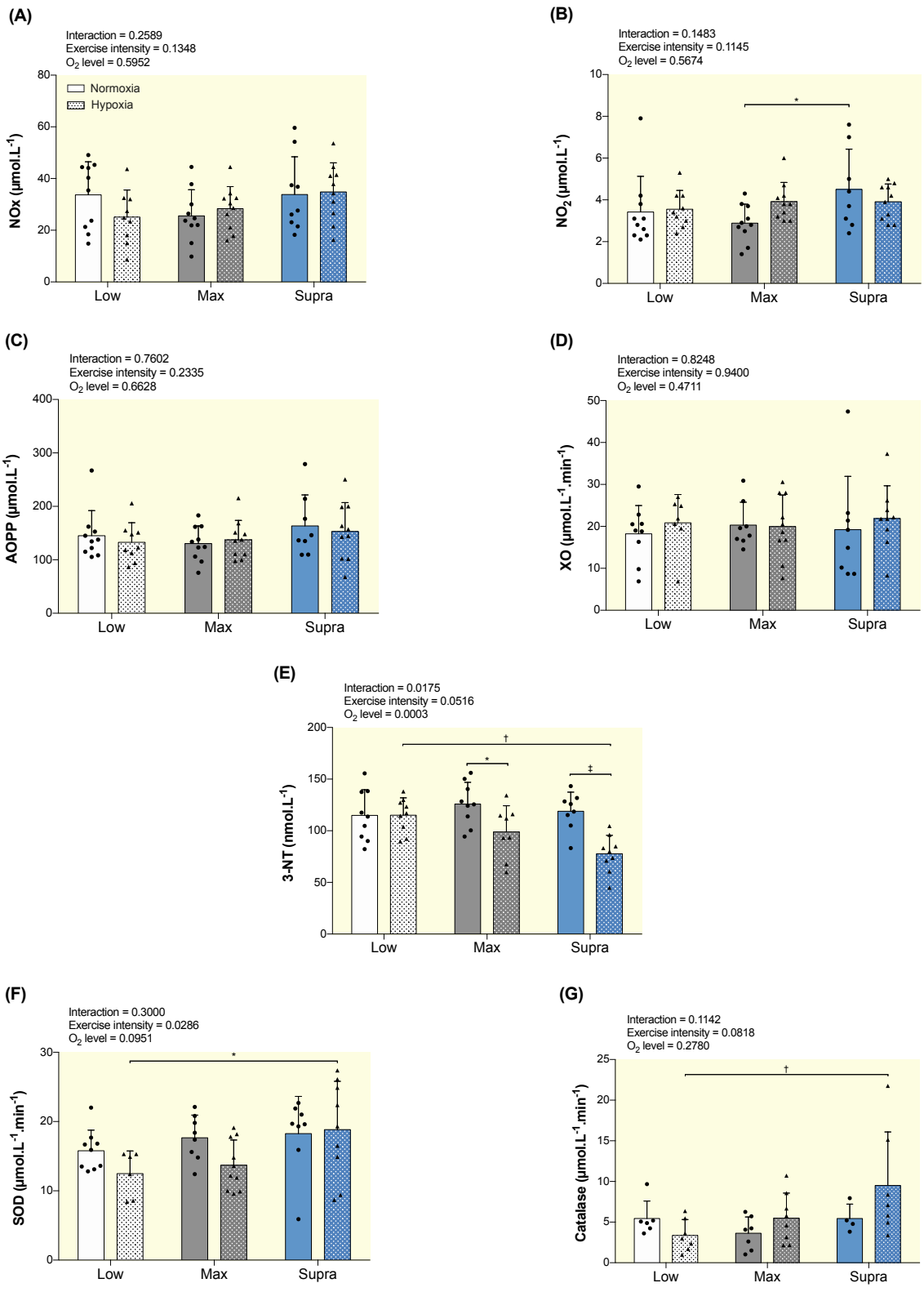


Figure 6. Effect of hypoxic training at different intensities on circulating NO metabolites, oxidative stress markers and antioxidant markers. Plasma concentrations of NO metabolism end-product nitrite and nitrate (NOx, A), nitrite alone (NO_2, B), advanced oxidation protein products (AOPP; C), xanthine oxidase (XO; D) and nitrotyrosine (3-NT, E), and plasma superoxide dismutase (SOD; F) and catalase (G) activities in low intensity

training group in normoxia (LowN), low intensity training group in hypoxia (LowH), maximal intensity training group in normoxia (MaxN), maximal intensity training group in hypoxia (MaxH), supramaximal intensity training group in normoxia (SupraN) and supramaximal intensity training group in hypoxia (SupraH). Data are presented as mean ± SD. Two-way ANOVA with Sidak post-hoc test: * $p<0.05$; † $p<0.01$; ‡ $p<0.001$.

Discussion

The main findings of this study were as follows: 1/ When performed in hypoxia, independently of intensity, exercise training improved vascular reactivity (i.e., both vasorelaxation and vasoconstriction) to a larger extent than the same intensity in normoxia; 2/ High (i.e., maximal and supramaximal) exercise training intensities performed in hypoxia improved endothelium-dependent vasorelaxation to a greater extent than low-intensity exercise training in hypoxia; 3/ Low-intensity exercise in hypoxia induced larger endothelium-independent relaxation than the same intensity in normoxia; 4/ NO bioavailability was improved by the change in pro/antioxidant balance: this mechanism underlines the above reported benefits of high-intensity exercise training in hypoxia.

The beneficial effects of low intensity exercise training on endothelial function are well documented.[8,9,24,25] High-intensity exercise training has also been studied both in healthy and diseased humans, and data indicate a positive impact of high-intensity exercise training compared to low-intensity on vascular function.[12,13,26–28] In animal models however, the superiority of high- versus low-intensity remains debated. Although several studies showed that higher intensity exercise training improves vasorelaxation,[15,16,29] others reported no benefits, or even a deleterious effect, when compared to low intensity.[17–20] In the present study, we showed no significant difference in aortic vasorelaxation among different exercise training intensities in normoxia in healthy mice. Our data are in line with those of Kim et al.[30] who reported that aortic endothelial function remained similar between C57BL/6J mice performing either high (6 sets of running at 85% MAS for 8 minutes followed by 2 min of active rest at 50% MAS) or low-intensity (continuous running at 65% of MAS) training.

On the contrary, exercise training performed in hypoxia significantly improved vasodilation, compared to normoxia independently of exercise intensity. In addition, for the first time to date, we report a larger effect of higher intensities of exercise training in hypoxia on endothelium-dependent relaxation.

Only few studies compared the impact of exercise training in hypoxia on endothelium-dependent vasorelaxation. Reboul et al.[31,32] reported enhanced aortic endothelium-dependent vasorelaxation in rats that lived and trained in normoxia, but not in rats living and training in hypoxia (partial pressure of inspired O_2 = 105 mmHg). Authors speculated that these specific adaptations may be explained by the altitude-induced limitation in aortic blood flow and shear stress.[31,32] This is in line with the findings of Casey and Joyner[33] who reported in humans that the vasoconstriction activity in vascular beds of contracting muscles was blunted to a larger extent during exercise training performed in hypoxia compared to normoxia ("functional sympatholysis"), to the point where vasodilation would prevail. Moreover, post-exercise reduction in total peripheral resistance is known for being enhanced in hypoxia (F_iO_2 = 0.15),[34] suggesting a larger hypotensive effect of exercise in hypoxia (F_iO_2 = 0.145) than in normoxia.[35] At the peripheral level, the main phenomenon driving blood flow response to exercise training is the vasomotor control, which translates into vasodilation and vasoconstriction.[23]

At low intensity, an important part of the observed compensatory vasodilation is mediated by vascular smooth muscle cells (VSMCs) β-adrenergic receptors, triggering NO release. However, as exercise training intensity increases, although NO still appears to play a role in vasodilation, the β-adrenergic-NO pathway is taken over by other NO-releasing mechanisms,[33,36] raising interest regarding the effects of exercise training intensity combined with hypoxia on vascular response.

During high-intensity exercise in hypoxia, one may speculate that the compensatory vasodilation (with an increase in blood flow and shear stress), aiming at constantly maintaining total O_2 delivery to tissues, is enhanced since exercise intensity is essential for the amplitude of this mechanism.[33]

Interestingly, the endothelium-independent relaxation induced by the NO-donor DEA/NO was higher at low-intensity exercise training performed in hypoxia compared to normoxia. This indicates that the observed increased relaxation in low-intensity exercise training combined

with hypoxia may be due to an increase in soluble guanylate cyclase activity (sGC), and/or subsequent formation of the second messenger cyclic guanosine monophosphate (cGMP), causing VSMCs relaxation and in turn vasodilation. However, no significant difference between our six experimental groups of mice was found with regards to aortic sGCα1 mRNA expression (Supplementary Figure 2). Therefore, further investigations are needed to determine the adaptative molecular mechanisms occurring in the VSMCs in response to low intensity exercise training in hypoxia compared to normoxia.

It is important to note that the endothelium-independent relaxation to the NO donor was not significantly different between the higher intensities exercise training in hypoxia versus normoxia (MaxH vs MaxN, and SupraH vs SupraN). The present findings suggest that the improved vasorelaxation with high intensity exercise training in hypoxia was not a consequence of a change in vasodilating capacity of VSMCs, but potentially of the production and/or bioavailability of NO.

At the molecular level, it is well known that the NO pathway is paramount in the enhancement of vasorelaxation in response to low intensity exercise, via eNOS activation and NO production.[1,33,36,37] Based on that, one could hypothesize that the improved ACh-mediated vascular relaxation observed in response to exercise training in hypoxia is associated with increased eNOS expression in hypoxic compared to normoxic exercise training groups, as well as in the high-intensity hypoxic compared to low-intensity hypoxic exercise training groups. We therefore determined aortic mRNA and/or protein expression of eNOS and phospho-eNOS. Our data revealed increased eNOS mRNA expression in hypoxic versus normoxic exercise training only with the Supra intensity. However, this result was not confirmed at the protein level. Surprisingly, we observed a lower eNOS mRNA level in MaxH versus LowH, while SupraH and LowH were not different. Again, no changes at the translational level were observed. Moreover, neither phospho-eNOS protein content, nor the ratio of phospho-eNOS to eNOS ratio were modulated by hypoxia and/or training intensity. In agreement with these data, circulating NO metabolism end-products nitrate and nitrite did not reveal any significant

differences among groups. Taken together, our findings indicate that the observed improved ACh-induced relaxation may not be the result of an increased eNOS activation and/or NO production, but rather of mechanisms involved in NO bioavailability.

In order to further investigate the underlying mechanisms of this bioavailability, we looked at markers of oxidative stress and antioxidant defenses, since endothelial NO inactivation is determined by the balance between pro- and antioxidant status. The major source of oxidative stress in the arterial wall is NADPH oxidase (NOX), which is involved in the production of ROS, in particular of superoxide anion ($O_2^{\bullet-}$).[38] $O_2^{\bullet-}$ reacts with NO to generate a more potent oxidant peroxynitrite ($ONOO^-$).[39] A decrease in NOX expression and/or activity associated with less $O_2^{\bullet-}$ production in the endothelium and subsequent scavenging of NO may result in increased NO availability and endothelium-dependent vasorelaxation.

In the present study, mRNA and protein expression of p47phox – i.e., a subunit of NOX2 that has been shown to be involved in pro-oxidant activity and regulated by exercise training[40] – was not modulated by the present exercise training modalities. Besides, circulating pro-oxidants AOPP and XO were not significantly different among groups. Very interestingly, we observed a lower nitrotyrosine (3-NT) level with hypoxic high intensities exercise training (MaxH and SupraH) compared to the same intensities performed in normoxia, as well as in SupraH compared to LowH, indicating that oxidation of NO by ROS is decreased. This finding suggests that a limiting NO inhibition at the systemic level may be a potential mechanism for the improved endothelium NO-dependent vasorelaxation in these mice.

SOD isoforms represent a major defense against NO inactivation and $ONOO^-$ formation. SOD isoforms expression upregulation has been shown to be associated with improved endothelial function in response to exercise training in mice.[41] Here, we did not observe any difference in aortic mRNA and/or protein expression of SOD1, SOD2 and SOD3. At the plasma level however, there was an increase in SOD and catalase activities in SupraH when compared to LowH. Based on the present 3-NT results, one could also expect a higher SOD and catalase

plasma concentration in MaxH compared to MaxN and in LowH compared to LowN. The fact that the increases in SOD and catalase activities occurred only in SupraH versus LowH suggests that this mechanism (i.e., higher systemic antioxidant defense) is likely to underlie the larger improvement in endothelium-dependent relaxation observed specifically in SupraH versus LowH.

In order to produce NO, eNOS needs the cofactor tetrahydrobiopterin (BH_4). In its absence, eNOS will produce $O_2^{\bullet-}$ instead of NO (i.e., eNOS uncoupling), impairing its bioavailability.[37] Studies have shown a cardioprotective role of ischemic preconditioning (i.e., intermittent exposure of tissues to short bouts of localized hypoxia),[42] which is partly physiologically comparable to our SupraH training. This phenomenon would prevent an increase in $O_2^{\bullet-}$ derived from eNOS uncoupling, probably because of an increased BH_4 availability. However, this eNOS uncoupling could be a way to limit the amount of eNOS-dependent ROS production. By limiting the synthesis of NO, eNOS uncoupling prevents the reaction between NO and $O_2^{\bullet-}$ that in turn produces $ONOO^-$,[43] which may explain the decreased 3-NT in SupraH and MaxH compared to the same intensities in normoxia.

Finally, we explored the possible role of hydrogen sulfide (H_2S) in the observed vasorelaxation, since it has been previously reported that this molecule can be modulated by exercise training.[44] Like NO, H_2S is a ubiquitous second messenger molecule with important roles in the vessel wall, including vasoreactivity. To this end, we looked at the mRNA levels of the three enzymes responsible for H_2S generation, i.e., cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate (3MST; Supplementary Figure 3). The mRNA levels of CBS, CSE and 3MST did not differ between any of the groups. Collectively, our data suggest that the H_2S pathway is not likely involved in the improved endothelium-dependent vasodilation observed after exercise training in hypoxia compared to the same intensities in normoxia, and higher intensities compared to the lowest intensity in hypoxia.

Another important finding of the present study is that, alongside an improved vasorelaxation, we observed an increased Phe-induced vasoconstriction in hypoxic conditions, independently of the intensity, as well as between SupraH and LowH. Our results are partially in line with the increased sympathetic vasoconstrictor activity directed towards skeletal muscle as observed with hypoxic training.[33] Despite this increased vasoconstriction, we propose that the degree of vasodilatation prevails over the vasoconstrictor response in our mice, in accordance with previous studies.[33]

Because Phe induces VSMCs contraction and vessels vasoconstriction by binding to α_1-AR on VSMCs,[45] we hypothesized that exercise training in hypoxia would lead to increased α_1-AR expression. To gain insights into the molecular mechanisms, we therefore looked into aortic α_1-AR mRNA and protein expression. No significant differences were noted in α_1-AR mRNA expression between hypoxia and normoxia for the same exercise training intensities. Contrary to our expectations, α_1-AR mRNA expression was downregulated in MaxH and SupraH compared to LowH. However, the fact that no change was observed at the protein level indicates that an increase in α_1-AR expression is unlikely to explain the functional effect of hypoxic training on vasoconstriction. One might not exclude however an increased sensitivity of α_1-AR as a mechanism of the observed increased Phe-induced vasoconstriction. In addition, other mechanisms, such as enhanced sympathetic activation, or increased production of endothelin-1 might likely be involved.[46]

In conclusion, the present study provides the first experimental evidence that high-intensity exercise training in hypoxia improves vascular reactivity. Mechanistically, it appears that benefits of high intensity exercise in hypoxia on endothelium-dependent vasorelaxation are not mediated by an increased NO production, but rather by increased NO bioavailability secondary to an increased antioxidant status.

Clinical Applications

Our findings highlight that high-intensity exercise in hypoxia may represent a novel therapeutic strategy to improve and/or preserve endothelial function. High-intensity training in hypoxia may bring new promising perspectives in terms of physical activity prescription amongst the general population for primary prevention of endothelial dysfunction and cardiovascular diseases, including hypertension.

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Conflict of interest

None.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter 7

Study 2 - Effect of high intensity exercise training in hypoxia on atherosclerosis development and progression in ApoE$^{-/-}$ mice

Study 2 – Effect of high intensity exercise training in hypoxia on atherosclerosis development and progression in ApoE$^{-/-}$ mice

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Animals

A total of 123 8 to 10-week-old male C57BL/6J ApeE$^{-/-}$ mice were used. Mice were purchased from Charles River Development, Inc. (Beijing, China) and housed under a 12-h light/dark cycle, at a temperature of 22±2°C and humidity of 50-70%. Throughout the study, mice had free access to a high fat diet (HFD), containing 21% (w/w) fat and 1.5% (w/w) cholesterol (Beijing Keao Xieli Feed Co., Ltd, China), and water. All experiments were approved by the Animal Care and Use Committee of Beijing Sport University.

Study Design

Preventive protocol: effects of hypoxic exercise training on atherosclerotic plaque development

For this part of the study, 59 mice were used. The following protocol has been designed based on a previously published one (Riopel et al., 2019). At the beginning of the study, mice were randomly divided into six groups: 1. Sedentary in normoxia (**SedN**, n = 10); 2. Sedentary in hypoxia (**SedH**, n = 10); 3. Low-intensity continuous training in normoxia (**LowN**, n = 10); 4. Low-intensity continuous training in hypoxia (**LowH**, n = 9); 5. High-intensity interval training in normoxia (**HighN**, n = 10); 6. High-intensity interval training in hypoxia (**HighH**, n = 10) (Figure 16).

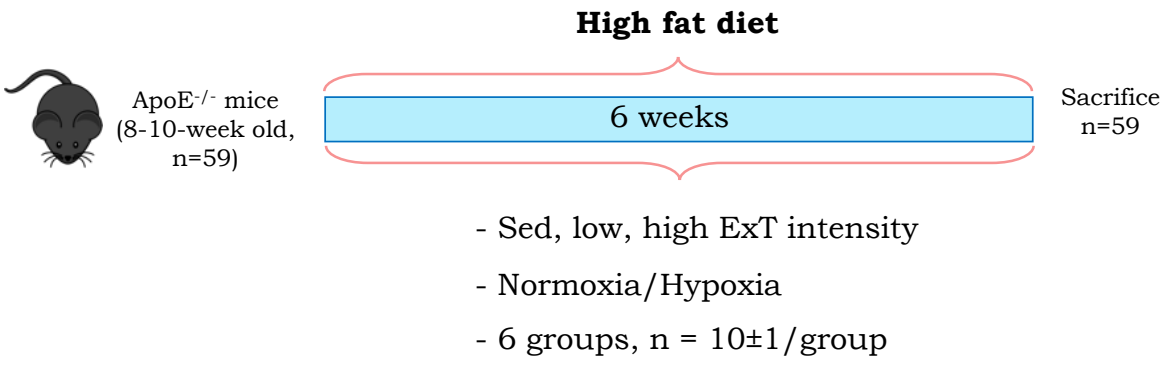

Figure 16 : Experimental design of the prevention protocol.

Therapeutic protocol: effects of hypoxic exercise training on pre-existing atherosclerotic plaque regression

For this part of the study, 64 mice were used. The following protocol has been designed based on a previously published one (Zimmer et al., 2016). Mice were all fed a high fat diet for 12 weeks before the beginning of the intervention to induce atherosclerotic plaque development. At baseline (i.e., at the beginning of the intervention), 7 mice were sacrificed to have a timepoint reference of the development of atherosclerotic plaques (i.e., atherosclerotic plaque development control mice). The rest of the mice were randomly divided into six groups: 1. Sedentary in normoxia (**SedN**, n = 10); 2. Sedentary in hypoxia (**SedH**, n = 9); 3. Low-intensity continuous training in normoxia (**LowN**, n = 8); 4. Low-intensity continuous training in hypoxia (**LowH**, n = 9); 5. High-intensity interval training in normoxia (**HighN**, n = 11); 6. High-intensity interval training in hypoxia (**HighH**, n = 10) (Figure 17).

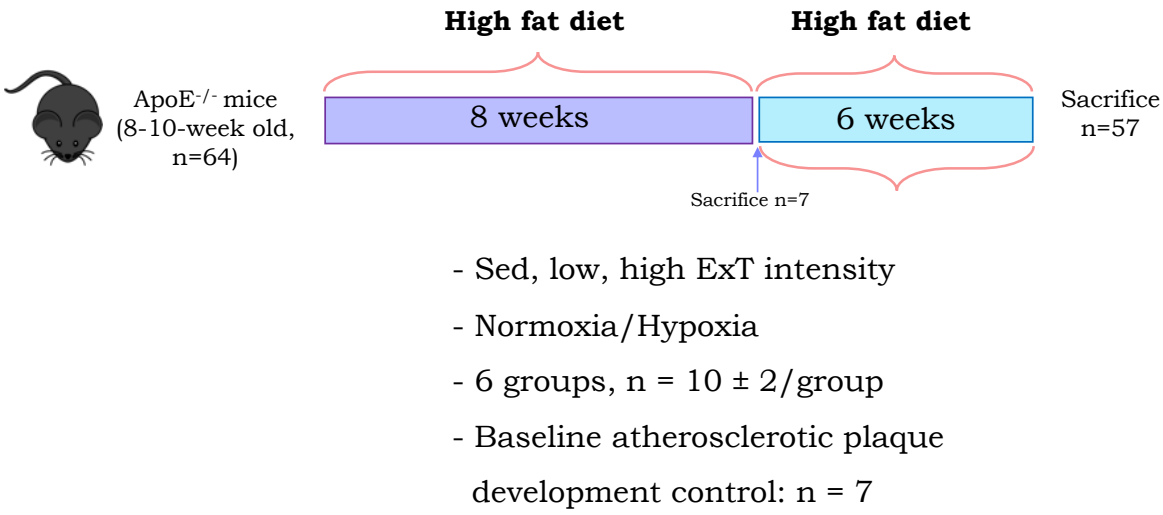

Figure 17. Experimental design of the therapeutic protocol.

Exercise Protocols

SedH mice were submitted to passive exposure to hypoxia for 6 weeks. ExT mice were acclimated to treadmill running for 4 days before the beginning of the training protocols (10 min per day, starting at 8 cm/s on day 1 and progressively increasing until reaching 14 cm/s on day 4). ExT consisted in forced training running 3 times per week for 6 weeks on a mouse treadmill (Panlab LE-8710, Bioseb, France). Low voltage stimuli (0.2 mA) and cotton swabs were used to motivate mice to run throughout the training. Mice assigned to Low ExT (groups 3 and 4) ran continuously for 40 min at 18 cm/s (~40% of their maximal aerobic speed MAS). High ExT mice (groups 5 and 6) ran 4 sets of 5 x 10 s sprints at 45 cm/s (~100% of MAS), with 20 s of passive recovery between each sprint. The interset rest was of 5min of passive recovery. For all protocols, each training session began with a 5 min warm-up at 8 cm/s, followed by 5 min at 15cm/s. The treadmill was placed in a hypoxic chamber with an F_iO_2 of either 0.11±0.01 (hypoxic training) or 0.21 (normoxic training). Hypoxic exposure was matched between groups (1h).

At the end of the protocols and 48h after the last training session, blood was collected by cardiac puncture under chloral hydrate anesthesia, and the plasma was obtained by

centrifugation for 10 min at 2500 rpm at +4°C. Plasma samples were then snap frozen and stored at -80°C until further analysis. Immediately after cardiac puncture, mice were euthanized by cervical dislocation, then aortas were isolated and cleaned of fat and connective tissue in cold phosphate-buffered saline. The aortic arch as well as the upper part of the heart were stored in 3.7% formalin for further histological analyzes. The rest of the aorta was stored in RNA later (Ambion, Invitrogen, California, USA) at +4°C before storage at -80°C. The apex of the heart, the spleen, the pulmonary artery and the lungs were snap frozen in liquid nitrogen before storage at -80°C for further analyzes. Those samples were then shipped back to the angiology laboratory of the CHUV in Lausanne where they will be analyzed.

Left and right quadriceps, soleus, gastrocnemius, tibialis anterior muscles, as well as brown fat, subcutaneous fat and peritesticular fat were collected and frozen for analyzes by the laboratory of Professor Zhang. A first study entitled *"High-intensity interval training and moderate-intensity continuous training attenuates oxidative damage and promotes myokine response in skeletal muscle of ApoE KO mice on high-fat diet"* has been published (Wang et al., 2021) (see Annex 1).

Planned Analysis

Histological and immunohistochemical analysis

Hearts and aortic arches preserved in formalin will be used for histologic and immunohistochemistry analysis of plaque surface and plaque composition in the aortic sinus as previously described (Centa et al., 2019; Pellegrin et al., 2018). To assess plaque surface, the arch will be opened longitudinally, pinned on a black silicone-covered dish, and stained with Oil red O. Pictures of stained aortic arches will be taken with a digital camera, and the surface areas of total pinned arch and Oil red O-stained areas will be measured using a computer-assisted image analysis. Aortic arch plaque area will then be expressed as the

percentage of positive Oil red O area over the total area of the arch. To assess plaque surface and composition, the heart will be embedded in paraffin and cross-sectioned (3µm-thick) until reaching the aortic sinus. Sections will be stained with Movats pentachrome and photographed using a digital camera. Pictures will then be analyzed, and aortic sinus plaque will be expressed in μm^2. Macrophage and smooth muscle cells in plaque will be identified by immunostaining with primary anti-mouse monoclonal Mac-2 and α-smooth muscle actin antibodies, respectively, followed by the appropriate biotinylated secondary antibodies. Plaque total collagen content will be determined using Sirius red staining. Quantitative analysis will be performed using light microscopy.

Molecular analysis

RT-qPCR and western blot analysis will be performed to determine aortic gene and protein expression of target markers (to be defined).

Expected Results

Figure 18 shows three pictures taken when mice from the therapeutic group were sacrificed. The size of the developed atherosclerotic plaques is such that they are clearly visible to the naked eye. On the left picture, the arrow points to what is left of the lumen of the brachiocephalic artery. The middle picture shows the plaques that developed in the aortic arch and the right picture shows that these plaques are also present in the descending thoracic aorta. These pictures validate our atherosclerosis model.

The first expected outcome of these studies is for hypoxic training to be more effective than normoxic training in preventing plaque development (i.e., preventive) and reverse pre-existing plaques (i.e., therapeutic), independently of intensity. The second expected result is that HighH and HighN would prevent plaque development and increase regression of pre-existing plaques compared to LowH and LowN respectively.

These studies could help with the prescription of more effective exercise training regimens for primary and secondary prevention of many cardiovascular diseases associated with atherosclerosis.

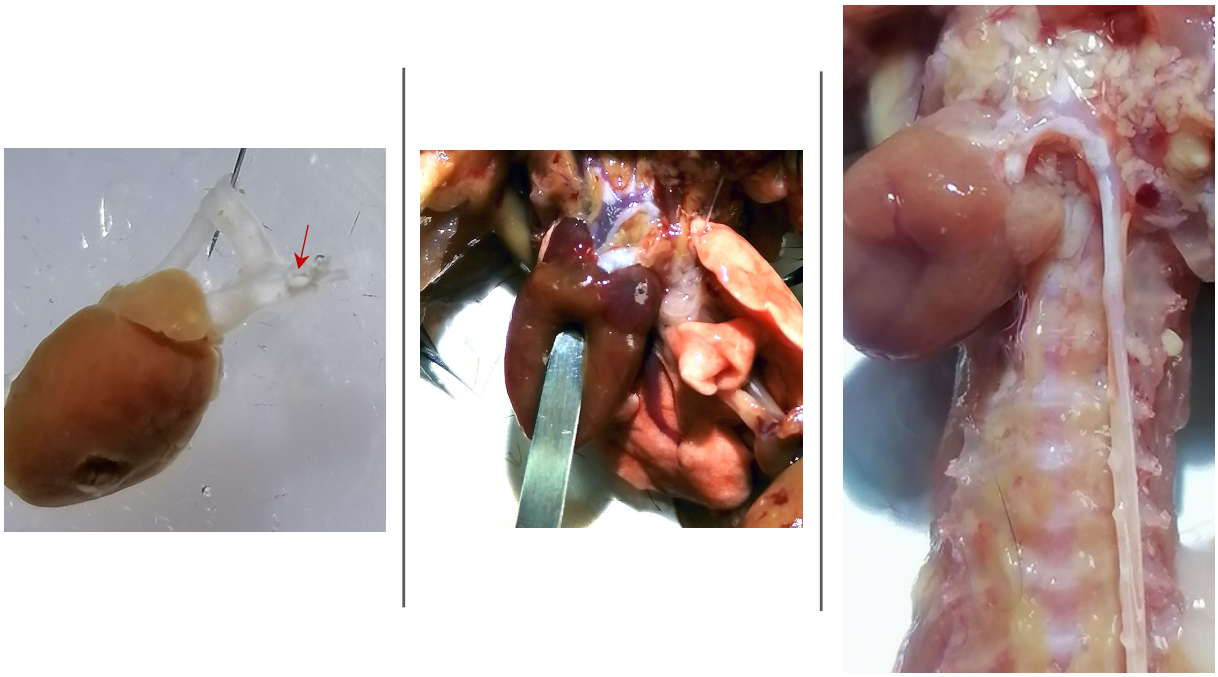

Figure 18. Atherosclerotic plaque in aortas and aortic arches.

Mice fed a high-fat diet for 18 weeks developed plaques visible during dissection in the aortic arch and the thoracic aorta. The arrow in the left picture points to the remaining lumen of the brachiocephalic artery, which appears greatly narrowed. *Personal data.*

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Chapter 8

Annex 1 - High-Intensity Interval Training and Moderate-Intensity Continuous Training Attenuate Oxidative Damage and Promote Myokine Response in the Skeletal Muscle of ApoE KO Mice on High-Fat Diet

Annex 1 – High-intensity interval training and moderate-intensity continuous training attenuate oxidative damage and promote myokine response in the skeletal muscle of ApoE KO mice on high-fat diet

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Abstract

The purpose of this study was to investigate the effects of high-intensity interval training (HIIT) and moderate-intensity continuous training (MICT) on the skeletal muscle in Apolipoprotein E knockout (ApoE KO) and wild-type (WT) C57BL/6J mice. ApoE KO mice fed with a high-fat diet were randomly allocated into: Control group without exercise ($ApoE^{-/-}$ CON), HIIT group ($ApoE^{-/-}$ HIIT), and MICT group ($ApoE^{-/-}$ MICT). Exercise endurance, blood lipid profile, muscle antioxidative capacity, and myokine production were measured after six weeks of interventions. $ApoE^{-/-}$ CON mice exhibited hyperlipidemia and increased oxidative stress, compared to the WT mice. HIIT and MICT reduced blood lipid levels, ROS production, and protein carbonyl content in the skeletal muscle, while it enhanced the GSH generation and potently promoted mRNA expression of genes involved in the production of irisin and BAIBA. Moreover, $ApoE^{-/-}$ HIIT mice had significantly lower plasma HDL-C content, mRNA expression of *MyHC-IIx* and *Vegfa165* in EDL, and ROS level; but remarkably higher mRNA expression of *Hadha* in the skeletal muscle than those of $ApoE^{-/-}$ MICT mice. These results demonstrated that both exercise programs were effective for the ApoE KO mice by attenuating the oxidative damage and promoting the myokines response and production. In particular, HIIT was more beneficial to reduce the ROS level in the skeletal muscle.

Introduction

Atherosclerosis can pathologically affect the large arteries of the heart, as well as the peripheral arteries in the human body [1,2]. In the peripheral arteries, atherosclerosis has a considerable effect on skeletal muscle structure and function. It has been reported that individuals with peripheral arterial disease (PAD) usually suffer a myopathy in the diseased limbs caused by the oxidative damage and mitochondrial disorder [3,4]. Apolipoprotein E (ApoE) is an important component of all plasma lipoproteins and serves as a ligand for the cell-surface lipoprotein receptors, such as the LDL-receptor. ApoE knockout (KO) mice spontaneously develop hypercholesterolemia and atherosclerosis when fed standard chow [5]. It has also been observed that a high-fat diet (HFD) can further exacerbate and accelerate these lesions [6]. Thus, ApoE KO is a commonly used animal model in atherosclerosis research.

Physical activity has been demonstrated to be a preventive tool for the development of atherosclerosis [7,8]; however, most current research has investigated the effects of exercise on the severity and composition of atherosclerotic plaque in cardiovascular diseases, whereas the impacts on the skeletal muscle are not well studied. Specifically focused on the ApoE KO mice with PAD, our team has found that neither running on the treadmill or wheel nor swimming enhanced vascularization and blood flow in the ischemic limb, as well as negligible changes in glucose metabolism and mitochondrial biogenesis markers in the skeletal muscles [9]. These results suggest that not all kinds of exercise are beneficial to the ApoE KO mice. Therefore, investigating the most effective exercise modality with different intensities and durations is worthy of interest.

The high-intensity interval training (HIIT) consists of brief intervals of vigorous activity interspersed with periods of low activity or rest and has been employed to im- prove athletic exercise performance since the mid-20th century. Contemporary protocols developed for non-athletes are intended to reduce exercise time and provide a greater stim- ulus for physiological

adaptation, compared with moderate-intensity continuous training (MICT) [10,11]. Recent studies have shown an improvement in both whole-body and skele- tal muscle metabolic health in different populations following HIIT [12]. In particular, HIIT has been touted as the most beneficial exercise intervention for patients with cardiovascular disease [13]. However, the effects of HIIT intervention on the skeletal muscle of ApoE KO mice on HFD, compared with MICT, have not been studied to date.

Oxidative stress is an important manifestation of peripheral atherosclerosis in the skeletal muscle [6,14]. At higher concentrations, reactive oxygen species (ROS) may lead to oxidative stress and oxidative damage of biomolecules [15]. The increased ROS production by dysfunctional mitochondria in the leg muscles of PAD patients has been recognized as the key mechanism of initiation and progression of the disease [3,16]. Previous studies have also observed elevated levels of 4-hydroxynonenal (4-HNE) and protein carbonyls in PAD muscle specimens [17,18]. NADPH oxidases (Noxs) are ROS-generating enzymes. Skeletal muscles express three isoforms of Noxs (Nox1, Nox2, and Nox4) that have been identified as critical modulators of redox homeostasis [19]. Among the three isoforms, Nox2 acts as the main source of skeletal muscle ROS during contractions [19–21] and the subunit p47phox is required for Nox2 activity [22]. It is well-known that the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is the master regulator of cellular antioxidant defense, because it regulates more than 200 cytoprotective genes in response to oxidative stress [23]. Thus, it is important to understand the effects of HIIT and MICT on redox homeostasis and the adaptative response of Nrf2 pathway expression. In addition, skeletal muscle morphology may be transformed by hyperlipidemia. ApoE deficiency and Western-type diet independently induced a higher capillary-to-fiber ratio in the glycolytic extensor digitorum longus (EDL) followed by increased mRNA levels of key angiogenic factors [24], leading to an enhanced transition to more oxidative myofibers i.e., the transition of fast-twitch glycolytic IIb to the fast-twitch IIx fibers, in ApoE KO mice [24,25].

The skeletal muscle is the major organ contributing to the whole-body metabolism, and the identification of exercise-induced myokines set a new paradigm in exercise biology and metabolic homeostasis. In the past few decades, a great number of myokines have been discovered, and among them are irisin, FNDC5/irisin, β-aminoisobutyric acid (BAIBA), and musclin [26,27]. A lack of exercise, which is associated with a network of diseases, including hyperlipidemia, type 2 diabetes, cardiovascular diseases, and others [28,29], leads to an altered response of the secretion of myokines and/or resistance to them. Thus, myokines could serve as the prognostic biomarkers, which reflect the benefits of exercise on metabolism [26]. However, the effects of exercise modality (HIIT vs. MICT) on the production of irisin, BAIBA, and musclin in skeletal muscles of ApoE KO mice still remains poorly understood. In this context, the purpose of the present study was to investigate and compare the effects of six weeks of HIIT and MICT on the hypercholesterolemia model of ApoE KO mice fed HFD, with a special focus on the changes in redox homeostasis and myokine production in the skeletal muscle. We hypothesized that the two training modalities would present remarkable impacts on the measured variables, with HIIT being superior to MICT for improving the pro-/antioxidant status of the skeletal muscle.

Materials and Methods

Animals

The protocols of this study were approved by the Animal Care and Use Committee of Beijing Sport University. Male wild-type (WT) C57BL/6J mice (9 weeks old, n = 10) and male C57BL/6J ApoE-KO mice (9 weeks old, n = 35) were purchased from Charles River Development, Inc. (Beijing, China), respectively. All mice were housed indoors under a temperature of 22 ± 2 ◦C, humidity of 50–70%, 12-h light/dark cycles and they had *ad libitum* access to deionized water and food.

Five ApoE KO mice were used to determine the maximal running speed on the treadmill and the remaining 30 ApoE KO mice were randomly allocated into three groups: ApoE KO control

(ApoE$^{-/-}$ CON), ApoE KO mice with HIIT (ApoE$^{-/-}$ HIIT), and ApoE KO mice with MICT (ApoE$^{-/-}$ MICT), with 10 mice in each group. All ApoE KO mice were fed the HFD, containing 21% (*w/w*) fat and 1.5% (*w/w*) cholesterol (Beijing Keao Xieli Feed Co., Ltd., Beijing, China), following a three-day standard chow then a three-day mixed diet of standard chow and a gradually increased proportion of HFD. In the meantime, the WT mice were fed standard chow, containing 4–5% (*w/w*) fat and no cholesterol (Beijing Huafukang Bioscience Co., Ltd., Beijing, China). In addition, in the ApoE$^{-/-}$ HIIT and ApoE$^{-/-}$ MICT groups, there was a 4-day adaptive training session before the start of the training program. The adaptive training program was conducted by running on a treadmill 10 min per day, and the speed was gradually increased every day from 10 cm/s, 12 cm/s, 14 cm/s to 16 cm/s from day 1 to day 4, respectively. Overall, the acclimatization of animals to their housing, chow, and treadmill running lasted one week.

Determination of the Maximal Running Speed on Treadmill

Five ApoE KO mice performed a treadmill running test, which started at 4.8 m/min for 10 min with an incline of 0◦ and the speed was progressively increased 1.2 m/min every 3 min until exhaustion. The exhaustion was judged when the mouse stayed still either for three seconds on the electric grid or received 100 shocks without moving [30]. The last speed was defined as the maximal running speed.

Training Protocols

The HIIT program was described before [30], with slight modification, which consisted of 4 sets of 5 × 10-s sprints with 20 s of rest between each sprint and the interset rest was 5 min. One training session took about 23 min in total. The exercise intensity of the sprint was about 100% of the measured maximal running speed. On the other hand, the ApoE$^{-/-}$ MICT group performed the continuous endurance running for 40 min with a speed at 40% of the determined maximal running speed. All training sessions for the two groups were carried out in the morning, three times per week, for six weeks.

Assessment of Endurance Exercise Performance

The mice in the $ApoE^{-/-}$ HIIT and $ApoE^{-/-}$ MICT groups performed an incremental treadmill running test to exhaustion after the training intervention. The protocol was the same as the one used to determine the maximal running speed. The running distance was recorded as the endurance exercise performance. After the incremental treadmill exercise, the mice rested for at least 48 h. Then they were anesthetized, and blood samples were collected by the percutaneous cardiac puncture. The muscle samples from gastrocnemius, soleus, and extensor digitorum longus (EDL) were removed, cleaned, and quick-frozen in liquid nitrogen, and then stored at −80 ◦C.

Plasma Lipid Profiles

Plasma total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured using the specific assay kits (A111-1-1, A110-1-1, A113-1-1, and A112-1-1, respectively, Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturer's protocols. Changes in absorbance were determined with Bio Tek Synergy H1 (Bio Tek Instruments, Inc., Winooski, VT, USA) at 510, 510, 546, and 546 nm for plasma TC, TG, LDL-C, and HDL-C assays, respectively.

Real-time Quantitative PCR Analysis

Total RNA was isolated from about 50 mg of crushed gastrocnemius muscle the using TRIzol reagent (TaKaRa, Japan) and about 1 μg total RNA was reverse-transcribed to cDNA using a kit (FSQ-101; Toyobo Co., Ltd., Japan) according to the manufacturer's instructions. Besides, total RNA was isolated from about 10 mg of soleus and EDL, respectively, using the RNA Isolation Kit by TransGen Biotech (Beijing, China) and about 1 μg of total RNA was reverse-transcribed to cDNA using the same kit (FSQ-101; Toyobo Co., Ltd., Japan). Moreover, the real-time qPCR was performed in an ABI 7500 Real-time PCR System (Thermo Scientific, Inc.,

Waltham, MA, USA) using the SYBR Green Real-time PCR Master Mix kit (Toyobo Co., Ltd., Osaka, Japan) with the previously synthesized cDNA as a template in a 20 μL reaction volume. Glutamate-cysteine ligase catalytic subunit (*Gclc*; gene ID: 14629; QT00130543), glutathione reductase (*Gsr*; gene ID: 14782; QT01758232), and 18S ribosomal RNA (*Rn18s*; gene ID: 19791; QT02448075) commercial primers from Qiagen (Germany) were used.

In addition, the primer sequences of glutamate-cysteine ligase modifier subunit (*Gclm*), glutathione synthase (*Gss*), NADPH oxidase 2 (*Nox2*) (also called *gp91phox*), neutrophil cytosolic factor 1 (Ncf1) (also called *p47phox*), NADPH oxidase 4 (*Nox4*), fibronectin type III domain containing 5 (*Fndc5*), acyl-CoA dehydrogenase short chain (*Acads*), hydroxyacyl- CoA dehydrogenase trifunctional multienzyme complex subunit alpha (*Hadha*), and hydroxyacyl-coenzyme A dehydrogenase (*Hadh*) were listed in Table 1 and these primers were synthesized by Invitrogen Trading Co., Ltd. (Shanghai, China). The difference in expression between control and experimental samples was calculated using the $2^{-\Delta\Delta Ct}$ method, as described previously [31].

Table 1. Description of primers used for quantitative real-time PCR.

Gene Name	Gene ID	Forward Primer	Reverse Primer
Gclm	14630	5′-AGGAGCTTCGGGACTGTATCC-3′	5′-GGGACATGGTGCATTCCAAAA-3′
Gss	14854	5′-CAAAGCAGGCCATAGACAGGG-3′	5′-AAAAGCGTGAATGGGGCATAC-3′
Gclc	14629	5′-GGGGTGACGAGGTGGAGTA-3′	5′-GTTGGGGTTTGTCCTCTCCC-3′
Gsr	14782	5′-CACGGCTATGCAACATTCGC-3′	5′-GTGTGGAGCGGTAAACTTTTTC-3′
Nox2	13058	5′-TGAATGCCAGAGTCGGGATT-3′	5′-CGAGTCACGGCCACATACA-3′
p47phox	17969	5′-ACACCTTCATTCGCCATATTGC-3′	5′-TCGGTGAATTTTCTGTAGACCAC-3′
Nox4	50490	5′-TCCATCAAGCCAAGATTCTGAG-3′	5′-GGTTTCCAGTCATCCAGTAGAG-3′
Fndc5	384061	5′-TTGCCATCTCTCAGCAGAAGA-3′	5′-GGCCTGCACATGGACGATA-3′
Acads	11409	5′-GACTGGCGACGGTTACACA-3′	5′-GGCAAAGTCACGGCATGTC-3′
Hadha	97212	5′-TGCATTTGCCGCAGCTTTAC-3′	5′-GTTGGCCCAGATTTCGTTCA-3′
Hadh	15107	5′-TGCATTTGCCGCAGCTTTAC-3′	5′-GTTGGCCCAGATTTCGTTCA-3′
Vegfa165	22339	5′-TGCAGGCTGCTGTAACGATG-3′	5′-GAACAAGGCTCACAGTGATTTTCT-3′
MHC-IIa	17886	5′-CAGCTGCACCTTCTCGTTTG-3′	5′-CCCGAAAACGGCCATCT-3′
MHC-IIx	17879	5′-GGACCCACGGTCGAAGTTG-3′	5′-CCCGAAAACGGCCATCT-3′
MHC-IIb	77579	5′-CAATCAGGAACCTTCGGAACAC-3′	5′-GTCCTGGCCTCTGAGAGCAT-3′

The measurement samples for mRNA expression of vascular endothelial growth factor a 165 (*Vegfa165*) were from soleus and EDL, respectively; the samples for mRNA expression of myosin heavy chain *(MHC)-IIa, IIx, and IIb* were from EDL. Their primers were also synthesized by Invitrogen Trading Co., Ltd. (Shanghai, China) and were listed in Table 1.

Western Blotting

Total proteins were isolated from 50 mg of gastrocnemius using RIPA protein extraction reagents (P0013B; Beyotime, Inc., Shanghai, China). Protein concentration was measured using the BCA protein assay kit (Pierce 23225; Thermo Fisher Scientific, Inc.). Twenty micrograms of proteins were separated on Bolt 4–12% Bis-Tris PlusGels (NW04125BOX; Thermo Fisher Scientific, Inc., Waltham, MA, USA.) by electrophoresis, and the fractionated proteins were subsequently transferred to a nitrocellulose membrane using iBlot Gel Trans- fer Stacks Nitrocellulose (IB23001; Thermo Fisher Scientific, Inc.). The blots were probed using the following antibodies: Nuclear factor erythroid-derived 2-like 2 (Nrf2) (1:200, sc-365949; Santa Cruz Biotechnology, Dallas, TX, USA), Ser40-phosphorylated (p)-Nrf2 (P-Nrf2) (1:200, bs-2013R; Bioss, Beijing, China), 4-HNE-modified protein (1:500, ab-46545; Abcam, Cambridge, MA, USA), catalase (CAT) (1:10,000, 66765-1-Ig, Proteintech, Huhan, China), superoxide dismutase 1 (SOD1) (1:500, sc-11407; Santa Cruz Biotechnology, USA), NAD (P)H quinone oxidoreductase 1 (NQO1) (1:500, sc-32793; Santa Cruz Biotechnology, USA), glutathione peroxidase 1 (GPX1) (1:500, ab-108427; Abcam, USA), and β-actin (1:1000, sc-47778, Santa Cruz Biotechnology, Dallas, TX, USA). The density of protein bands was analyzed using Bio-Rad imaging software (Bio-Rad Laboratories, Hercules, CA, USA). The individual values were originally expressed as a ratio of a standard (β-actin content) and then expressed as a fold change of the control group value.

Reactive Oxygen Species (ROS) Generation

Following the manufacturer's instructions, 50 mg of gastrocnemius muscles were homogenized with reagent C in the kit (GMS10016.3; GENMED, Shanghai, China). The supernatants were used to yield ROS samples (2 g protein/L). Then ROS samples were incubated with the chloromethyl derivative (CM-H2DCFDA) of 5-(and-6)-chloromethyl- 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) at 37 ◦C for 20 min in the 96-well plates, and

the ROS levels were detected by a fluorescence plate reader at λexc: 490 nm and λem: 520 nm (Bio Tek Synergy H1, Bio Tek Instruments) as previously described [32].

Glutathione Redox State and Protein Carbonyl Content of the Skeletal Muscle

Glutathione redox state (the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG); GSH/GSSG) was measured from 50 mg of the gastrocnemius muscle by GSH and GSSG commercial kits from Solarbio (BC1175 and BC1185, Beijing, China), according to the manufacturer's protocols. Protein carbonyl content was assayed in the homogenate supernatant of 50 mg of gastrocnemius tissue using the commercial assay kit purchased from Solarbio (BC1275, Beijing, China), according to the manufacturer's instructions.

Plasma Irisin and Muscle Musclin Concentration

Plasma irisin and musclin concentration in the gastrocnemius were assessed according to the manufacturer's instructions with the mouse irisin and musclin ELISA kits (Gene lab., Beijing, China), respectively. The plates were read at 450 nm (Bio Tek Synergy H1, Bio Tek Instruments, Inc., Winooski, VT, USA).

Statistical Analysis

All values were presented as the mean ± standard error (SE). Statistical analyses were performed using SPSS Statistical software V 19.0 (IBMCorp., Armonk, NY, USA). Comparisons between the means of the WT mice and ApoE$^{-/-}$ CON groups were made using the independent sample t-test. The one-way ANOVA was used to analyze the impact of different interventions on the ApoE$^{-/-}$ mice followed by the least significant difference (LSD) post hoc test at $p < 0.05$ level of significance.

Results

Body Weight, Running Distance, and Plasma Lipid Profile

There were no significant differences in body weight and running distance between the ApoE$^{-/-}$ CON group and WT mice and among CON, MICT, and HIIT groups of ApoE$^{-/-}$ mice (Figure 1A, B). However, significantly higher levels of plasma TC, TG, and LDL-C were observed in the ApoE$^{-/-}$ CON group than those of the WT group (Figure 1C–E). Meanwhile, the ApoE$^{-/-}$ HIIT and ApoE$^{-/-}$ MICT groups had significantly lower plasma TC and TG levels, while the ApoE$^{-/-}$ MICT group had a significantly higher plasma HDLC level than those of the ApoE$^{-/-}$ CON group (Figure 1C, D). In addition, the ApoE$^{-/-}$ MICT group had a significantly higher plasma HDL-C level than that of the ApoE$^{-/-}$ CON and ApoE$^{-/-}$ HIIT groups, respectively (Figure 1F).

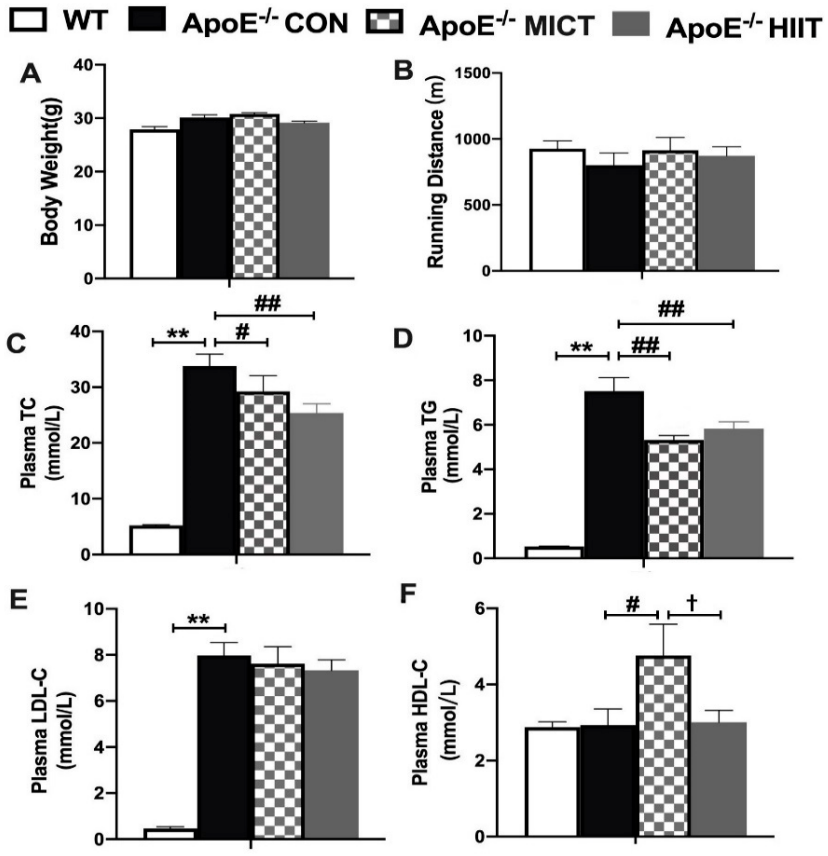

Figure 1. Effects of HIIT and MICT on body weight (A), running distance (B), and plasma lipid

profiles on HFD. Values are displayed as the mean ± SEM (n = 10/group). ** $p < 0.01$ vs. WT; # $p < 0.05$ vs. ApoE$^{-/-}$ CON; ## $p < 0.01$ vs. ApoE$^{-/-}$ CON; † $p < 0.05$ vs. ApoE$^{-/-}$ MICT.

The mRNA Expression of Vegfa165, MyHC-IIa, MyHC-IIx, and MyHC-IIb in Soleus or EDL

The mRNA expression of *Vegfa165* in soleus and EDL was significantly higher, and *MyHC-IIb* in EDL was significantly lower in the ApoE$^{-/-}$ CON group, compared to those of WT mice (Figure 2A, B, E). However, six weeks of the HIIT and MICT induced a significantly lower mRNA expression of *Vegfa165* in EDL, respectively, and the HIIT group had a markedly lower mRNA expressions of *MyHC-IIx* in EDL, compared with those of the Apo KO control mice (Figure 2B, D). Moreover, the mRNA expression of *Vegfa165* and *MyHC-IIx* in EDL of ApoE$^{-/-}$ HIIT mice was significantly lower than those of ApoE$^{-/-}$ MICT mice (Figure 2B, D). There were no significant differences in *MyHC-IIxa* between the ApoE$^{-/-}$ CON group and WT mice and among CON, MICT, and HIIT groups of ApoE$^{-/-}$ mice (Figure 2C).

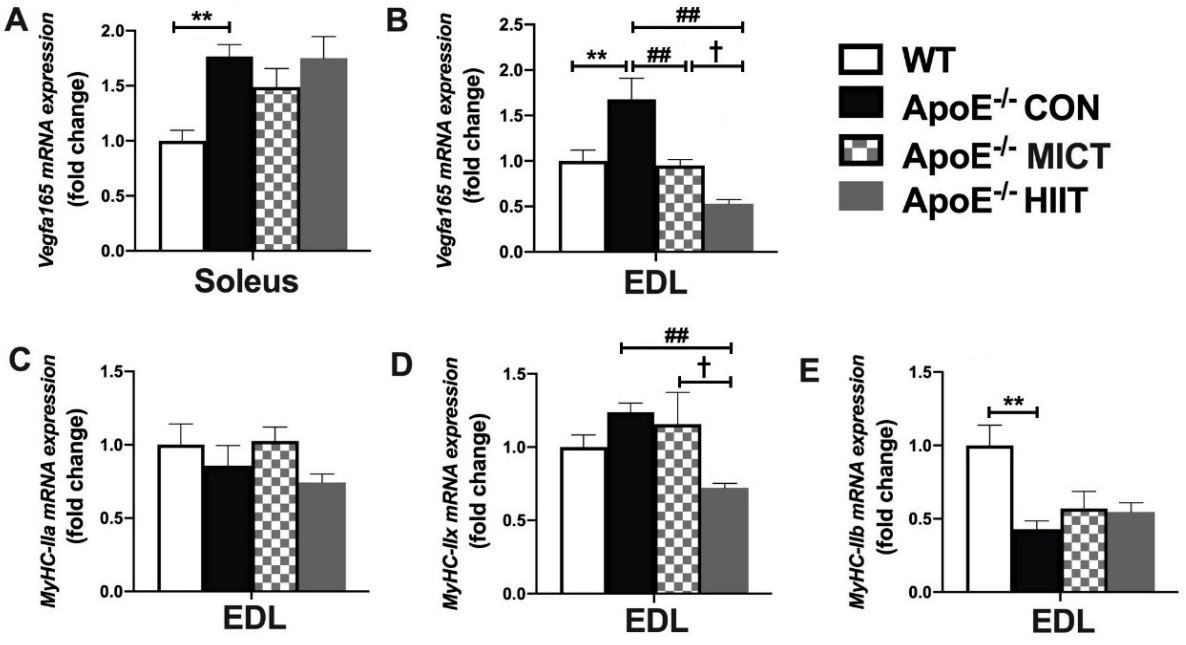

Figure 2. Effects of HIIT and MICT on the mRNA expression of Vegfa165 in soleus (A) and EDL (B), and MyHC-IIa, MyHC-IIx, and MyHC-IIb in EDL (C–E) of WT and ApoE KO mice on HFD. Values are displayed as the mean ± SEM (n = 10/group). ** $p < 0.01$ vs. WT; ## $p < 0.01$ vs. ApoE$^{-/-}$ CON; † $p < 0.05$ vs. ApoE$^{-/-}$ MICT.

Muscle ROS, Protein Carbonyl, 4-HNE Modified Proteins, and the mRNA Expression Levels of Nox2, p47phox and Nox4

There were higher levels of ROS and protein carbonyl and mRNA expression of *Nox2* and *Nox4* in skeletal muscles of ApoE$^{-/-}$ CON mice than those of WT mice (Figure 3A, B, D, F); while the expressions of 4-HNE protein and p47 phox mRNA were not different between the ApoE$^{-/-}$ CON group and WT mice and among CON, MICT, and HIIT groups of ApoE$^{-/-}$ mice (Figure 3C, E). Moreover, the treatments of HIIT and MICT resulted in significantly lower levels of the muscle ROS, protein carbonyl, and mRNA expression of *Nox4*, compared with those of the ApoE$^{-/-}$ CON mice (Figure 3A, B, F). In addition, ROS level was significantly lower and the mRNA expression of *Nox2* and *Nox4* were significantly higher in gastrocnemius muscles of ApoE$^{-/-}$ HIIT mice, compared with those of ApoE$^{-/-}$ MICT mice (Figure 3A, D, F).

The mRNA Expression of Genes Involved in the Production of GSH, GSH, GSSG Levels, and GSH/GSSG Ratio

The mRNA expression of genes (*Gsr* and *Gclm*) involved in the production of GSH was significantly lower in the skeletal muscle of ApoE$^{-/-}$ CON mice, compared to those of the WT mice (Figure 4B, D). Moreover, the treatment of HIIT and MICT produced significantly higher expression in all measured genes involved in the production of GSH (*Gss*, *Gsr*, *Gclc*, and *Gclm*) in the skeletal muscle, compared with those of the Apo KO control mice (Figure 4A–D). The lower level of GSH and the higher level of GSSH resulted in a reduced GSH/GSSG ratio in the ApoE$^{-/-}$ CON group, compared to those of the WT mice (Figure 4E–G). Moreover, the HIIT and MICT treatments did not exhibit a significant difference in the GSH/GSSG ratio, compared with that of the ApoE$^{-/-}$ CON group, although the GSH levels were increased in the ApoE$^{-/-}$ HIIT and ApoE$^{-/-}$ MICT groups, and GSSG level was decreased in the ApoE$^{-/-}$ HIIT group (Figure 4E–G). In addition, there was no significant difference in these measured indexes between ApoE$^{-/-}$ HIIT and ApoE$^{-/-}$ MICT mice (Figure 4A–G).

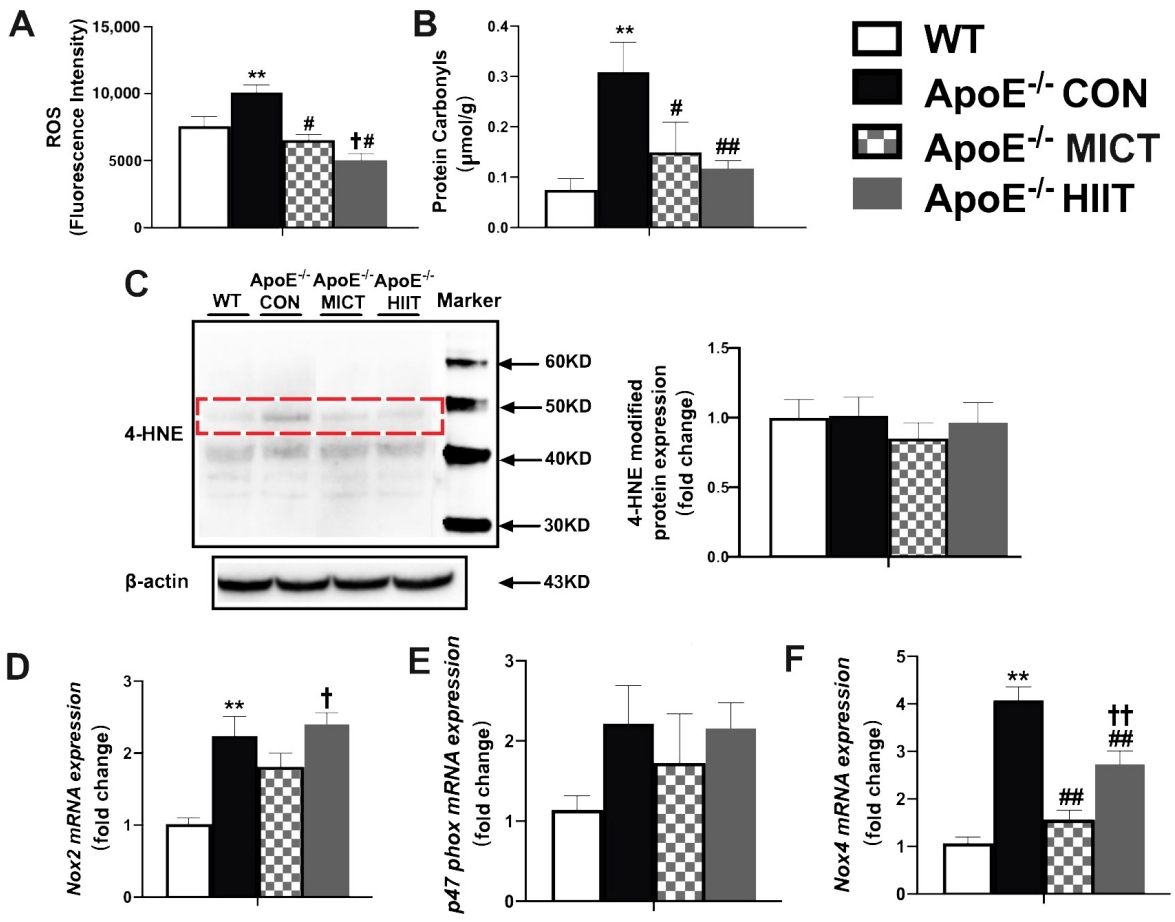

Figure 3. Effects of HIIT and MICT on ROS generation (A), protein carbonyl (B), 4-HNE-modified proteins (C), and the mRNA expression levels of *Nox2*, *P47phox*, and *Nox4* (D–F) in skeletal muscles of WT and ApoE KO mice with HFD. Values are displayed as the mean ± SEM (n = 10/group). ** $p < 0.01$ vs. WT; # $p < 0.05$ vs. ApoE$^{-/-}$ CON; ## $p < 0.01$ vs. ApoE$^{-/-}$ CON; † $p < 0.05$vs. ApoE$^{-/-}$ MICT; †† $p < 0.01$ vs. ApoE$^{-/-}$ MICT.

The Protein Expression of Nrf2, p-Nrf2 (ser40), and Antioxidants

There were no significant differences in the protein expression of Nrf2, p-Nrf2 (ser40), and all measured antioxidants between ApoE$^{-/-}$ CON and WT mice (Figure 5A-F). Moreover, there was significantly higher protein expression of p-Nrf2 (ser40) and GPX1 in ApoE$^{-/-}$ HIIT mice, and a prominently higher protein expression of NQO1 in ApoE$^{-/-}$ MICT mice, compared with ApoE$^{-/-}$ CON mice, respectively (Figure 5B, D, E). There was no significant difference in these measured protein expression levels between ApoE$^{-/-}$ HIIT and ApoE$^{-/-}$ MICT (Figure 5A-F).

The mRNA Expression of Fndc5, Hadh, Acads, and Hadha in Gastrocnemius, Plasma Irisin Level, as well as Muscle Musclin Concentration

There was no significant difference in the mRNA expression of *Fndc5*, *Hadh*, *Acads*, and *Hadha*, and musclin content in the gastrocnemius, as well as plasma irisin level between the WT and ApoE$^{-/-}$ CON groups (Figure 6A-F). However, significantly higher mRNA expression of *Fndc5* (Figure 6A), *Hadh*, and *Hadha* in the gastrocnemius (Figure 6D, F), as well as plasma irisin level (Figure 6B), were observed in the ApoE$^{-/-}$ MICT and ApoE$^{-/-}$ HIIT groups than those of the ApoE$^{-/-}$ CON group. Meanwhile, there was a significantly higher musclin level of the gastrocnemius of ApoE$^{-/-}$ MICT than that of the ApoE$^{-/-}$ CON group (Figure 6C). In addition, there was a significantly higher mRNA expression of *Hadha* in the gastrocnemius of ApoE$^{-/-}$ HIIT mice than that of ApoE$^{-/-}$ MICT mice (Figure 6F).

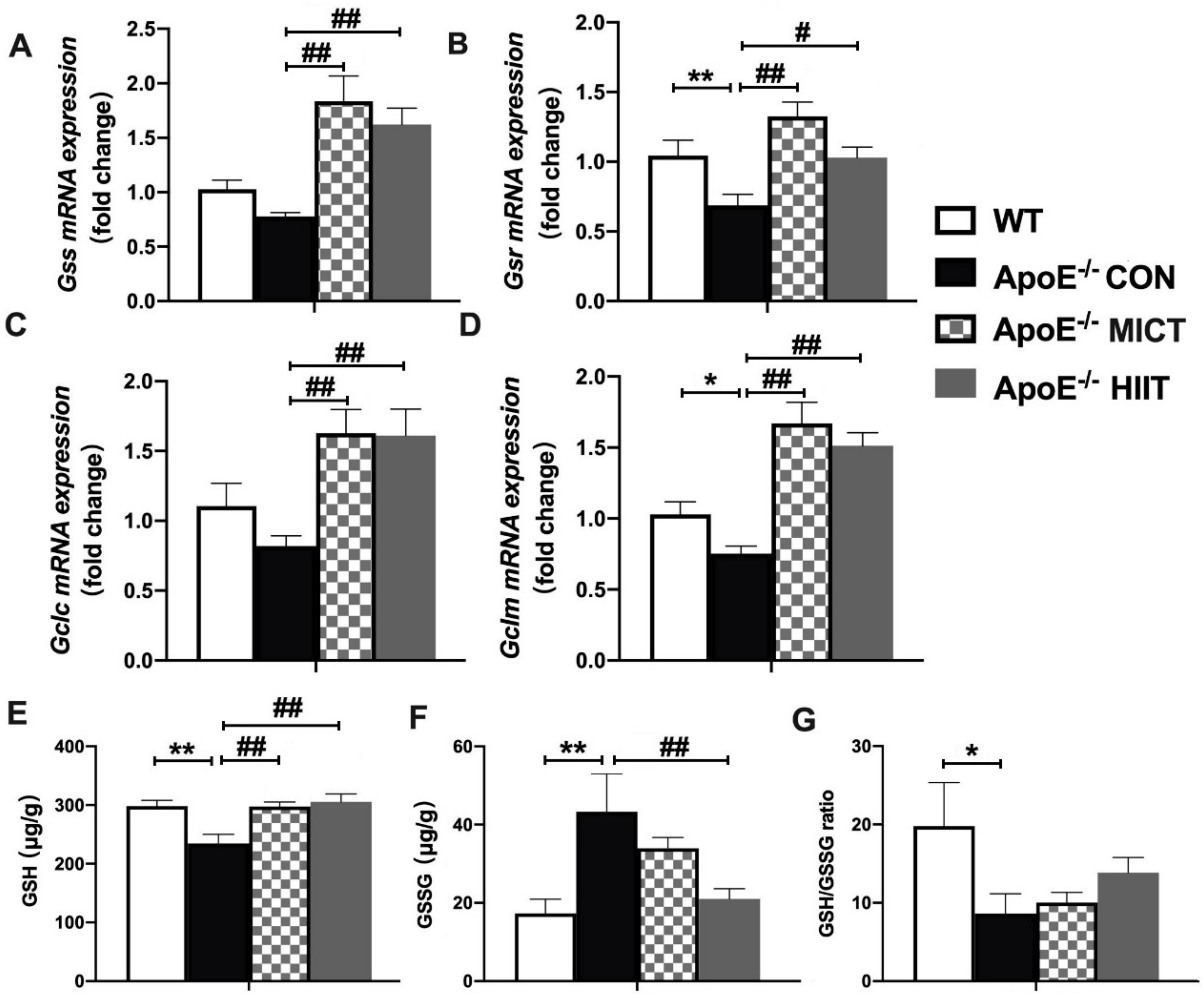

Figure 4. Effects of HIIT and MICT on the mRNA expression of genes involved in the production of GSH (A–D), GSH (E), GSSG (F) levels, and GSH/GSSG ratio (G) in skeletal muscles of WT and ApoE KO mice with a high-fat diet. Values are displayed as the mean ± SEM (n = 10/group). * $p < 0.05$ vs. WT; ** $p < 0.01$ vs. WT; # $p < 0.05$ vs. ApoE$^{-/-}$ CON; ## $p < 0.01$ vs. ApoE$^{-/-}$ CON.

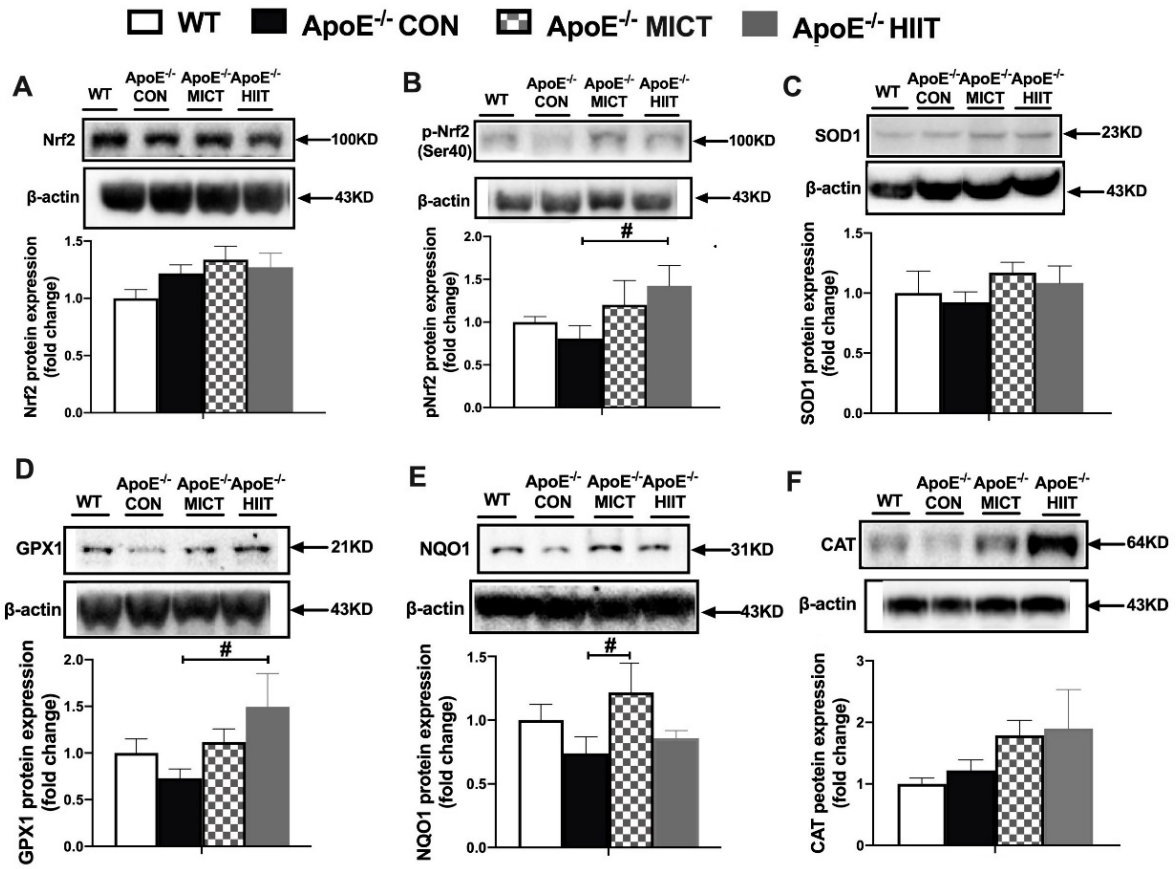

Figure 5. Effects of HIIT and MICT on the protein expression of Nrf2 (A), p-Nrf2 (ser40) (B), and antioxidants (C-F) in skeletal muscles of WT and ApoE KO mice with HFD. Values are displayed as the mean ± SEM (n = 10 / group). # $p < 0.05$ vs $ApoE^{-/-}$ CON.

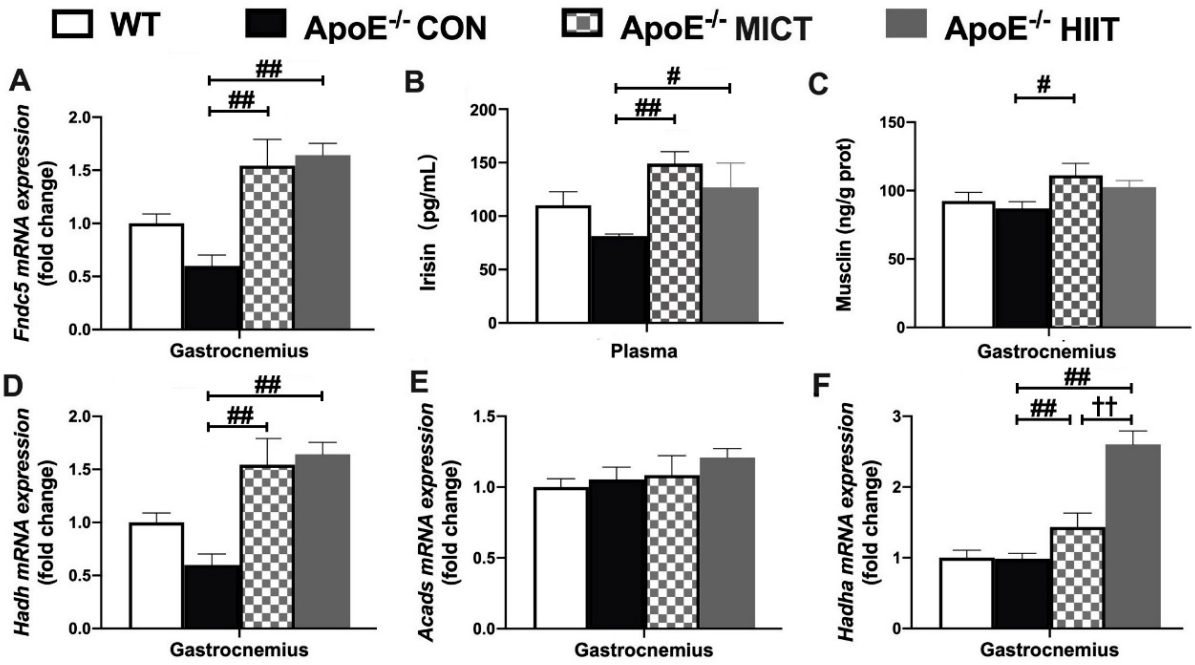

Figure 6. Effects of HIIT and MICT on the mRNA expression of Fndc5 (A), Hadh, Acads, and Hadha (D-F), and musclin level (C) in the gastrocnemius as well as plasma irisin level (B) of WT and ApoE KO mice with HFD. Values are displayed as the mean ± SEM (n = 10 / group). $^{\#}$ $p < 0.05$ vs. ApoE$^{-/-}$ CON; $^{\#\#}$ $p < 0.01$ vs. ApoE$^{-/-}$ CON; †† $p < 0.01$ vs. ApoE$^{-/-}$ MICT.

Discussion

The main findings revealed that concurrent 6-week HIIT and MICT protocols improved blood lipid profiles, counteracted ROS production and protein carbonylation in the gastrocnemius muscle and decreased the mRNA level of the angiogenic gene *Vegfa165* in the EDL muscle. At the same time, both HIIT and MICT enhanced the GSH generation and potently promoted mRNA expression of genes involved in the production of irisin and BAIBA in the gastrocnemius muscle of ApoE KO mice. Comparison of the two training outcomes indicated that HIIT was more efficient than MICT in decreasing the ROS level of the skeletal muscle, whereas MICT was more efficient in increasing the plasma HDL-C level. To our knowledge, this is the first study to report and compare that HIIT and MICT, as potential adjuvant treatments, can attenuate oxidative damage and promote the myokine response in skeletal muscles of ApoE KO mice on HFD. These results support the hypothesis of the present study.

Our results of body weight and blood lipid profiles were in line with previous studies with ApoE KO mice on HFD exhibiting significant increases in plasma TC, TG, and LDL-C levels [33] but not becoming obese [24,34]. This may come from both lower synthesis and increased hydrolysis of triacylglycerols from the $ApoE^{-/-}$ adipocytes [34,35]. It confirms that this mouse model is a valid model of hyperlipidemia. The present study also provided relevant evidence that ApoE KO with HFD resulted in increased mRNA expression of *Vegfa165*, and decreased mRNA expression of *MyHC-IIb*, especially in EDL. These changes in the skeletal muscle represent a functional adaptation to a hyperlipidemic environment or compensation for the excess fat. Furthermore, the ROS production and protein carbonyl in the $ApoE^{-/-}$ CON group were higher, and the value of GSH generation was lower as compared with the WT group, confirming that hyperlipidemia increases oxidative stress in the skeletal muscle. This finding was further supported by the evidence of increased mRNA expression of Noxs genes (*Nox2*, *p47 phox*, and *Nox4*) in response to ApoE KO on HFD. The $ApoE^{-/-}$ CON mice of the present study showed increased markers of muscular oxidative stress, the same as the mouse model

of the previous studies [16,24]. However, our ApoE$^{-/-}$ CON mice did not have a higher level of 4-HNE-modified proteins (the end products of lipid peroxidation) in the skeletal muscle, compared to that of the WT mice.

It is well-known that regular exercise has preventive effects on various organs of atherosclerosis prone ApoE KO mice [33,36]. However, until now, there were no reports about the benefits of exercise on the skeletal muscle of ApoE KO mice on HFD. Investigating the modalities of exercise treatment (i.e., exercise duration and exercise intensity) is therefore paramount when evaluating its effects. In the present study, we applied HIIT and MICT programs and found that both of them improved plasma lipid profiles and counteracted the compensatory enhanced EDL capillarization caused by ApoE KO with HFD. Importantly, while ApoE$^{-/-}$ CON mice impaired muscle redox homeostasis, the two training programs did attenuate the oxidative damage as shown by decreased ROS production, protein carbonyl content, and mRNA expression of *Nox4*, and also increased mRNA expression of genes involved in GSH production, GSH level, and the protein expression of some antioxidase in the skeletal muscle of these mice. This means that the two training modalities could induce adaptive responses, which were beneficial for the organism.

We found that ROS level was remarkably lower in HIIT mice than MICT mice, which implied that the magnitude of training adaptation was in part dependent upon exercise intensity, so that higher training intensities induced greater changes in the antioxidant defense [37], although there were no significant differences in measured variables of pro/antioxidant balance, including the levels of protein carbonyl, 4-HNE, GSH, and the protein expression of some antioxidase in the skeletal muscle between the two groups. Even for the Noxs, a key ROS generator during muscle contractions, the mRNA expression of *Nox2* and *Nox4* was significantly higher in ApoE$^{-/-}$ HIIT mice than ApoE$^{-/-}$ MICT mice. It indicated that in the skeletal muscle of HIIT mice, the antioxidant system could rapidly remove ROS before they caused cellular dysfunction and was more robust than that of the MICT group. As shown in Figure 5, HIIT mice had a significantly higher protein expression of p-Nrf2 (ser40) in the skeletal muscle,

whereas MICT mice did not, compared with the ApoE$^{-/-}$ CON group. Therefore, based on the current results, we speculated that the lower ROS level could be closely linked to the higher protein expression of p-Nrf2 (ser40) in HIIT mice. However, there might be other proteins and muscle antioxidant enzymes, other than our measured antioxidants, involved in reducing the muscle ROS level of the HIIT group. Further research is needed to address them.

Furthermore, in the present study, it was found that the mRNA expression of *MyHCIIx* and *Vegfa165* in EDL of ApoE$^{-/-}$ HIIT mice was significantly lower than those of ApoE$^{-/-}$ MICT mice. This result may suggest that HIIT could have more potent effects on the resistance to the transition to slower myofibers and enhanced capillarization caused by ApoE deficiency and HFD. However, it was interesting to note that HIIT was not superior to MICT in altering blood lipids of ApoE KO mice on HFD, especially in the change of HDL-C. The change of HDL-C seems to be sensitive to training volume rather than exercise intensity.

Analyses of the skeletal muscle secretome revealed that numerous myokines are produced in response to muscle contraction, and then these factors not only regulate energy demand, but also contribute to the broad beneficial effects of exercise [27]. Myokines may be useful biomarkers for monitoring exercise prescription [38]. It has been reported that endurance exercise training upregulates peroxisome proliferator-activated receptor coactivator 1 (PGC-1) in the skeletal muscle [39,40] and the PGC-1 overexpression in the skeletal muscle increases the production of Fndc5, a precursor form of irisin, and irisin then stimulates the transformation of white adipose tissue to brown adipose tissue [41]. A prospective population-based study showed that higher serum irisin levels are associated with lower prevalence and progression of coronary atherosclerosis [42]. Protective effects of irisin on atherosclerosis were reported in two different ApoE KO mouse models [43,44]. BAIBA was also revealed to induce browning of the white adipocyte and stimulate hepatic oxidation. In humans, plasma BAIBA levels were increased with exercise and inversely associated with metabolic risk factors, such as fasting glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), and the levels of TG and TC [45]. In addition, musclin is an exercise-stimulated myokine [46]

and its expression level is tightly regulated by nutritional changes, and its physiological role could be linked to glucose metabolism [47]. In the present study, we did not find significant changes in the mRNA expression of *Fndc5* and genes required for BAIBA biosynthesis (*Hadh*, *Acads*, and *Hadha*), musclin content in the skeletal muscle, and plasma irisin level between ApoE$^{-/-}$ CON and WT mice. Meanwhile, the mRNA expression of *Fndc5*, *Hadh*, and *Hadha*, and musclin content in the skeletal muscle and blood irisin were upregulated in response to HIIT or MICT. Our results are in accordance with previous research that showed that muscle contraction stimulated myokine (irisin, BAIBA, and musclin) production [46,48,49]. However, it is worth noting that the mRNA expression of *Hadha*, the key gene involved in BAIBA biosynthesis, in the skeletal muscle of HIIT mice, was significantly higher than that in the MICT group. Since few studies have compared the effects of HIIT and MICT training programs on BAIBA production in muscle tissue, it was only speculated that the HIIT could be superior to MICT in BAIBA production, although BAIBA content in the skeletal muscle was not measured directly in the present study.

There are some limitations in this study. We only investigated the impacts of HIIT and MICT on ApoE KO mice with HFD for six weeks. Future studies should consider a longer duration, such as 12 weeks. We also only investigated the mRNA expression of many genes, such as *MyHC-IIa*, *MyHC-IIx*, and *MyHC-IIb*, but the immunohistochemical staining or Western blots would provide further results on the morphological changes. In addition, we focused on the changes in protein expression of antioxidant enzymes in the skeletal muscle, but we did not measure the possible changes in their activity. Further studies are also needed to determine whether antioxidant enzyme activity changes for a comprehensive evaluation of the pro-/anti-oxidant balance.

Conclusions

Six weeks of HIIT or MICT programs exerted beneficial effects on ApoE KO mice on HFD by attenuating oxidative damage and promoting myokines production in the skeletal muscle. Both training modalities could decrease plasma TC and TG levels, ROS production, and protein carbonylation in the skeletal muscle; simultaneously, they increased GSH generation and mRNA expression of genes involved in the production of irisin and BAIBA. Furthermore, HIIT was more beneficial than MICT for reducing the ROS level in the skeletal muscle.

Author Contributions

G.P.M. and M.P. designed the experiment; L.W. and J.L. performed experiments and they contributed equally to this work as joint first authors; W.H., Y.W., L.G., and H.W. helped with experiments; L.W. and Y.W. analyzed data; Y.Z. wrote the manuscript; J.W., G.P.M., and M.P. edited and revised manuscript; G.P.M. and Y.Z. obtained funding. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Institute of Sport Sciences of the University of Lausanne for the ApoE KO mice with high-fat diet experiments; and the Animal Care and Use Committee of Beijing Sport University for the WT mice and laboratory experiments (2020ZJ007).

Informed Consent Statement

Not applicable.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interest

The authors declare no conflict of interest.

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Chapter 9

Annex 2 – Ma thèse en 180 secondes

Annex 2 – Ma thèse en 180 secondes

Le concours « Ma thèse en 180 secondes » est un concours de communication et vulgarisation inspiré du concours australien « Three minute thesis ». Ce sont les Québécois qui ont initié le premier concours francophone en 2012. En 2016, la CUSO a coordonné, pour la première fois, la participation de la Suisse au concours.

Le concours est ouvert à tous les doctorantes et les doctorants. Les participant·e·s doivent présenter, en français, leur sujet de recherche en termes simples et convaincants, à l'aide d'une seule diapositive, et devant un auditoire profane, provenant de divers horizons. Pour gagner, il s'agit de se montrer clair, concis, enthousiaste, communicatif, quels que soient la discipline et le sujet que l'on a choisis.

Une compétition interne dans les hautes écoles universitaires participantes (UNIFR, UNIGE, UNIL, UNINE, EPFL, UZH) est organisée. Au terme de ces concours, un à trois lauréat-e-s par établissement sont sélectionné·e·s pour participer à la seconde phase : la finale nationale du concours. Le ou la gagnant·e de cette finale suisse est ensuite sélectionné·e pour représenter son pays lors de la finale internationale du concours qui réunit une vingtaine de concurrent-e-s issu-e-s de différents pays.

Photo: © ChristMarcelle

LAVIER Jessica

UNIVERSITÉ DE Lausanne
Faculté de Biologie et Médecine
sous la direction du Prof. Grégoire Millet (co-directeur Maxime Pellegrin)

VIDÉO

https://www.youtube.com/watch?v=Cs7XLikzbx0
20:05

«Effets thérapeutiques de l'exercice à différentes intensités et en hypoxie chez des modèles murins de pathologies humaines»

Jessica est entrée à l'Université de Lausanne en 2010, où elle a obtenu un Bachelor en Biologie puis un Master en Biologie Médicale avec un projet de mémoire sur la thématique « exercice et maladies cardiovasculaires ». Suite à ça, elle se lance dans un doctorat pour poursuivre ses recherches dans cette thématique sport/santé. Le sport, elle en fait une thèse, mais c'est également une passion. En effet Jessica pratique le karaté depuis son plus jeune âge et en 2015 elle obtient le Prix des Sports de l'Université de Lausanne pour s'être distinguée par sa pratique sportive.