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THE REGULATION OF SKELETAL AND CARDIAC MUSCLE BLOOD FLOW IN HUMANS

Studies by positron emission tomography with special reference to exercise, adenosine and nitric oxide

by

Ilkka Heinonen

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From Turku PET Centre and the Department of Clinical Physiology and Nuclear Medicine, University of Turku, Turku Finland

Supervised by

Adjunct Professor Kari Kalliokoski, PhD
Turku PET Centre
University of Turku
Turku, Finland

and

Assistant Professor Robert Boushel, DSc
Department of Biomedical Sciences, Centre for Healthy Aging
University of Copenhagen
Copenhagen, Denmark

Reviewed by

Professor Russell Richardson, PhD
Center on Aging, University of Utah
Salt Lake City, Utah, USA

and

Professor Dirk Duncker, MD, PhD
Experimental Cardiology, Thoraxcenter, Erasmus MC Cardiovascular
Research Institute, University Medical Center Rotterdam
Rotterdam, Netherlands

Dissertation opponent

Professor Michael Joyner, MD
Mayo Clinic
Rochester, Minnesota,USA

To my family

ABSTRACT

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Turku PET Centre and the Department of Clinical Physiology and Nuclear Medicine,
University of Turku, Turku, Finland

Virtually every cell and organ in the human body is dependent on a proper oxygen supply. This is taken care of by the cardiovascular system that supplies tissues with oxygen precisely according to their metabolic needs. Physical exercise is one of the most demanding challenges the human circulatory system can face. During exercise skeletal muscle blood flow can easily increase some 20-fold and its proper distribution to and within muscles is of importance for optimal oxygen delivery. The local regulation of skeletal muscle blood flow during exercise remains little understood, but adenosine and nitric oxide may take part in this process. In addition to acute exercise, long-term vigorous physical conditioning also induces changes in the cardiovasculature, which leads to improved maximal physical performance. The changes are largely central, such as structural and functional changes in the heart. The function and reserve of the heart's own vasculature can be studied by adenosine infusion, which according to animal studies evokes vasodilation via its A_{2A} receptors. This has, however, never been addressed in humans in vivo and also studies in endurance athletes have shown inconsistent results regarding the effects of sport training on myocardial blood flow. This study was performed on healthy young adults and endurance athletes and local skeletal and cardiac muscle blod flow was measured by positron emission tomography. In the heart, myocardial blood flow reserve and adenosine A_{2A} receptor density, and in skeletal muscle, oxygen extraction and consumption was also measured. The role of adenosine in the control of skeletal muscle blood flow during exercise, and its vasodilator effects, were addressed by infusing competitive inhibitors and adenosine into the femoral artery. The formation of skeletal muscle nitric oxide was also inhibited by a drug, with and without prostanoid blockade. As a result and conclusion, it can be said that skeletal muscle blood flow heterogeneity decreases with increasing exercise intensity most likely due to increased vascular unit recruitment, but exercise hyperemia is a very complex phenomenon that cannot be mimicked by pharmacological infusions, and no single regulator factor (e.g. adenosine or nitric oxide) accounts for a significant part of exercise-induced muscle hyperemia. However, in the present study it was observed for the first time in humans that nitric oxide is not only important regulator of the basal level of muscle blood flow, but also oxygen consumption, and together with prostanoids affects muscle blood flow and oxygen consumption during exercise. Finally, even vigorous endurance training does not seem to lead to supranormal myocardial blood flow reserve, and also other receptors than A_{2A} mediate the vasodilator effects of adenosine. In respect to cardiac work, atheletes heart seems to be luxuriously perfused at rest, which may result from reduced oxygen extraction or impaired efficiency due to pronouncedly enhanced myocardial mass developed to excel in strenuous exercise.

Keywords: skeletal muscle, myocardium, blood flow, exercise, adenosine, nitric oxide

TIIVISTELMÄ

Ilkka Heinonen

LUURANKO- JA SYDÄNLIHAKSEN VERENVIRTAUKSEN SÄÄTELY IHMISELLÄ
Tutkimuksia positroniemissiotomografialla erityishuomiona liikunnan, adenosiinin ja typpioksidin vaikutukset

Lähes kaikkien elimistön solujen ja elinten normaali toiminta on riippuvainen hyvästä hapensaannista. Hapenkuljetuksesta huolehtii verenkiertoelimistö, joka kierrättää verta kudoksiin tarkasti niiden tarpeiden mukaan. Fyysinen rasitus on verenkierrollisesti haastavimpia tilanteita, joita ihmisen elimistö voi kohdata. Fyysisessä rasituksessa lihaksen verenvirtaus voi helposti nousta jopa 20-kertaiseksi ja sen jakautuminen lihaksissa on tärkeää optimaalisen hapensaannin kannalta. Lihasten verenvirtauksen paikallinen säätely ihmisillä rasituksessa ei ole kuitenkaan vielä hyvin selvillä, mutta adenosiini ja typpioksidi saattavat osallistua tähän prosessiin. Akuutin rasituksen lisäksi myös pitkäaikainen tehokas liikunta- tai urheiluharjoittelu aiheuttaa muutoksia verenkiertoelimistössä ja suorituskyky paranee. Muutokset ovat paljolti keskeiseverenkierrollisia, kuten rakenteelliset ja toiminnalliset muutokset sydämessä. Sydämen oman verenkierron toimintaa ja reserviä voidaan tutkia adenosiini-infuusiolla, joka eläintutkimusten mukaan aiheuttaa verisuonten laajenemisen A_{2A} reseptoreidensa kautta. Tätä ei ole kuitenkaan selvitetty aikaisemmin ihmisillä ja myös sydämen verenvirtaustutkimukset kestävyysurheilijoilla ovat johtaneet ristiriitaisiin tuloksiin urheiluharjoittelun vaikutuksista sydämen verenvirtaukseen. Tässä terveillä aikuisilla ja kestävyysurheilijoilla suoritetussa tutkimuksessa luurankolihaksen ja sydämen paikallista verenvirtausta mitattiin posintroniemissiotomografialla. Sydämestä määritettiin lisäksi verenvirtausreservi ja adenosiini A_{2A} reseptoritiheys, ja luurankolihaksen kohdalla mitattiin hapen irtoamista verestä sekä hapenkulutusta. Adenosiinin roolia luurankolihaksen verenvirtauksessa tutkittiin estämällä lääkeaineilla sen normaalia toimintaa ja infusoimalla adenosiinia suoraan reisivaltimoon. Luurankolihaksen typpioksidin muodostuminen estettiin myös lääkeaineella, yksin ja yhdessä prostanoidien muodostuksen eston yhteydessä. Tutkimuksen tuloksina ja johtopäätöksinä voidaan todeta, että rasituksessa lihaksen verenvirtauksen heterogeenisyys vähenee intensiteetin mukaisesti johtuen todennäköisesti uusien kapillaariyksiköiden rekrytoinnista, mutta luonnollista rasituksenkaltaista verenvirtausta ei voida jäljitellä lääkeaineilla ja mikään yksittäinen tekijä (adenosiini tai typpioksidi) ei yksin ole vastuussa verenvirtauksen säätelystä rasituksessa. Typpioksidi on kuitenkin merkittävä lihaksen verenvirtauksen säätelijä levossa, ja rasituksessa yhdessä prostanoidien kanssa. Tässä tutkimuksessa ensimmäistä kertaa ihmisillä sen havaittiin vaikuttavan myös lihaksen lepotilan hapenkulutukseen, ja rasituksen hapenkulutukseen yhdysvaikutuksena prostanoidien kanssa. Kovakaan kestävyysharjoittelu ei kuitenkaan näytä johtavan ylinormaaliin sydämen verenvirtausreserviin ja todennäköisesti myös muut adenosiinireseptorit kuin A_{2A} välittävät adenosiinin verisuonivaikutuksia. Urheilijan sydämessä näyttää kuitenkin virtaavan levossa enemmän verta suhteessa työtasoon, joka voi johtua vähäisemmästä hapen irtoamisesta verestä, tai epätaloudellisuudesta johtuen kovaa rasitusta kestämään rakentuneesta paksusta sydänlihaksesta.

TABLE OF CONTENTS

ABBREVIATIONS

ADO	Adenosine
$[^{15}O]H_2O$	$[^{15}O]$-water
$[^{15}O]O_2$	$[^{15}O]$-oxygen
A	Peak atrial flow velocity
BMI	Body mass index
E	Early peak flow velocity
ECG	Electrocardiography
ECHO	Echocardiography
HCM	Hypertrophic cardiomyopathy
HR	Heart rate
LV	Left ventricle
LVEF	Left ventricular ejection fraction
MAP	Mean arterial pressure
MBF	Myocardial blood flow
MRI	Magnetic resonance imaging
NO	Nitric oxide
PET	Positron emission tomography
QF	Quadriceps femoris
RF	Rectus femoris
ROI	Region of interest
RPP	Rate pressure product
SD	Standard deviation
SV	Stroke volume
VI	Vastus intermedius
VL	Vastus lateralis
VM	Vastus medialis
VO_{2max}	Maximal oxygen uptake

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, which are referred to the text by the corresponding Roman numerals, I – V.

I   Heinonen Ilkka, Nesterov Serge V, Kemppainen Jukka, Nuutila Pirjo, Knuuti Juhani, Laitio Ruut, Kjaer Michael, Boushel Robert, Kalliokoski Kari K. Role of adenosine in regulating the heterogeneity of skeletal muscle blood flow during exercise in humans . *J Appl Physiol*, Dec 2007; 103: 2042 - 2048.

II Heinonen Ilkka, Nesterov Serge V, Liukko Kaisa, Kemppainen Jukka, Någren Kjell, Luotolahti Matti, Virsu Pauliina, Oikonen Vesa, Nuutila Pirjo, Kujala Urho M, Kainulainen Heikki, Boushel Robert, Knuuti Juhani, Kalliokoski Kari K. Myocardial blood flow and adenosine A_{2A} receptor density in endurance athletes and untrained men. *Journal of Physiology* 2008, 586.21: 5193-5202.

III Heinonen Ilkka, Kemppainen Jukka, Kaskinoro Kimmo, Peltonen Juha E., Borra Ronald, Lindroos Markus, Oikonen Vesa, Nuutila Pirjo, Knuuti Juhani, Hellsten Ylva, Boushel Robert, Kalliokoski Kari K. The comparison of exogenous adenosine and voluntary exercise on human skeletal muscle perfusion and perfusion heterogeneity. *J Appl Physiol,* Feb 2010; 108: 378-386.

IV Heinonen Ilkka, Kemppainen Jukka, Kaskinoro Kimmo, Peltonen Juha E., Borra Ronald, Lindroos Markus, Oikonen Vesa, Nuutila Pirjo, Knuuti Juhani, Boushel Robert, Kalliokoski Kari K. Regulation of skeletal muscle perfusion and its heterogeneity during hypoxic exercise in humans. *Submitted.*

V   Heinonen Ilkka, Saltin Bengt, Kemppainen Jukka, Sipilä Hannu, Oikonen Vesa, Nuutila Pirjo, Knuuti Juhani, Kalliokoski Kari K. Hellsten Ylva. The effects of nitric oxide and cyclooxygenase inhibitions on human skeletal muscle blood flow and oxygen consumption at rest and during exercise: a PET study. *Submitted.*

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1   INTRODUCTION

The regulation of blood flow has a very rich history of investigation, but still the control of cardiac and skeletal muscle blood flow in humans is little understood. Since virtually every organ in a body relies on the aerobic metabolism and is thus critically dependent on a continuous supply of oxygen, the proper function of the cardiovascular system and the precise regulation of blood flow are of great importance, especially in tissues with high metabolic demand, such as in cardiac and skeletal muscle during physical exercise. It is, however, not sufficient to supply oxygen to the tissues as a whole, but it has to also be distributed properly to precisely match the local metabolic demand of the working muscle. Indeed, blood flow heterogeneity is a functionally important paramenter that can affect matching oxygen supply to tissue metabolism and nutrient exchange (Duling & Damon, 1987). Both previous animal and human studies have shown that skeletal muscle blood flow is heterogeneously distributed, but it is basically largely unknown how muscle blood flow heterogeneity is regulated in humans, especially in response to voluntary exercise. One of the main aims of the present thesis is to elucidate this aspect in human muscle blood flow regulation.

Nobel Laurate Szent-Gyorgyi with his coworker Drury showed as early as 1929 that adenosine and related compounds among others, dilate the coronary arteries, but it was Robert Berne, who in the 1960s, really advanced the concept that adenosine is a metabolic regulator that is activated especially in ischemic and hypoxic conditions to restore oxygen supply back to proper levels. It is currently acknowleged that metabolic vasodilation serves as a link between the cellular metabolism and oxygen supply, but the physiological importance of the adenosine hypothesis especially in skeletal muscle remains largely undefined in humans. As a metabolic signal, adenosine could however account for exercise-induced blood flow increase and also affect the heterogeneity of muscle blood flow, and this was therefore addressed in the present thesis.

Reduced heart rate is perhaps one of the most obvious signs of physical conditioning and indicates cardiac adaptation to chronically increased levels of physical activity. The most pronounced cardiac adaptations are seen in endurance athletes whose heart regularly faces both volume and pressure work in training and competitions with large muscle groups, such as in cross-country skiing. In these individuals the athlete's heart is frequently observed, which typically means increased cavity dimensions and thicker cardiac walls. However, despite these structural adaptations being rather well elucidated in humans, studies on functional adaptations in the cardiac vasculature and blood flow have proved inconclusive. For instance, studies that have addressed the myocardial blood flow reserve in endurance athletes have given rather inconsistent results (Radvan *et al.*, 1997;Toraa *et al.*, 1999;Kalliokoski *et al.*, 2002;Kjaer *et al.*, 2005). Myocardial blood flow reserve is usually studied by pharmacological means, typically with adenosine infusion that induces maximal or near maximal cardiac blood flow. In addition to degree of cardiac hypertrophy, the density of adenosine A_{2A} receptors may have affected the inconsistencies in myocardial blood flow results. A large body of animal studies suggests that out of four G-protein coupled cell membrane adenosine receptors, A_{2A} receptors are the primarily pathway

by which adenosine-induced blood flow is mediated. This has, however, never been investigated by any means in humans in vivo.

In addition to adenosine, it is currently well established that limb blood flow (Vallance *et al.*, 1989) and blood pressure (Rees *et al.*, 1989) are largely dependent on the formation and actions of nitric oxide (NO). There are three forms of nitric oxide synthases, and while resting blood flow is solely dependent on neuronal-derived NO, increased blood flow-induced arterial vessel dilatation depends on endothelial-derived NO (Seddon *et al.*, 2008). Despite NO accounting for a large part of blood flow regulation in various physiological conditions and its reduced bioavailability explaining vascular impairments in several diseases, in the exercising limb NO seems to have only a minor if any role (Tschakovsky & Joyner, 2008), even if blood flow can easily increase some 10-20-fold. However, synergistically with prostanoids NO importantly affects limb blood flow, although their effect on sole exercising muscle blood flow remains unexplored. Moreover, during the last decade, many in vitro studies have suggested that NO could also directly affect cellular aerobic respiration, and with respect to oxygen, the primary effect of NO on mitochondrial activity is reversible and competitive inhibition of cytochrome oxidase activity (Brown, 1999;Brown, 2001;Moncada & Erusalimsky, 2002;Erusalimsky & Moncada, 2007;Cooper & Giulivi, 2007). Although some animal studies have subsequently found evidence that NO indeed would tonically inhibit mitochondrial respiration also in vivo (Shen *et al.*, 1994;Shen *et al.*, 1995;Shen *et al.*, 2000), there has not been any evidence for this in humans (Radegran & Saltin, 1999;Frandsenn *et al.*, 2001). However, some of the methodological issues may have affected the different outcomes in human studies.

Although there is currently a substantial amount of analytical in vitro and animal work available on vessel tone regulation, it is also acknowledged by the leading contributors in the field that similar studies are much more complicated, and difficult, to perform on humans (Vanhoutte, 2009). Thus, rather than becoming a lost art and purely of academic interest, it is of importance to test the physiological relevance of in vitro derived observations and ideas on humans to be able to better understand the basic human physiology and mechanisms possibly affecting human vascular disorders.

2   REVIEW OF THE LITERATURE

2.1   Circulation and blood flow distribution in humans

It has been known since the times of William Harvey that blood returning from the peripheral tissues and organs through the great veins enters a thin-walled collecting chamber, right atrium of the heart, and that when this chamber is full it contracts and blood flows into a thicker-walled chamber, the right ventricle. The subsequent contraction of this ventricle expels the blood through the pulmonary artery into the lungs, from which it returns to the left side of the heart. A similar succession of events as in the right side brings the blood from the left atrium to the thick-walled left ventricle. The latter propels the blood into the large scale channel, the aorta, which conveys it to the peripheral organs of the body (Shepherd & Vanhoutte, 1979). The aorta branches to the smaller and smaller arteries, ultimately to smallest branches called arterioles, from which capillaries originate. Although the individual vessels become smaller in diameter as the branching occurs, their number multiplies to such an extent that the total cross-sectional area of each consecutive section of the vasculature tree increases, reaching the maximum at the capillary level (Figure 2.1.1, modified from Shepherd & Vanhoutte, 1979).
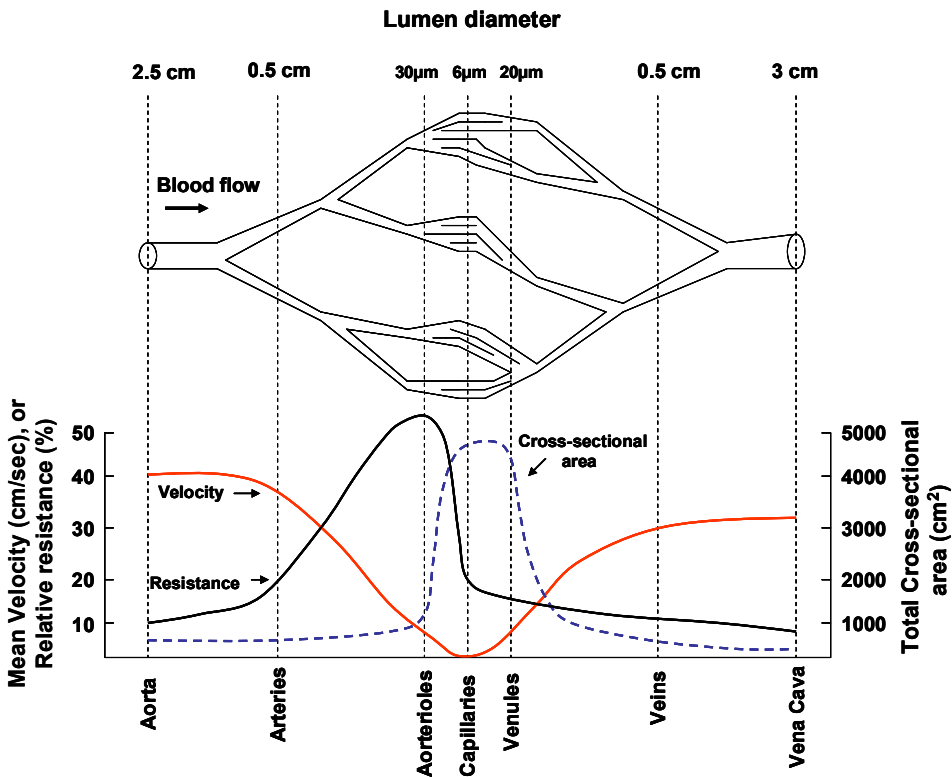
Figure 2.1.1. Changes in the estimated cross-sectional area, mean velocity of flow, and vascular resistance in consecutive segments of the systemic blood vessels.

When the capillaries reunite to form venules and veins, there is a progressive decrease in total cross-sectional area. Since the amount of blood passing through each cross-section per unit of time is the same, the velocity of flow decreases progressively toward the periphery and is the lowest at the capillary level optimizing oxygen and nutrient exchange in the capillaries (Shepherd & Vanhoutte, 1979). Approximately 70 % of blood volume resides on the venous side of the circulation. With the help of valves in these veins, this blood 'reservoir' can be redistributed to the central circulation by sympathetic nerves and muscle pump constricting veins. After having circulated through the lungs, oxygenated blood comes under the greater control of the arterial resistance at the level of the arterioles. Arterioles are thick-walled vessels capable of marked changes in diameter. Inasmuch as the resistance to flow is inversely proportional to the radius of the blood vessels raised to fourth power, even small changes in radius can yield large changes in resistance and therefore in blood flow. The relationship between flow and resistance is governed by a hydraulic analog of Ohm's law; thus perfusion pressure equals flow times resistance. Important perfusion pressure affecting tissue blood flow is the pressure gradient between the arterial and venous sites of the capillaries where the oxygen and nutrient exchange between blood and tissues mainly happens.
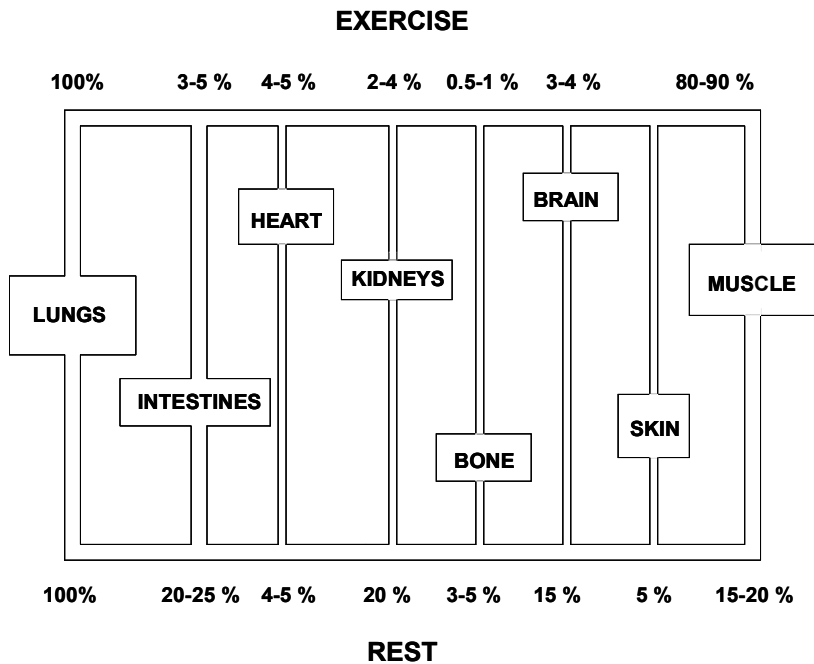
Figure 2.1.2. Schematic drawing of blood flow distribution to main organ vascular beds at rest and exercise. Not included is an estimated blood flow of 5 % to fatty tissues at rest and about 1 % during exercise. Cardiac output is approximately 5-6 litres per minute and usually increases up to 20-25 litres per minute in healthy young men. Picture modified from Åstrand et al. 2003. (Åstrand *et al.*, 2003).

Skeletal muscle accounts for 20-30% of whole body resting oxygen uptake and receives approximately 15-20 % of cardiac output (1.2 L/min) while in heart it is about 5 % (Figure 2.1.2). The major sites for vascular conductance and resistance in resting

humans are in the splanchnic, renal, and skeletal muscle circuits. These comprise 65 % of the total. Since skeletal muscle is the largest organ in the body and thus represents approximately 40 % of total body weight in a normal adult, it is also possibly the most important organ for blood pressure control (Shepherd, 1983). It is also of note that organs that have the largest importance to total systemic resistance also have the smallest metabolic demands relative to their blood flow; that is, the arterial-venous oxygen difference or the percentage of oxygen extracted in one pass through these organs is low so that large changes in blood flow do not compromise these organs' oxygen supply (Rowell, 1986e). The relationship of oxygen requirements to the blood supply for various vascular beds is shown in Figure 2.1.3. In is noteworthy that in contrast to the heart, skeletal muscle is relatively luxuriously perfused.

Total oxygen consumption = 234ml / min
Cardic output = 5,800 ml / min
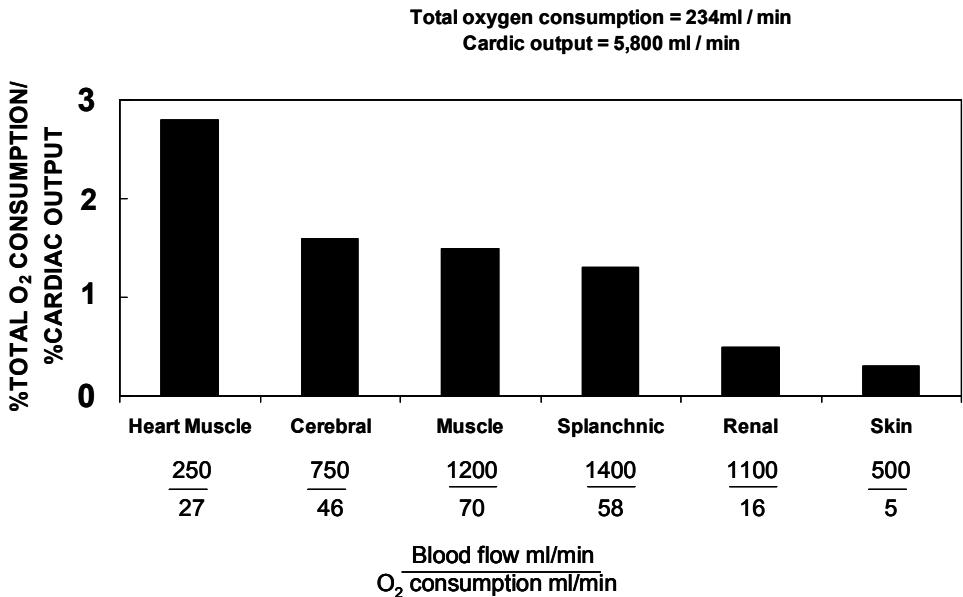
Figure 2.1.3. Relation of oxygen requirements to blood supply for various vascular beds. Heart muscle extracts large amounts of oxygen relative to blood flow compared to many other tissues, where flow is greatly in excess of metabolic requirements (Shepherd & Vanhoutte, 1979).

2.2   Overview of the cardiovascular adjustments in response to exercise

Dynamic exercise presents one of the greatest challenges to cardiovascular control. In response to increasing workloads and oxygen consumption (for example running, bicycling and cross-country skiing), heart rate increases progressively towards maximal values, usually around 200 beats per minute. This tachycardia coupled with sustained or modestly increased stroke volume (amount of blood pumped per one heart beat) causes some four-to-five-fold increases in blood flow to the systemic circulation, termed cardiac output, which is the driving force for enhancements in peripheral blood flow. On the other hand marked vasodilation also occurs in active skeletal muscles such that virtually all of this increase in cardiac output (some 80 %) is directed to working skeletal muscles, although skin blood flow may also rise if internal

temperature increases sufficiently (Figure 2.1.2). Especially in heat, skin blood flow may 'steal' some blood flow from working muscles, although this prevents heat accumulation and improves exercise tolerance. Blood flow to inactive areas (especially splanchnic and renal circulations) decreases as a result of substantial activation of the sympathetic nervous system, and this flow is also redistributed to the active skeletal muscle. With the marked changes in autonomic nervous activity, increases in cardiac output, and vasoconstriction in inactive vascular beds, arterial pressure also rises.

In contrast to dynamic exercise, with strenuous static muscle contraction, increases in heart rate and cardiac output also occur, as does vasoconstriction to inactive vascular beds. Vasodilation within the active muscles, however, becomes mechanically restricted since strong static contractions cause large increases in tissue pressure within the muscle. This compresses the blood vessels that are never released, and arterial systolic pressure increases, sometimes up to 400 mmHg extreme levels if the statically contracting muscle mass is large enough.

The mechanisms mediating these substantial cardiovascular and autonomic adjustments to exercise involve the action and interaction of central and peripheral inputs, mainly central command and feedback from muscle afferents (Rowell, 1986f;O'Leary & Potts, 2006). Central command is the concept that volition to exercise can elicit cardiovascular responses. It is a feed-forward system meaning that with activation of the motor cortex, there is a parallel activation of pathways that descend into brainstem areas controlling sympathetic and parasympathetic activities. Thus, the initial rapid increase in heart rate at the initiation of exercise stems from activation of central command causing rapid decreases in parasympathetic activity and the subsequent increase in heart rate is due to increases in sympathetic drive. However, skeletal muscles are also extensively supplied with afferent nerves that sense both the mechanical (group III) and metabolic (group IV) environment. Activation of especially the muscle metaboreflex especially with large muscle mass exercise is capable of eliciting profound increases in sympathetic activity, arterial pressure, heart rate and cardiac output, ventricular performance and also peripheral vasoconstriction presumably in an attempt to increase blood flow to the ischemic muscle. Less is known about the normal role of mechanoreceptors, but their activation (stress of muscle due to contraction) also increases the physiological responses mentioned above. Afferent feedback from muscles finally tunes the central cardiovascular adjustments for a given level of exercise. As a result of central command and afferent modulation arterial pressure increases, which would normally be blunted by arterial baroreflex, but during exercise, increase in arterial blood pressure together with increased heart rate is allowed due to arterial baroreflex resetting to higher pressures rather than being turned off by exercise (O'Leary & Potts, 2006).

2.3   Anatomy and function of blood vessels in skeletal muscle and heart

The manner in which the myocardium is supplied with blood is different from that seen in skeletal muscle. In the larger muscles of the trunk and extremities, the feeding artery or arteries can present half of the total resistance to blood flow. They, however, are not in direct contact with muscle and therefore under immediate vasoactive stimuli produced by

muscle (Segal & Bearden, 2006). Feed arteries penetrate deep within the muscle mass, subsequently giving off branches in all directions that distribute blood throughout the muscle and thereby control regional tissue perfusion. There can also be anastomoses between these branches that provide alternative pathways for the delivery of blood and from which collaterals can arise. Ultimately, capillaries lie longitudinally, but circuitously with muscle fibres going in both directions forming also interconnective branches with each other (Segal & Bearden, 2006). The smallest unit of blood flow control is the microvascular unit that consists of all capillaries (typically 20) arising from the terminal arteriole. The volume of muscle tissue within a microvascular unit is about 0.1 mm^3, with average dimensions of 1 to 2 mm long, 0.5 mm wide and 0.2 mm thick (Segal, 2005;Segal & Bearden, 2006). The capillary density in skeletal muscle is the highest in fibres called red, or slow-twitch fibres. These fibres have a high oxidative capacity, are usually deep muscles located near the bone to keep body posture, and are thus designed for long-term muscular work. They have about three times the number of capillaries per fibre (accounting in differences in fibre size) than fast-twitch white fibres, which are designed for high-speed and forceful contractions. In general, capillary density appears to match the oxidative capacity of the fibres and blood flow both at rest and during exercise is higher in capillary-rich fibres/muscles (Johnson *et al.*, 1973;Edgerton *et al.*, 1975;Laughlin & Armstrong, 1985).

Perfusion of the heart is supplied mainly by the left and right coronary arteries. The larger branches of the coronary arterial system of the heart, in contrast to skeletal muscle, generally run superficially in the epicardium, giving off numerous small branches that then penetrate perpendicularly to the myocardium. (Berne & Rubio, 1979;Van Mierop, 1979). Since cardiac muscle is perhaps the most aerobic organ in the body, its vasculature is therefore very rich in capillaries. One of the classical studies on human heart stated that 1) myocardial capillaries multiply during postnatal growth to compensate for the increase in muscle fiber size, 2) the muscle-to-capillary ratio attains a value of approximately 1:1 at maturity, and 3) this ratio persists throughout life (Roberts & Wearn, 1941). Compared to skeletal muscle (vastus lateralis), myocardial capillary density is approximately 10-fold higher (Berne & Rubio, 1979;Zumstein *et al.*, 1983;Laughlin & Tomanek, 1987).

Largely due to the anatomical organization of the vessels, muscle contraction greatly affects blood flow. The interplay between cardiac muscle contraction and vascular function has been described in detail in a recent review written by Westerhof et al. and here only a simplistic view is presented (Westerhof *et al.*, 2006). Cardiac contraction (systole) impedes coronary blood flow, which mostly thus happens during diastole (picture 2.3.1). Even backflow can occur during systole. Impediment of coronary blood flow increases with heart rate and contractility and also the time spent in diastole is markedly reduced with increasing heart rate as during exercise. Even if this is the case, it is the current understanding that this does not limit myocardial performance even during maximal effort (Duncker & Bache, 2008).

Skeletal muscle contraction similarly affects blood flow. Thus, during contraction blood flow in the femoral artery is stopped, and perfusion occurs during relaxation

phase (Saltin *et al.*, 1998). Therefore, effective blood flow may be mechanically hindered with increasing force production and contraction frequency (Lutjemeier *et al.*, 2005), but, however, may not disturb oxygen diffusion in capillaries since it is not restricted to relaxation phase only but also happens during contractions (Lutjemeier *et al.*, 2008). It remains to be determined whether red cell flux also occurs in human skeletal muscle during contraction. Short-term mechanical and functional disturbances may not necessarily lead to poor oxygenation within a tissue since oxygen can also diffuse from the arterioles and from capillary to capillary (Pittman, 2000;Segal, 2005).
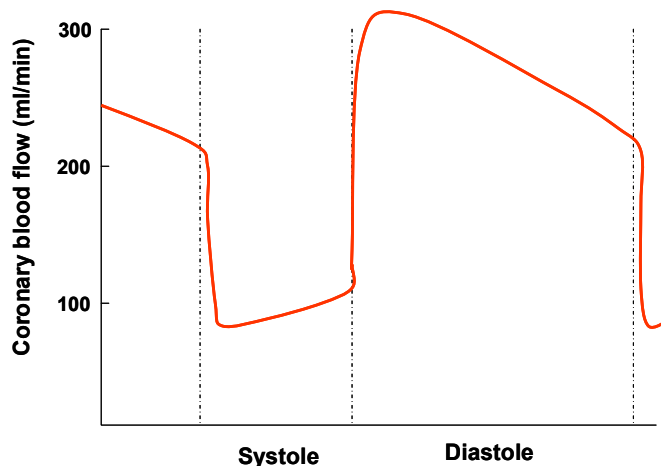
Figure 2.3.1. The effect of cardiac muscle contraction-shortening on coronary blood flow (modified from Guyton & Hall 2000, page 227). During systole, cardiac muscle contraction impairs blood flow in the arteries supplying the myocardium and there is flow only in the epicardial layer. During diastole, however, compression is released and all the layers of the left venriculum are supplied by the blood rich in oxygen (Guyton & Hall, 2000). A similar pattern of flow occurs in skeletal muscle during the contraction-relaxation cycle.

2.4 Basic principles of blood flow regulation in the human body

The tone of vascular smooth muscle, or broadly speaking the diameter of the vessel, is regulated by a myriad of vasoactive substances that may reach the vessel wall intraluminally, thus through the bloodstream, or may be released by endothelial, myocyte and blood cells, or adrenergic, cholinergic or other nerve terminals abluminally close to the vessel wall. In essence, they all work together to meet the local needs of continuously changing bodily requirements. The most important known vasoactive substances and general integrative picture is presented in the following sections.

2.4.1 Autonomic nervous regulation

Both cardiac and skeletal muscle blood vessels are richly innervated with sympathetic and parasympathetic nerve endings, although the existence and role of parasympathetic innervation in human skeletal muscle is debatable. With increasing sympathetic activation there is a release of norepinephrine, neuropeptide Y and adenosine triphosphate from the nerve endings, which all mostly cause vasoconstriction.

Sympathetic activation also leads to release of adrenaline from the adrenal medulla, which acts to vasodilate vascular beds rather unspecifically. Complete abolition of sympathetic activity to the resting limb muscles results in a two-to threefold increase in blood flow; when the noradrenergic nerves are activated maximally the resting flow is decreased by about 75 % or more (Shepherd, 1983). Thus, compared to local metabolic mechanisms that are soon to be introduced, in relative terms noradrenergic nerves control a relatively small portion of the maximal flow capacity of the muscle. However, when its large mass is taken into account, the absolute changes are important to total systemic resistance (Shepherd, 1983). In animals there are also sympathetic vasodilator nerves important for proper defence responses, and although this is also the case especially in cutaneous circulation in humans, they do not appear to play a much role in skeletal muscle blood flow regulation in humans (Joyner & Halliwill, 2000;Joyner & Dietz, 2003). From the evolutionary perspective they thus seem to be better preserved in animals. It may, however, be that there is some yet unknown vasodilator substance that is released from these nerves.

Acetylcholine, which is released (solely) from parasympathetic nerve endings, but in skeletal muscle also from motor nerves, is known to be a potent vasodilator, especially when affecting blood vessels intraluminally with intact endothelium. In general, it is commonly considered that acetylcholine has limited or no role in controlling blood flow increase in humans (Clifford & Hellsten, 2004). However, though cholinergic, neurally mediated vasodilation remains controversial, atropine sensitive vasodilation has been shown in the human forearm at the beginning of exercise (Sanders *et al.*, 1989) and findings from the animals studies have implied that acetylcholine released from activation of motor end plates can 'spillover' to initiate dilatation of arterioles and trigger ascending vasodilation of feed arteries (Welsh & Segal, 1997;Vanteeffelen & Segal, 2006). There are also non-adrenergic non-cholinergic (NANC) neurons that release, for instance, nitric oxide. Finally, in general, similar reasoning as presented here also applies to cardiac muscle, although studying the importance of autonomic regulation on coronary vascular tone is severely hampered by direct effects of nerves on heart muscle function, which by metabolic regulation directly affects vascular tone (Duncker & Bache, 2008). However, in the heart it is well established that there is dominance of α-1 aderenoceptors on the large coronary arteries and on the other hand dominance of β-aderenoceptors on the small resistance arterioles, and symphatetic noradrenergic stimulation for instance during exercise causes these small vessels to dilate and while constricting the large vessels (Tune *et al.*, 2002;Tune *et al.*, 2004). This nervous effect can account for some 25 % of exercise-induced blood flow increase and it also prevents back flow coupled to systole.

2.4.2   *Local regulation*

Current knowledge of local blood flow regulation has recently been extensively reviewed in the latest Microcirculation textbook in a chapter including 1383 references (Davis *et al.*, 2008), and here only the most essential parts are presented. Local regulation of microcirculation can be subdivided into many subparts, which are however naturally in interaction with each other.

Metabolic vasodilation means that tissue sends signals to the resistance vessels in the vicinity of metabolically active cells and by so doing, modulates the contractile state of the smooth muscle cells. As early as the late 1870s, Gaskell was one of the first who proposed that muscle vascular tone is inhibited by metabolites released during contractions. Metabolic vasodilation is generally agreed to play the largest role in controlling vascular tone especially when an organ's metabolic demands increase, such as in cardiac and skeletal muscles during exercise. Its main purpose is thus to match blood oxygen supply to cellular metabolic demand. A host of factors have been proposed to serve the function of metabolic regulators such as O_2, K^+, H^+, adenosine, H_2O_2, CO, CO_2, lactate, P_i, osmolarity, and reactive oxygen species, among others. Endothelium-derived factors, which will be soon described in more detail, can also act as metabolic regulators. Every vasodilator substance ultimately leads to a decrease in smooth muscle cell intracellular free calcium, which is the major determinant of smooth muscle cell contractile state (Horowitz *et al.*, 1996). Metabolic vasodilation is discussed again later in the light of the adenosine hypothesis.

There is an important point to be considered in regard to the theory of metabolic vasodilation (Saitoh *et al.*, 2006). It is namely usually considered to work as a negative feedback loop, when oxygen demands exceed oxygen supply, which leads to the production of a metabolic dilator. Having restored the blood flow to match oxygen demands, the metabolic dilator returns back to normal levels since oxygen supply has been rectified via dilation. There is, however, one factor that argues against this scheme since an error signal to sustain blood flow is then missing (Saitoh *et al.*, 2006). Thus, it must be the case that the factors that initiate (metabolic) vasodilation are different from those that sustain it.

Myogenic response. As evidence of their ability in autoregulation, smooth muscle cells, especially in resistance arteries, constrict in response to increased transmural pressure and dilate to pressure reduction. This phenomenon is generally referred as myogenic response and it is an important determinant of peripheral vascular resistance, blood pressure, and regional blood flow control in several vascular beds, including skeletal and cardiac muscle (Davis & Hill, 1999). Although the myogenic response has been recognized for more than a century after the first findings of Bayliss' in the early 1900s, the basic signalling mechanisms are still incompletely elucidated. It is, however, known that myogenic response is an intrinsic property of the vascular smooth muscle cells of resistance arteries and occurs in the absence of endothelial or neuronal input (Davis & Hill, 1999).

Vasomotion means rhythmic oscillations in vascular tone due to changes in smooth muscle contractile state and according to Clough & Egginton (2009), has been known since the earliest days of microscopical observations by Jones in 1850s (Clough & Egginton, 2009). Vasomotion is thought to be generated in the vascular wall and not to be a consequence of heart beat, respiration, or under neuronal regulation (Newman *et al.*, 2009). Studies in humans have been limited to the skin, but it is also likely that it happens also in muscle and is likely to affect temporal blood flow heterogeneity.

2.4.3   Vascular endothelium in the control of vessel diameter: key observations

The inner layer of all blood vessels is surrounded by endothelial cells. For a long time they were thought only to act as a semimembrane allowing oxygen and nutrients from the blood stream to diffuse to the underlying tissues without letting proteins and blood cells escape. Cuurently it is known that vascular endothelial cells regulate the tone of the underlying vascular smooth muscle cells by releasing various relaxing and also contracting factors. They also importantly affect the coagulation of blood and if intact and healthy, inhibit thrombosis formation.

The discovery of the functionality of endothelium and subsequent understanding of its importance on cardiovascular health is definitely one of the most fascinating stories in physiology. Before the late 1970s, it was well established, based on the original observations of Henry Dale (Dale 1954), that acethylcholine (Ach), a common neurotransmitter in vivo, induces marked vasodilation in various isolated vascular beds, but it however also constricts many isolated preparations of blood vessels (Furchott *et al.*, 1981). One of these preparations was helical strips of rabbit thoracic aorta, which showed concentration-dependent contractions as a response to Ach, regardless of whether the strip was initially at resting tone or in tonic contraction. It was then accidentally discovered by Robert Furchgott's student that Ach, and also muscarinic antagonist carbachol, was able to produce relaxation of tonically contracted rings of the aorta at concentrations lower than those needed to cause contraction of the strips. Higher concentrations caused both strings and strips to contract. It was, however, soon esablished that the loss of relaxing response in the helical strip was the result of unintentional rubbing of its intimal surface against foreign surfaces when preparing the strips for investigation. Importantly, if care was taken to avoid rubbing of the intimal surface during aortic preparation, it always relaxed in response to lower concentrations to Ach. Studies also showed that the relaxations to Ach, as well as contraction responses were mediated via muscaric receptors and these early findings for the first time suggested that the relaxation of the smooth muscles of the rabbit aorta by Ach required the presence if intact endothelial cells on the intimal surface (Furchott *et al.*, 1981). It became obvious that endothelial cells released some factor(s) that relax underlying smooth muscle cells, and it was named endothelial derived relaxing factor (EDRF).

In 1986, Ignarro and Furchott independently suggested that EDRF was nitric oxide (NO) and Salvador Moncada and collegues first showed this experimentally in 1987 (Palmer *et al.*, 1987). Before the 1980s, it was already known that similar to organic nitrates, NO is capable of activating soluble guanylyl cyclase in smooth muscle cells, and thus induce vasorelaxation (Moncada *et al.*, 1988). Hence, during the 1980s, the two came together and the effects organic nitrates, which had already been used to treat angina pectoris since 1867, were also able to be explained by the formation of nitric oxide (Moncada *et al.*, 1988). The biggest breakthrough in human physiology was the developed understanding that NO was also formed *endogenously*. It is known today that the main physiological stimulus of NO generation is physical force against the vessel wall (shear stress) and when formed, NO randomly diffuses in every direction. It thus also acts intraluminally and inhibits platelet aggregation and adhesion in addition

to its smooth muscle cells relaxing effects. The early studies especially, by Moncada's group, paved the way for the understanding that blood pressure depends very much on local vasodilator tone and that conductance might be more important than resistance in regulating blood pressure. Overproduction of NO leads to vasodilation, hypotension, vascular leakage, and distruption of cell metabolism such as increased free radical production, while reduced formation of NO leads to vasoconstriction, elevated blood pressure and thrombus formation (Moncada & Higgs, 2006). NO has been implicated in a number of cardiovascular diseases and virtually every risk factor for these appear to be associated with a reduction in endothelial generation or action of NO. Reduced formation or action of NO is termed endothelial dysfunction, which in general is predictive of cardiovascular disease and observed prior to any other evidence of disease in subjects with a family history of essential hypertension or other risk factors for atherosclerosis (Moncada & Higgs, 2006). Endothelial (dys)function can be measured non-invasively in humans by measuring flow mediated dilatation, which is induced by increased shear stress and largely due to NO (Joannides *et al.*, 1995). In 1998, Ignarro, Furchott and Murad received a Nobel Prize for their contributions to elucidating the physiological importance of NO.

Endothelial cells not only release NO, but also release endothelial hyperpolarizing factors, which cause smooth muscle cell relaxation by increasing its membrane potential, and for instance endothelin-1, which is the most potent vasoconstrictor in the human body. Endothelial cells also release prostanoids/prostaglandins that either vasodilate or vasoconstrict underlying smooth muscle cells. In this respect it is noteworthy that it was actually through prostaglandins via the possibility of endothelial cells control the tone of the underlying smooth muscle cells was first realized. Moncada and colleagues reported in 1976 that they had discovered prostacyclin while studying the metabolism of arachidonic acid in the vessel wall (Moncada *et al.*, 1976). Prostacyclin is a powerful vasodilator generated preferentially by the vascular endothelium, although smooth muscle cells can also produce it (Vane, 1994). It belongs to prostanoids, which are a group of vasoactive lipid mediators that are synthesised from membrane-derived arachidonic acid by prostaglandin H synthase-1 (cyclooxygenase 1, COX-1) and prostaglandin H synthase-2 (cyclooxygenase 2, COX-2). They include the prostaglandins and thromboxanes (Figure 2.4.3). They belong to eicosanoids, which also include lipooxygenase-derived and cytochrome 450-derived products formed from arachidonic acid metabolism. The formation pathways of eicosanoids are shown in Figure 2.3.2. As clear evidence that prostanoids could affect vascular tone is the fact that inhibition of COX enzymes with non-steroidal anti-inflammatory drugs is generally known to increase blood pressure (Wallace *et al.*, 2009). The most common drug to inhibit COX enzymes is acetylsalicylic acid, which was synthesized industrially in 1987 and marketed as Aspirin since 1899 (Patrono & Rocca, 2009). It was, however, only in the early 1970s when Vane and Moncada reasoned its precise mechanism of action. Another common drug which inhibits COX products is Indomethacin, which started to be used to treat rheumatoid arthritis in 1963 (Patrono & Rocca, 2009). Vane, Samuelsson and Bergström received a Nobel Prize in 1982 for their contributions in elucidating the physiological importance of prostanoids.
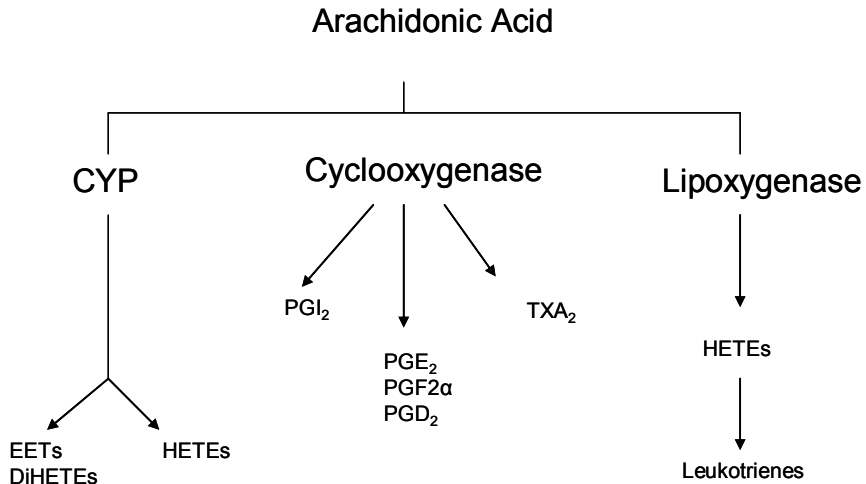
Arachidonic Acid

Figure 2.4.3. Pathways for the metabolism of arachidonic acid. Arachidonic acid is metabolized via cyclooxygenase, lipooxygenase, and cytochrome P-450 (CYP) enzymes to prostaglandins (PG), prostacyclin (PGI$_2$), thromboxane A$_2$ (TxA$_2$), or a series of hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs), and dihydroxyeicosatrienoic acids (DiHETEs).

2.5 The effect of acute exercise on skeletal muscle blood flow

Muscle blood flow increases immediately after the first contraction due to the mechanically increased tissue pressure that causes smooth muscles to vasodilate after pressure release (Clifford & Tschakovsky, 2008), due to both endothelium dependent- and independent mechanisms (Clifford *et al.*, 2006). After the first few contractions metabolic vasodilation takes over and accounts for the major part of the decrease in resistance during exercise (Clifford & Hellsten, 2004). There is no significant dilation of the common femoral artery during leg exercise (Radegran & Saltin, 2000;Wray *et al.*, 2005). The capacity of skeletal muscle to increase its blood flow is very high. Muscle blood flow can be increased from the resting value of ~1-6 ml/100g/min to well above 300 ml/100g/min (Andersen & Saltin, 1985;Richardson *et al.*, 1993). The study by Andersen & Saltin (1985) is frequently cited as the first human study showing the great capacity for blood flow increase per 100g of muscle, but a few years before Klausen et al. had already measured and reported maximal limb blood flow of 8 L/min in one-leg knee extensor exercise (Klausen *et al.*, 1982), but did not normalize it to working muscle mass. Afterwards, Richardson and collegues reported the value of 385 ml/100g/min in trained subjects that is now considered as maximal in humans.

Thus, the capacity of muscle to increase its blood flow is obvious, and it is actually the greatest of all organs in the body (in the myocardium maximal values are the same or even higher, but resting blood flow is also ten-times higher). This is why many researchers call human musculature the sleeping giant (Joyner, 2004). In fact, if all the muscles are being activated maximally, their need for blood flow is substantially higher than heart can supply and peripheral resistance falls so low blood pressure

cannot be maintained. Careful calculations predicted already some two decades ago that with a normal maximal cardiac output of 25 litres per min in young healthy men, maximal muscle blood flow during whole body (cycling) exercise could 'only' be 73 ml/100g/min (Rowell, 1988). Interestingly, a recent study that measured respiratory but also skeletal muscle blood flow with near infrared spectroscopy produced almost exactly the same measured value for maximal leg muscle (m. vastus lateralis) blood flow of ~75 ml/100g/min during maximal cycling in young healthy men (Vogiatzis *et al.*, 2009). Muscle pump is of great importance in achieving high muscle blood flow during exercise (Laughlin, 1987). It however remains to be determined whether blood flow in deep slow-twitch muscle fibres would be even higher since especially several animal (Laughlin *et al.*, 1997), but also human studies (Frank *et al.*, 1999;Kalliokoski *et al.*, 2000;Richardson *et al.*, 2001;Laaksonen *et al.*, 2003;Hannukainen *et al.*, 2006) have shown that there are marked differences both between and within muscle blood flow heterogeneity in different muscular beds. Finally, after cessation of exercise limb blood flow decreases rather rapidly (seconds) close to the resting baseline. However it remains elevated sometimes for more than 30 mins, which also suggest that muscle blood low is not necessarily directly regulated by the need for oxygen in exercising muscles (Bangsbo & Hellsten, 1998).

2.5.1 *Muscle blood flow heterogeneity*

Blood flow heterogeneity can be defined as nonuniform distribution of total blood flow among the perfused vessels in an organ (Duling & Damon, 1987). Variability of blood flow with time within each vessel has been termed temporal heterogeneity while the variability between vessels or tissue regions spatial heterogeneity. Blood flow heterogeneity is regarded as an important parameter which is believed to affect the matching of oxygen and nutrient supply to cellular demands (Kalliokoski *et al.*, 2006b). Its importance is illustrated by the fact that it is possible to alter capillary perfusion heterogeneity while maintaining constant mean blood flow (Duling & Damon, 1987). In animal studies it has been shown with microspheres that there can be substantial blood flow heterogeneity between muscles and muscle groups (Armstrong, 1988a;Armstrong, 1988b). With recent advances in imaging techniques it has been possible to show that also in humans blood flow is different between different types of muscles, being the highest in the deep m. intermedius (Kalliokoski *et al.*, 2000), which most likely stems from differences in capillary densities between muscles. Thus, vessel anatomy affects blood flow heterogeneity. When studying muscle blood flow heterogeneity, the standard deviation of blood flow is used as an index of absolute flow dispersion and is increased in direct proportion to mean blood flow. The coefficient of the variation of blood flow is used as a measure of relative dispersion or true flow heterogeneity and it is thought to reflect the extent that some vessels receive more and some less flow than their appropriate fraction of the total (Duling & Damon, 1987). In addition to anatomical explanations, both motor and vascular unit recruitments and vasodilator substances may affect muscle blood flow heterogeneity, but these are little studied in humans.

2.5.2 *The effect of endurance training on skeletal muscle or limb blood flow*

In general, resting skeletal muscle blood flow is not affected after endurance training (Delp, 1998). Limb blood flow at submaximal exercise intensities in an experimental one-leg exercise was, however, significantly lower after endurance training (Lawrenson *et al.*, 2003). Reduced blood flow is enabled because of increased limb oxygen extraction fraction (Lawrenson *et al.*, 2003). Interestingly, untrained younger subjects initially have higher limb blood flow than older ones, but after a period of endurance training this difference is abolished (Lawrenson *et al.*, 2003). This suggests that younger subjects normally have relatively luxurious limb blood flow during small mass exercise, but endurance training improves the distribution of flow and it becomes more efficient to supply similar oxygen demands of the working limb.

Endurance training also increases the total capacity of limb blood flow (Laughlin & Roseguini, 2008). This is evident in terms of experimental flow capacity determinations such as pharmacological tests or reactive hyperemia, but it is also clear that endurance-trained persons are able to reach higher limb blood flow during maximal exercise because of more extensive vasculature, and as a result, reach higher maximal workloads (Richardson *et al.*, 1993). Despite our knowledge of increased limb blood flow capacity after exercise training, it remains however to be determined whether skeletal *muscle* blood flow capacity specifically is related to systemic maximal oxygen consumption (VO_{2max}) in untrained subjects. This knowledge would be of importance in establishing the common belief that muscle capillary blood flow capacity is a determinant of VO_{2max} in healthy untrained subjects, which in contrast to trained athletes, tend to be more peripherally than centrally limited in whole body VO_{2max}.

The capillary surface area in human skeletal muscles is estimated to be around 7 m^2-kg or 210 m^2 in a 75 kg person with 30 kg muscle (Rowell, 1986d). Ever since Andersen & Henriksson first reported that endurance training increases capillary density in muscles engaged in exercise (Andersen & Henriksson, 1977), this enhancement in muscle exchange surface area leading to better performance has been confirmed by numerous studies (Prior *et al.*, 2004). However, not only is there an increase in capillary density, but also the respective arterioles and bigger arteries grow in size. Current understanding on the effects of long-term endurance training on limb vascular adaptations was recently reviewed by Green (Green, 2009) and in more depth by Prior et al. (2004) and Laughlin & Roseguini (2008). Essentially, enlargement of an arterial vessel in response to regular intermittent physical conditioning occurs in response to elevated internal pressure, which increases wall stress and in addition, also shear stress on the endothelial surface is elevated due to increased blood flow. Vessels enlarge to regulate shear stress back to normal. This is largely dependent on nitric oxide and exercise training also elevates its content, production and bioavailability in the vessel wall. Conversely, if blood flow is reduced by inactivity, the diameter of the artery decreases. Importantly, it has recently been shown that functional changes in conduit arteries occur rapidly and precede arterial remodelling in vivo. Finally,

structural vascular changes are closely adjusted to the metabolic needs of the corresponding extremity musculature (Huonker *et al.*, 2003).

2.5.3 *The effect of reduced arterial oxygen content on limb and muscle blood flow*

Acute hypoxia in the inspired air is a circulatory, respiratory, and metabolic challenge for the human body. Since arterial partial pressure is lowered in hypoxia, acute systemic hypoxia evokes an increase in ventilation and heart rate, although they may wane when the period of hypoxia is maintained (Dempsey & Forster, 1982). The effect of hypoxia on human cardiovascular adjustments (to exercise) has been recently reviewed by Calbet (Calbet, 2000) and also from an interesting integrative (evolutional) biological point of view by Arjamaa & Nikinmaa (Arjamaa & Nikinmaa, 2009). In essence, the effect of systemic hypoxia on thigh blood flow at rest and during leg exercise is however the best summarized by the classical study of Rowell (Rowell *et al.*, 1986). While leg blood flow does not change at rest in hypoxia, it increases substantially to (fully) compensate the reduced arterial-to venous oxygen difference during exercise (Figure 2.5.3). Thus, oxygen supply and consumption are well preserved and there is no impairment in performance in hypoxia during small muscle mass exercise. In this regard it has to be however pointed out that when well trained subjects perform the same one-leg knee extension exercise, blood flow is not enhanced since increased oxygen extraction compensates reduced arterial oxygen content, and maximal normoxic work performance is not reached (Richardson *et al.*, 1995). Moreover, in general, various different experiments in several species have shown that there is little if any increase in limb blood flow until partial pressure of arterial oxygen is reduced below 40 mmHg (95 mmHg normally), but blood flow in coronary and cerebral circulations is enhanced substantially before that (Rowell, 1986b). The reason for this is chemoreflex-triggered increase in sympathetic nervous activation, which constricts blood flow in many other organs securing normal oxygen supply to the most critical organs, thus heart and brain, and also helps to maintain blood pressure.

During whole body exercise in physiological hypoxia at 3000-4000 metres, cardiac output is increased at submaximal exercise levels and reaches similar maximal values as in normoxia. However, since arterial oxygen content is reduced, maximal oxygen consumption diminishes (Rowell, 1986b). In severe hypoxia (~5000m) cardiac output also decreases leading to decreased limb blood flow and oxygen consumption (Calbet, 2000). However, despite all this knowledge already available, relatively little is known regarding the effects of hypoxia on skeletal muscle blood flow distribution especially during exercise. It is for instance undefined whether increased blood flow during exercise occurs unspecifically, thus both in exercising and non-contracting muscles, or specifically only in exercising muscle, which is more likely. Moreover, when superimposed on exercise, hypoxia has been shown to potentiate muscle sympathetic activation (Seals *et al.*, 1991), which may affect the distribution of muscle blood flow and its heterogeneity within the exercising limb. In regard to exercising muscles, there is paucity of human data how especially combined hypoxia and exercise might affect capillary recruitment and blood flow heterogeneity (Bourdillon *et al.*, 2008). However, both hypoxia (Granger *et al.*, 1975;Pradhan *et al.*, 2007) and exercise (Honig *et al.*,

1980;Laughlin *et al.*, 1996) can be expected to increase vascular capillary recruitment, which should be seen as more homogenous blood flow within exercising muscle as compared to baseline condition, but this has never been addressed in humans.
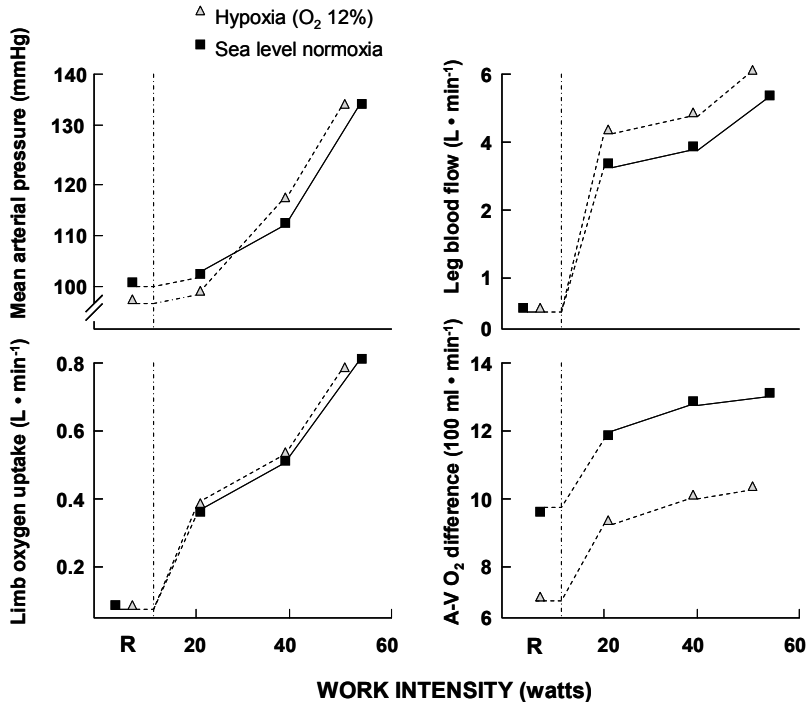
Figure 2.5.3. Circulatory responses to small muscle mass exercise (dynamic knee extensions) up to peak workloads in stimulated systemic hypoxia of 12 % of oxygen and normoxic sea level. While leg blood flow does not change at rest (R) in hypoxia, it increases substantially to (fully) compensate the reduced arterial-to venous (A-V) oxygen difference during exercise. Thus, oxygen supply and consumption are well preserved and there is no impairment in performance in hypoxia during small muscle mass exercise (Modified from Rowell et al. 1986).

2.5.4 Is there a capillary recruitment from rest to exercise?

Understanding of the role capillaries in the tissue oxygenation has advanced substantially from the earliest observations of August Krogh (Krogh, 1919a;Krogh, 1919b), who in the 1920s received a Nobel Prize due to his contributions to this field. The studies of Krogh led to the idea that capillaries must be recruited whenever there is a fall in muscular tissue PO_2 such as during exercise. This idea is still supported by most of the experimental data (Duling & Damon, 1987;Laughlin *et al.*, 1996). In this respect, relatively recently a whole new concept has been proposed, which states that there can be increase in capillary recruitment in skeletal muscle despite no change in mean bulk blood flow since blood could be redirected from non-nutritive pathways to capillaries (Clark *et al.*, 2000a;Clark *et al.*, 2000b). Part of the actions of insulin for instance may also be accounted by this mechanism. The idea of non-nutritive vasculature initially arose from the studies of Pappenheimer who noticed that total

blood flow into muscle did not correlate with either metabolic and heat transfer responses (Pappenheimer, 1941). It seems that there are no clear direct arterial-to-vena anastomoses in skeletal muscle and non-nutritive vessels are capillary-like. However, they are two times larger than "normal" nutritive capillaries and thus have a much higher blood conducting capacity (Grant & Wright, 1970). Animal studies suggest that over 50 % of resting blood flow is non-nutritive (Harrison *et al.*, 1990;Newman *et al.*, 2007). This blood flow redirection and capillary recruitment has, however, been hotly debated in recent years (Clark *et al.*, 2008) (and subsequent point-counterpoint discussion) and awaits further experimentation, especially in humans. It is also evident that capillaries of true nutritive nature are not all open at rest, but are recruited, for instance, in response to exercise (Segal, 2005) for which there is extensive evidence from laboratory animals (Duling & Damon, 1987;Laughlin *et al.*, 1996). Finally, even the existence of direct arterial-to-venous anastomoses in muscle is sometimes mentioned as textbook knowledge (Åstrand *et al.*, 2003).

From the anatomical point of view it is known that vascular units are not precisely coupled to muscle fibre units, which are distributed and can also be activated fairly heterogeneously especially during low intensity exercise. Indeed, the volume of muscle supplied by each microvascular unit usually contains segments of 20-30 muscle fibres (Segal, 2005). Moreover, capillaries are called into action in groups rather than individually (Pittman, 2000;Segal, 2005) and it thus follows that blood flow during submaximal exercise is likely to be increased around those muscle fibres that are not activated at all (Fuglevand & Segal, 1997;Emerson & Segal, 1997). Since skeletal muscle fibres can be several centimetres long and microvascular units (capillaries) only 1-2 mm, it follows that with fibre activation, many more vascular units must be activated that also supply inactive fibres. It must, however, be acknowledged that this arrangement also promotes oxygen diffusion (which also happens not only in capillaries, but also in arterioles) between the capillaries of neighbouring microvascular units that can offset heterogeneities in oxygen delivery between capillaries (Pittman, 2000;Segal, 2005;Segal & Bearden, 2006). Additionally, the distribution of red blood cells within a microvascular unit is not uniform, and this also affects oxygen supply of the muscle.

2.5.5 *Specific features of skeletal muscle blood flow*

Conductive vasodilation means that vasodilation that began in resistance arterioles travels upstream in a coordinated manner to control blood flow to metabolically active tissue parts. It happens by electrical communication that is made possible by gap junctions, intercellular channels located in endothelial and smooth muscle cells that permit ions to flow down their electrochemical gradient. Segal and Duling (1986) were the first to describe this functional cell-cell communication in resistance arteries (Segal & Duling, 1986). Before them, Hilton had described in 1959 that contractile activity initiates vasodilation that ascends from arterioles into the arterial supply (Hilton, 1959). Such coordinated relaxation of smooth muscle cells along the resistance network minimizes the restriction of blood flow by proximal segments and allows subjects to attain peak levels of tissue perfusion. When tissue metabolism decreases,

the arterial network also constricts back to its initial tone in a similar coordinated fashion by which it was dilated. The phenomenon of conductive of vasodilation is not that precisely defined in cardiac muscle as it is in skeletal muscle.

Functional sympatholysis is a characteristic feature especially for exercising skeletal muscle. It means that despite increased vasoconstrictive sympathetic nervous drive to muscle with increasing exercise intensity, blood flow is still able to increase. Thus, sympathetic drive is blunted locally in active muscle (Remensnyder *et al.*, 1962). Locally released vasoactive substances most likely account for functional sympatholysis, in addition to their direct relaxing effects on smooth muscle. One of these agents may be adenosine (Vanhoutte *et al.*, 1981;Smits *et al.*, 1991), although not every study supports its importance in the phenomenon. Moreover, even if studies in rodents suggest that nitric oxide is a symphatolytic substance, human studies have not been able to confirm these findings (Tschakovsky & Joyner, 2008). In addition to locally derived substance, many of which may not even be known yet, neurotransmitters itself are likely to induce sympatholysis by negative feedback loop (Vanhoutte *et al.*, 1981). A strong body of animal studies performed already decades ago suggests that for instance norepinephrine (NE) inhibits its own release presynaptically, but overall, these nervous mechanisms have been very little studied in humans. However, it was very recently shown in accordance with this evidence that muscular contractions appear to modulate the effect of NE rather than its release (Mortensen *et al.*, 2009). Moreover, although the majority of the data seems to support the concept of locally released substances other that neurotransmitters, there are also concerns and studies that argue against this idea (Laughlin *et al.*, 1996). Finally, it must be pointed out that sympathetic vasoconstriction within the exercising muscle may not necessarily be as bad it is sometimes considered since it creates a better perfusion pressure and also leads to better perfusion distribution only to those vascular units that are supplying active muscle fibres, hence, blood flow to less active regions is inhibited by this vasoconstriction. In contrast to skeletal muscle, in myocardium it is not clearly determined whether α-adrenergic vasoconstriction is beneficial or detrimental in healthy human heart.

2.6 Myocardial blood flow at rest and during exercise

Current understanding of the effects of acute and long-term exercise on coronary blood flow has recently been comprehensively reviewed by Duncker and Bache (Duncker & Bache, 2008). Since it is generally considered that myocardial oxygen extraction is always relatively high, around 75 %, oxygen supply to cardiac muscle is dependent almost solely on blood flow. Therefore, myocardial blood flow increases in direct proportion to increases in myocardial workload while oxygen extraction is elevated only modestly (Laughlin *et al.*, 1996;Knaapen *et al.*, 2007;Duncker & Bache, 2008). This view still holds even if it seems that in humans oxygen extraction is somewhat lower, about 60-65 % (Heiss *et al.*, 1976;Iida *et al.*, 1995;Pernow *et al.*, 1996;Pernow *et al.*, 1997;Bergman *et al.*, 2009) than the textbook value of 75 % derived from on animal studies, but the physiological variation is also high, from 51-80 % (Binak *et al.*, 1967). The reason for this variation is undefined, but may depend on the degree of

physical fitness. True maximal myocardial blood flow remains to be determined in humans. It also remains to be determined in humans how vigorous endurance training affects maximal myocardial blood flow. Nevertheless, it seems to be established that myocardial blood flow does not limit exercise performance since there is always a reserve in blood flow even during maximal exercise (Duncker & Bache, 2008).

2.6.1 *The effect of endurance type exercise-training on the myocardium, its vasculature and blood flow*

It must be appreciated that no single part (for example heart, metabolism, muscles) can exclusively limit the maximal physical endurance performance in healthy humans since they are critically dependent on each other. However, the capacity of skeletal muscle to consume oxygen far exceeds the capacity of the heart to supply oxygen rich blood, and it is thus generally agreed that the improvements in short-term maximal endurance performance (maximal oxygen consumption) by physical conditioning are critically dependent on adjustments in central factors, that is in heart and cardiac output (Rowell, 1986a), although better oxygen extraction in muscles also plays a role. Enlargement of the heart and its stroke volume is one of the most important factors in enhancing cardiac output since maximal heart rate usually remains the same or slightly decreases, but on the other hand, the heart cannot pump more that it receives. Thus, during exercise the heart is dependent on peripheral factors such as the muscle pump and the autonomic nervous system that increases central blood volume and right atrial filling pressure (Frank-Starling mechanism). This enables the trained heart to take advantage of its size in the form of a high stroke volume of ~200 ml typical for highly trained endurance athletes. The important interplay between peripheral factors and cardiac performance is also nicely elucidated by the knowledge of the effects of cardiac pacing. Although heart rate and oxygen consumption can be substantially raised 'artificially' by pacing, there is a depletion of stroke volume and cardiac output that does not change from rest in healthy human heart (Bergman *et al.*, 2009). As evidence of peripheral contribution to cardiac remodelling, it is known that large muscle mass exercise leads to larger hearts (Price *et al.*, 2000). If one truly wants to comprehensively understand the important interplay between central and peripheral adjustments inducing cardiac adaptations in health and disease, the classical cardiovascular textbook published in 1986 by Rowell is highly recommended.

As a result of long-term athletic-type exercise training, symmetrical (Hauser *et al.*, 1985;Scharhag *et al.*, 2002) left-ventricular remodelling occurs, which includes increased dimensions in cavity size, wall thickness, and mass. These are regarded as physiological adaptation to the aforementioned increased hemodynamic load induced by chronic and intensive exercise training, and often called athlete's heart (Pelliccia *et al.*, 1999;Pluim *et al.*, 2000). In this regard, especially vigorous interval training typical for competitive level training has great potential to induce physiological cardiac remodelling (Wisloff *et al.*, 2009). In some endurance sports such as in cross-country skiing both training and competition consists of natural interval training due to the continuously changing terrain (uphill and downhill), which together with long distances produces high requirements for both volume and pressure work in the heart.

According to the theory first proposed by Morganroth (1975), athletes involved mainly in sports with a high dynamic component (e.g. running) develop predominantly increased left ventricular chamber size with a proportional increase in wall thickness caused by volume overload associated with the high cardiac output of endurance training (Morganroth *et al.*, 1975). Increased wall thickness normalizes the wall stress in this situation. On the other hand, athletes involved in mainly static or isometric exercise (e.g. weightlifting) develop predominantly increased left ventricular wall thickness with unchanged left ventricular chamber size, which is caused by pressure overload accompanying the high systemic arterial pressure found in this type of exercise. Therefore, especially in cross-country skiing pronounced left ventricle remodelling occurs. However, although many of the training effects and concepts of the athletes' heart have been confirmed by longitudinal training studies in humans and not just cross-sectionally, it must be acknowledged that genetic influence is also likely to be present in athletes.

The study that is frequently cited and really paved the way in elucidating exercise-induced structural vascular adaptations in the heart was the post-mortem case study from "Mister Marathoner" Clarence DeMar. He ran the Boston marathon 34 times up to the age of 66 years, won seven times, and at autopsy had unusually large coronary arteries (two to three times larger than normal) (Currens & White, 1961). This finding suggested for the first time that exercise increases the calibre of the coronary arteries, and this was confirmed by Haskell et al. by direct intracoronary vasodilator investigation indicating that endurance athletes had twice the vasodilated coronary artery cross-sectional area, although the resting calibre and the mass of the left ventricle was similar to controls (Haskell *et al.*, 1993). Dilating capacity was also interestingly positively related to maximal aerobic capacity among runners (Haskell *et al.*, 1993). Additionally, it was also observed in a study by Pellicia et al. (1992) that in physiological cardiac hypertrophy in athletes there is also an increase in coronary artery diameter (Pelliccia *et al.*, 1990), which is in line with current thinking that there is a concomitant increase in vasculature along cardiac hypertrophy. In general, there is a large body of evidence to say that coronary vasculature is tightly coupled to cardiac size in the healthy human heart (Rodriguez & Robbins, 1959;O'Keefe, Jr. *et al.*, 1987;Leung *et al.*, 1991;Dodge, Jr. *et al.*, 1992;Zandrino *et al.*, 2000). In animals that had undergone regular muscular exercise training, training-induced structural and functional dimensional adaptations of the coronary vascular system have been found (Laughlin *et al.*, 1998;White *et al.*, 1998). However, despite the fact that human data is missing, endurance training does not seem to increase myocardial capillary density, although studies are inconsistent due to the differences in species and training modalities (Wyatt & Mitchell, 1978;Scheuer, 1982;Hudlicka, 1982;Laughlin & Tomanek, 1987). Nevertheless, maximal coronary blood flow and transport capacity is greatly improved as a result of endurance-type training, which seems to be due to the enhanced capillary area available for exchange and capillary perfusion (Wyatt & Mitchell, 1978;Laughlin & Tomanek, 1987). It is, however, also known that arterioles grow in size and also capillary density may increase at some time point during the endurance training period, although ultimately due to enlargements in myocyte

capillary density may remain similar to pretraining values (White *et al.*, 1998). As is the case regarding the large conduit arteries in the human body (Miyachi *et al.*, 1998), it is known that endurance training increases the size (cross-sectional area) of epicardial arteries supplying the hypertrophied heart (Rodriguez & Robbins, 1959;Pelliccia *et al.*, 1990;Zandrino *et al.*, 2000) in order to meet the demands of larger cardiac outputs. However, this adaptation disappears with aging, that is with decreasing physical activity (Leung *et al.*, 1991). Nevertheless, a high possibility remains that even if having bigger epicardial arteries, which also show larger dilating capacity (Haskell *et al.*, 1993), coronary blood flow reserve may not be enhanced in highly trained endurance athletes. Thus, myocardial vasculature may simply increase in parallel with increases in the left ventricular mass to cope with the increased oxygen demand of vigorously beating hypertrophied hearts during maximal exercise in athletes.

It is well established that due to exercise-induced bradycardia, thus lowered heart rate, at rest and during exercise, both myocardial blood flow and oxygen consumption per gram of myocardium are lower at any given absolute level of exercise (Laaksonen *et al.*, 2007;Duncker & Bache, 2008). Previous human studies (all cross-sectional) have, however, resulted in different conclusions in regards to endurance training on myocardial blood flow reserve. Some have shown no improvements (Radvan *et al.*, 1997;Kalliokoski *et al.*, 2002;Hannukainen *et al.*, 2007) while others have detected supranormal reserve (Toraa *et al.*, 1999;Kjaer *et al.*, 2005). The degree of cardiac hypetrophy, genetics, training status, and/or the heart's work output may have affected the results and the issue needs further experimentation.

2.7   Adenosine: metabolic vasodilator in skeletal and cardiac muscle?

Adenosine was first recognized as a physiological regulator of coronary vascular tone by Drury and Szent-Gyorgyi in 1929. The main cardiac effects still known today are best summarized by their classical paper published in the Journal of Physiology (Drury & Szent-Gyorgyi, 1929). They reported that adenosine and related compound adenylic acid "slow the rate of heart beating, impair conduction from auricle to ventricle…" and "lower general arterial pressure as well as dilate the coronary vessels". It was, however, not until 1970 that Sattin and Rall showed that adenosine regulates cell function by binding to specific receptors on the cell surface (Sattin & Rall, 1970). Metabolic vasodilation is still considered to be the single most important regulator of skeletal and cardiac muscle blood flow, and one of the most important candidates to mediate this vasodilatation is adenosine. Adenosine is derived mostly from the breakdown of high-energy compound adenosine triphosphate (ATP). Intravenously infused adenosine is promptly cleared from the circulation by cellular uptake, predominately by erythrocytes and endothelial cells. Once inside the cell, adenosine is quickly metabolised either via phospholyration by adenosine kinase to adenosine monophosphate or via demamination by adenosine deaminase in the cytosol to iminase. Rapid clearance of adenosine results in a very short half-life (< 10s). Functions other than possible mediation of metabolic regulation for adenosine are neurotransmission (CNS effects of caffeine are thought to result from adenosine

receptor antagonism), modulation of cardiac conductance (intravenous preparations of adenosine are licenced for clinical use for the treatment of supraventricular tachycardias) and inhibition of inflammation (Cronstein, 2004).

2.7.1 Adenosine receptors

All the biological effects of adenosine are mediated through the four different adenosine receptor subtypes; A_1, A_{2A}, A_{2B} and A_3, which signal mainly through coupling with G proteins. A_1 and A_3 receptors are inhibitory G protein-coupled and thus inhibit adenylyl cyclase, whereas A_{2A} and A_{2B} are coupled to stimulatory G proteins. Both the high affinity A_{2A} and the low-affinity A_{2B} receptors activate adenylyl cyclase, resulting in increases in intracellular cyclic AMP (cAMP) (Jacobson & Gao, 2006). Elevation of cAMP levels induces smooth muscle cell vasorelaxation. Another main mechanism by which adenosine exerts its effects is the opening of K_{ATP} channels. A_2 receptors are the main receptors expressed in coronary circulation and predominantly account for vasodilation, while A_1 and A_3 receptors are expressed in cardiomyocytes and serve as cardioprotective receptors (Mubagwa & Flameng, 2001). Adenosine is well known to be an antiadrenergic agent and negative chronotrophic, the effects mediated primarily via A_1 and A_3 receptors (Mubagwa & Flameng, 2001).

Although it has been commonly held that adenosine-induced vascular relaxation is endothelium independent, in addition to vascular smooth muscle, adenosine receptors are also expressed both in human skeletal muscle (Lynge & Hellsten, 2000) and coronary (Olanrewaju et al., 2000) endothelium and endothelium-derived NO partly mediates adenosine-induced flow increase, again both in muscle (Smits et al., 1995) (See Costa et al. 1998 for controversial finding) and the myocardium (Buus et al., 2001) (see Kaufmann et al. 2004 for discrepancy (Kaufmann et al., 2004)). Infused exogenous adenosine activates mostly its endothelial receptors since endothelium is considered to be a highly active barrier for adenosine and adenosine concentration does not increase in muscle interstitial space upon infusion (Mo & Ballard, 2001;Gamboa et al., 2003). Endothelial-evoked adenosine responses seem to be mediated predominantly via A_1 receptors (Marshall, 2007), but also A_2 receptors are involved (Olanrewaju & Mustafa, 2000). According to a recent study, prostanoids are also involved in mediating flow increase in response to exogenous adenosine (Mortensen et al., 2007). This pattern of intraluminal adenosine infusion may, however, differ from exercise-induced adenosine receptor activation, which stimulates mostly smooth muscle cell receptors abluminally.
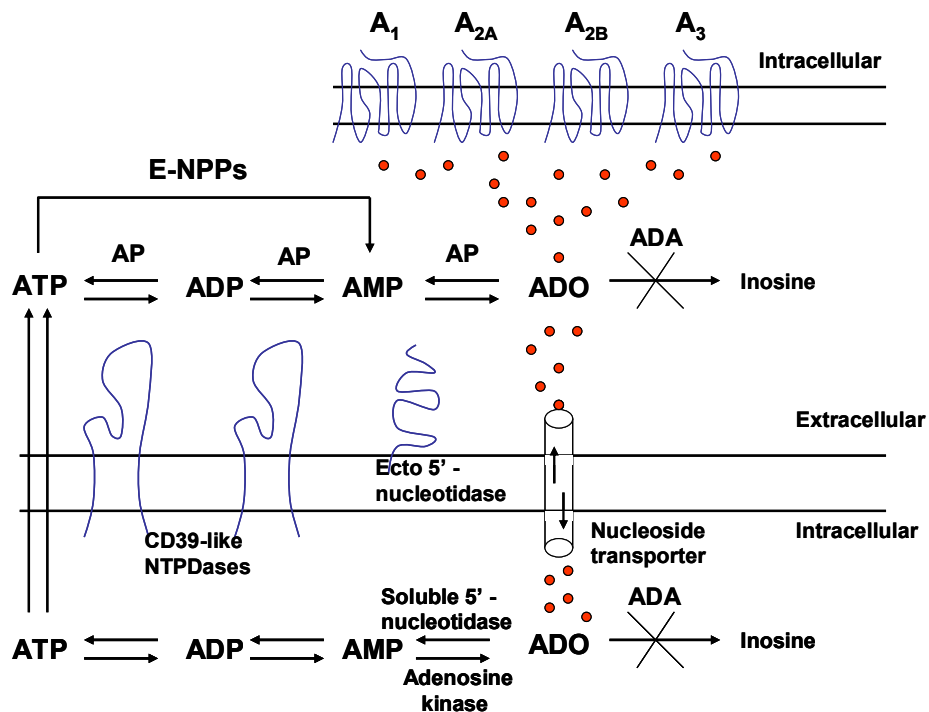
Figure 2.7.1. Adenosine production and metaobolism. Extracellular adenosine (ADO) is mainly produced by the metabolism of ATP released from different cells. ATP is sequentially dephosphorylated by a series of membrane-bound and soluble ectonucleotidases to produce adenosine. After having formed (intra- or extracellularly), adenosine can act at four distinct seven-transmembrane G-protein coupled receptors present of the surfaces on literally all the cells in the human body. ADA (adenosine deaminase) is primarily the catabolic enzyme for adenosine, and it is inhibited by local hypoxia or ischemia, thus allowing adenosine concentration to increase and restore cellular homeostasis. E-NNPs = ectonucleotide pyrophosphatase/phospho-diesterases; AP = alkaline phosphatase; NTPDdases ectonucleoside triphosphate-diphosphohydrolases. Modified from Tilley & Boucher 2005 (Tilley & Boucher, 2005).

2.7.2 *The role of adenosine in the regulation of skeletal muscle blood flow*

The vasodilatory effects of adenosine in the human limb were first reported by Born and collegues (Born *et al.*, 1965). The study was performed in a forearm and flow was determined by venous occlusion plethysmography. Hellsten and collegues showed in 1998 for the first time in humans that adenosine concentration increases in muscle interstitium from rest to exercise and also with increasing exercise intensities (Hellsten *et al.*, 1998) and this was also confirmed in the exercising forearm (Costa *et al.*, 2001). Later, Rådegran & Calbet found that intravenously infused unspecific adenosine receptor blocker theophylline reduced exercising limb blood flow by 20 % suggesting adenosine contributes to exercise hyperemia (Radegran & Calbet, 2001). Additionally, the concentration of adenosine has also been shown to increase in the resting forearm in systemic hypoxia (MacLean *et al.*, 1998) and there is evidence that this resting

hypoxic vasodilation is blocked by aminophylline (Leuenberger *et al.*, 1999), which like theophylline is an unspecific competitive adenosine receptor inhibitor. More recently, Martin and collegues found with using aminophylline that exercising muscle blood flow is reduced by 15 %, but this occurs only in the highest exercise intensity (Martin *et al.*, 2006b). Thus, there is evidence that adenosine might contribute to exercise hyperemia in humans, but it has not been determined whether the adenosine mediation explains exercise-induced blood flow increase in skeletal muscle capillaries, or whether adenosine mediation is pronounced when physiological systemic hypoxia is superimposed in exercise. Additionally, it has not been studied whether adenosine contributes to the regulation of blood flow heterogeneity in (exercising) human skeletal muscle. In this regard, some animal studies have suggested (Clark *et al.*, 1995) that endogenously released substances such as adenosine would act to homogenize muscle blood flow and thus match blood flow to oxygen consumption, rather than controlling limb blood flow levels.

In general, there are studies to both support (Tabaie *et al.*, 1977;Metting *et al.*, 1986;Poucher *et al.*, 1990;Persson *et al.*, 1991) and to suggest that the adenosine hypothesis does not play a role in metabolic mediation in exercise hyperemia in laboratory animals (Honig & Frierson, 1980;Koch *et al.*, 1990). Contrary to cited human observation, Mo et al. and Lo et al. did not see any increase in muscle interstitial adenosine concentration in systemic hypoxia (Lo *et al.*, 2001;Mo & Ballard, 2001). In addition to the relatively few human and animal studies, there is huge amount of in vitro findings regarding the cellular metabolism of adenosine, which seem to depend much on experimental conditions and thus give contradictory results. Interestingly, however, Lynge et al. have shown that during muscular contractions, adenosine is formed extracellularly and taken up by muscle cells rather than released from muscle, which is somewhat against the classical adenosine signalling idea in metabolic vasodilation (Lynge *et al.*, 2001). Finally, having studied contraction-induced muscle hyperemia and reviewed the evidence for adenosine-mediation until 2002, Valic and collegues from Clifford's lab went on to conclude that despite long-standing interest in adenosine as a mediator of metabolic vasodilator, there is strong evidence against its involvement in exercise hyperemia (Valic *et al.*, 2002).

2.7.3 The role of adenosine in the regulation of cardiac muscle blood flow

According to Tune (2007), Robert Berne argued as early as 1957 that the lack of significant coronary vasodilation to hypoxemia above coronary venous oxygen content of 5.5 ml/100 ml supports the role for a myocardial metabolic mechanism rather than direct vascular effects of oxygen partial pressure as the critical regulator of coronary blood flow (Berne *et al.*, 1957). This later led to Berne's seminal paper on the role of cardiac nucleotides in hypoxia (1963), in which Berne found that cardiac hypoxia resulted in a decrease in coronary vascular resistance that was closely correlated to metabolic byproducts of adenosine, that is inosine and hypoxhanthine, from isolated cat hearts and intact open-chest dog hearts (Berne, 1963). Largely based on these results, Berne proposed that the adenosine hypothesis,

which says that reduction in arterial PO_2 and/or increases in myocardial oxygen consumption decrease myocardial PO_2 thereby stimulating the breakdown of intracellular adenine nucleotides and the release of adenosine from myocytes. The resulting increase in cardiac interstitial adenosine concentration would relax coronary arteriolar smooth muscle cells thereby increasing coronary flow and oxygen delivery that would act to restore myocardial tissue PO_2 back to normal level in a classical negative feedback manner (Berne, 1980).

In contrast to skeletal muscle, the picture is clearer when cardiac muscle is considered regarding the attractive adenosine hypothesis as a mediator of physiological metabolic vasodilation. While it is clear that the formation of adenosine increases with increased cardiac workload and reduced arterial oxygen supply, there is no indication that endogenous adenosine would contribute importantly to increases in myocardial blood flow in response to increase in cardiac work (Tune *et al.*, 2004;Tune, 2007;Duncker & Bache, 2008). Without providing a large amount of single evidence from numerous studies it is stated here that it is generally agreed that adenosine does not contribute to myocardial blood flow during exercise in dog, swine or humans, but does contribute to control of myocardial blood flow when it becomes uderperfused/ischemic; thus O_2 delivery is not sufficient to meet myocardial requirements for O_2 (Tune *et al.*, 2004;Tune, 2007;Duncker & Bache, 2008). It must, however, be acknowledged that the experiments addressing the role of adenosine in elevating the cardiac muscle blood flow in response to exercise are difficult to interpret since it is generally held that other vasodilator systems may well compensate if one system fails (such as during pharmacological blockade), which also illustrates how fundamental process blood flow increase is to meet the continuous demands of oxygen. Moreover, there is also human evidence that adenosine contributes to coronary resistance at rest (Bottcher *et al.*, 1995;Edlund & Sollevi, 1995) and during hypoxic exercise (Edlund & Sollevi, 1995).

Despite the fact that there is no conclusive proof of the importance of adenosine controlling cardiac muscle blood flow in physiologically relevant situations such as when myocardial oxygen demand is elevated naturally during exercise in normal healthy subjects, exogenous (infused) adenosine is extensively being used in clinical practise to diagnose coronary artery disease. The concentration of 140 mikrog/kg/min has been shown to induce maximal or near maximal myocardial blood flow (Chan *et al.*, 1992), but also a higher dose can be used (210 mikrog/kg/min) (Reyes *et al.*, 2008). The diagnosis is based on the ratio between resting and adenosine-induced blood flow, and in general terms, a ratio below 2 is considered clinically abnormal (Kaufmann & Camici, 2005;Camici & Crea, 2007;Bengel *et al.*, 2009) and needs further clinical investigation. Animal studies strongly suggest that exogenous adenosine relaxes smooth muscle cells by activating mostly its A_{2A} receptor (Belardinelli *et al.*, 1998;Hein *et al.*, 1999;Hein *et al.*, 2001), but there are no in vivo studies that have addressed this in any means in humans. Despite this situation, drug development for specific A_{2A} receptor agonists has been vigorous, and today there are A_{2A} agonists available for human studies (Druz, 2009). These specific agonists induce less side effects such as flushing, warmth, abdominal

discomfort, chest pain and dyspnoea, which are common for normal adenosine and favour specific agonist use. The A_{2A} receptor may not, however, be the only adenosine receptor by which the vasorelaxation effects of infused adenosine are mediated since at least one human coronary artery study performed ex vivo suggests that the A_{2B} receptor subtype may be even more important than the A_{2A} receptor to relax vessel tone (Kemp & Cocks, 1999).

2.7.4 *Adenosine, or ATP?*

In 1978, Burnstock suggested a subdivision of purinoceptors based on the potent order of nucleotides to bind different receptors (Burnstock, 2008). At the P_1 receptors a potent order of ADO> AMP> ADP > ATP and vice versa for P_2 receptors was proposed. Since this proposal, it has received much support and appears to have been widely accepted. The importance of these two receptor subdivisions has gained more attention after it has become clear that ATP by acting with P_2 receptors is a physiologically important signalling molecule. It is well established that adenosine triphosphate (ATP) is released from nerves (within the same vesicles as NE and NPY), muscle cells, red blood cells, and also from endothelial cells, and affects smooth muscle tone. The effects can be either vasoconstriction or vasodilatation (Burnstock & Kennedy, 1986). It has been shown that when infused into the peripheral limb locally (Rongen *et al.*, 1994) or intravenously to study the effects in the heart (Miyagawa *et al.*, 1995), ATP induces remarkable vasodilation and thus blood flow is increased. During the recent years it has also been shown that ATP is capable of effective blunt sympathetic tone induced by exercise or pharmacological infusions (Rosenmeier *et al.*, 2004). However, it also generally known that ATP is very rapidly degraded to adenosine already in the synaptic cavity and it may also be that it is actually adenosine that is responsible for many of the actions of ATP. One cardiac study, for instance, showed that coronary vasodilation induced by exogenous ATP was entirely mediated by adenosine by acting upon A_{2A} receptors (Erga *et al.*, 2000). Intracoronary or intravenous infusion of ATP in humans has been shown to be safe and induces coronary vasodilation comparable to adenosine infusion (Miyagawa *et al.*, 1995). The cell sources of ATP and adenosine are shown in Figure 2.7.4.
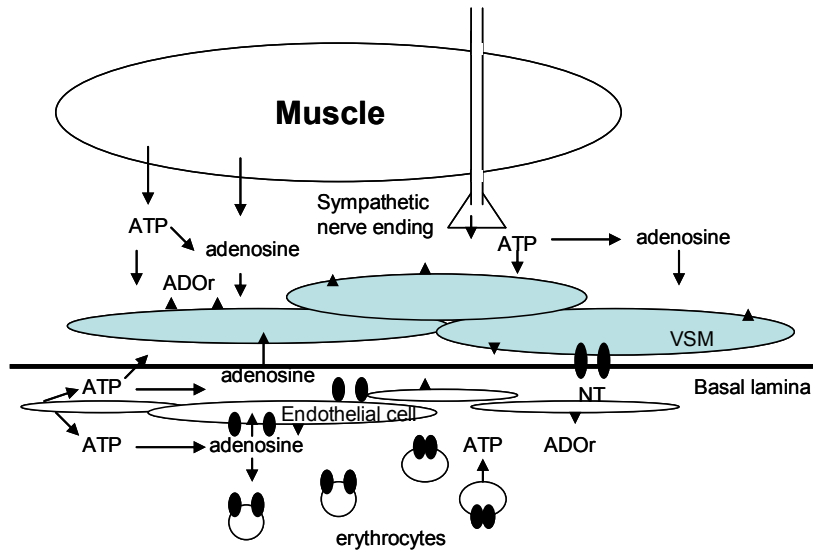
Figure 2.7.4. Schematic presentation of the possible sources of and the site of the effect of adenosine. Intraluminal adenosine is rapidly taken up by erythrocytes, endothelial cells, and smooth muscle cells, but when infused with a high concentration, some adenosine molecules can induce endothelium dependent and independent vasodilation. Naturally edogenous adenosine can be derived from muscle, and from endothelium and nerves as a result of rapid ATP degradation. NT = nucleoside transporter; ADOr = adenosine receptors; VSM = vascular smooth muscle cells.

2.8   Nitric oxide

Mammals express three main nitric oxide (NO) synthase (NOS) isoenzymes: (1) neuronal NOS (nNOS, NOS I), (2) inducible NOS (iNOS, NOS II), and endothelial NOS (eNOS or NOS III). NOS isoenzymes share approximately 50-60 % sequence homology and have similar structure and are only active when present as NOS dimers. The specific activity of nNOS is less than that of iNOS but approximately four times that of eNOS. The actual rate of NO production nNOS is twice as fast as iNOS and more than 30-times faster than eNOS (Fleming, 2008). nNOS is known to be widely distributed in specific neurons of the central and pheripheral nervous systems. NO derived from nNOS plays an important role not only in the modulation of physiological neuronal functions such as neurotransmitter release, but also in neural development, regeneration, synaptic plasticity and regulation of gene expressions. nNOS expression is not confined only to neuronal cells, but is also located, for instance, in the myocardium, skeletal and smooth muscles (Fleming, 2008). iNOS is normally mostly inactive, but is induced and produces large amount of NO during inflammatory conditions. The most important initiator for eNOS activation is fluid shear stress generates by the viscous drag of blood flowing over the endothelial cell surface. Despite the names that derive from the cells where each specific enzyme was first found, it is now clear that NOS enzymes can be found basically in almost every cell such as platelets, smooth muscle and red blood cells and cell organelles as such as mitochondrion (Fleming, 2008).

The NOS enzymes catalyse two sequential monooxygenease reactions: the first reaction involves the hydroxylaction of L-arginine to N-hydroxy-L-arginine which remains bound to the enzyme and is subsequently further oxidized in a second reaction to generate NO and L-citrulline. During the synthesis of NO, NADPH-derived electrons pass to flavins (FAD to FMN) in the reductase domain and must be transferred to the heme located in the oxygenase domain so that the heme iron can bind O_2 and catalyse the stepwise synthesis of NO from L-arginine. Soluble guanylyl cyclase (sGC) is the primary intracellular receptor for NO and was, until relatively recently considered to be located only in cytosol. However, it now appears that the sGC can colocalize with nNOS for instance in the sarcolemma of skeletal muscle fibres (Stamler & Meissner, 2001;Fleming, 2008). This allows the reduced possibility of inactivation of NO by intracellular O_2- and increases the effectiveness of NO signalling. Increase in the vascular production of O_2- which scavenges NO and decreases its bioavailability typifies endothelial dysfunction and can occur prior to any appreciable intimal thickening and is already apparent in patients with a family history of essential hypertension or atherosclerosis. However, when produced and in action, NO elicits relaxation of smooth muscle cells mostly by activating sGC to increase intracellular concentrations of cyclic GMP, which in turn activates the G kinase and elicits a decrease in intracellular Ca^{2+} levels. sGC-independent pathways have also been recognized and are now under intensive investigation (Fleming, 2008).

Interestingly, the relaxation to authentic and endothelium-derived NO is mediated by parallel cyclic GMP-dependent and independent pathways, while the relaxation mediated by NO donors is due solely to cyclic GMP-dependent mechanisms. Thus, NO has a rich chemistry and can affect cellular pathways by a number of mechanisms (most not mentioned here) including the classical pathway just described (sGC), as well as the direct NO-mediated inhibition of enzyme activity, as is the case among others for several heme containing enzymes and proteins with centers of iron-sulfur clusters (Fleming, 2008). NO is a highly unstable substance and in oxygenated solutions, is rapidly converted to NO_2- and NO_3-. NO synthase can be inhibited by drugs such as L-NMMA. This, and the formation of NO, is shown more specifically in Figure 2.8.
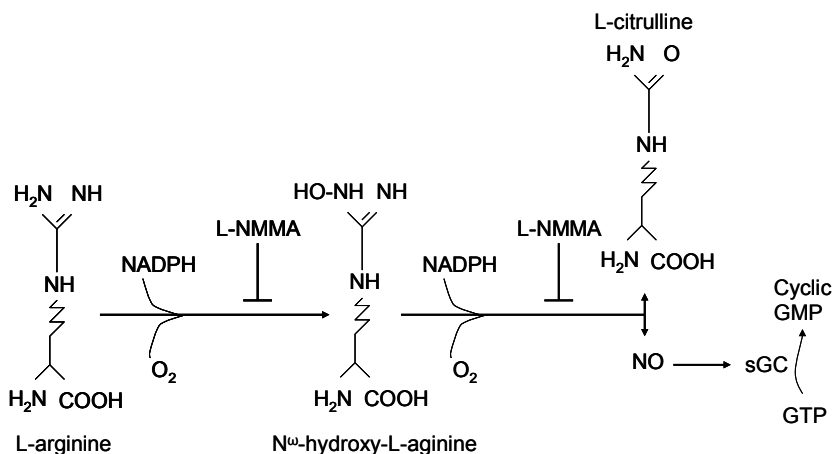
Figure 2.8. The formation and drug inhibition of NO. NO is synthesized from the guadino nitrogen atom(s) of amino acid L-arginine by the action of a family of nitric oxide synthase (NOS) enzymes. The process involves the incorporation of molecular oxygen (O_2) into unstable intermediate Nω-hydroxy-L-aginine and subsequently to L-citrulline, the co-product of the generation of NO. NG-monomethyl-L-arginine (L-NMMA) is a competitive inhibitor of NOS, and blocks the pathway at the points indicated. The many actions of NO are mediated by soluble guanylate cyclase (sGC). The binding of NO to the heme group of sGC induces a conformational change, a change in the stimulation of its catalytic activity and the enhanced production of cyclic GMP from GTP. This second messenger acts on cGMP-dependent protein kinases or cGMP-gated ion channels to mediate the physiological actions of NO, which induces amongst others vasodilation. Picture modified from Moncada & Erusalimsky (Moncada & Erusalimsky, 2002).

2.8.1 The role of nitric oxide in the regulation of limb blood flow during exercise

NO is an important regulator of basal vascular tone and its impact on human blood pressure regulation is substantial (Sander *et al.*, 1999). It is generally considered that this NO derives mostly from endothelium, but limb resting tone is, according to present reports, determined solely by neuronal NO (Seddon *et al.*, 2008). Flow mediated arterial dilatation, which is a widely used marker to depict endothelial (dys)function in various cardiovascular disorders, is known to depend largely on the formation of endothelium-derived NO (Seddon *et al.*, 2008). Resting hypoxic vasodilation (in forearm) is also largely dependent on NO (Blitzer *et al.*, 1996a;Blitzer *et al.*, 1996b). However, despite the importance of NO in mediating blood flow increase in all these conditions mentioned above, natural exercise-induced blood flow seems to be little explained by NO. It is currently generally accepted that NO contributes little if anything to exercise hyperemia (Radegran & Saltin, 1999;Frandsenn *et al.*, 2001). The key studies indicating this have recently been summarized by Tschakowsky & Joyner (2008). The earliest findings suggesting an important role for NO may have depicted blood flow after the early cessation phase of contractions since with blood flow cannot be measured during exercise plethymography. NO indeed contributes to blood flow in the early recovery phase of exercise (Radegran & Saltin, 1999). However, it should be mentioned that there are also convincing animal (Sheriff *et al.*, 2000) and human

(Schrage *et al.*, 2004) studies that still suggest an important role of NO in exercise hyperemia. Thus, it may be that differences in species, limb or muscle mass may partly explain the discrepant findings. Additionally, the effect of NO on pure skeletal muscle blood flow in humans has never been addressed.

Although the role of NO clearly seems to be of limited importance during exercise, there is a large body of evidence that NO works with prostanoids to control exercise hyperemia. From the basic biological point of view it has been well established that NO increases the activity of cyclooxygenase synthesis (Salvemini *et al.*, 1993;Salvemini, 1997) and combined inhibition of NOS and COX has been shown by several studies to reduce exercise-induced blood flow (Boushel *et al.*, 2002;Kalliokoski *et al.*, 2006a;Mortensen *et al.*, 2007;Schrage *et al.*, 2007). This was first shown by Boushel and collegues in 2002. As with NO, in general COX inhibition alone does not seem to affect exercise hyperemia in the leg (Mortensen *et al.*, 2007), but in the forearm COX products account for 12 % of exercise hyperemia (Schrage *et al.*, 2004). The important interplay between these regulatory pathways is illustrated in Figures 2.8.1.1 and 2.8.1.2.
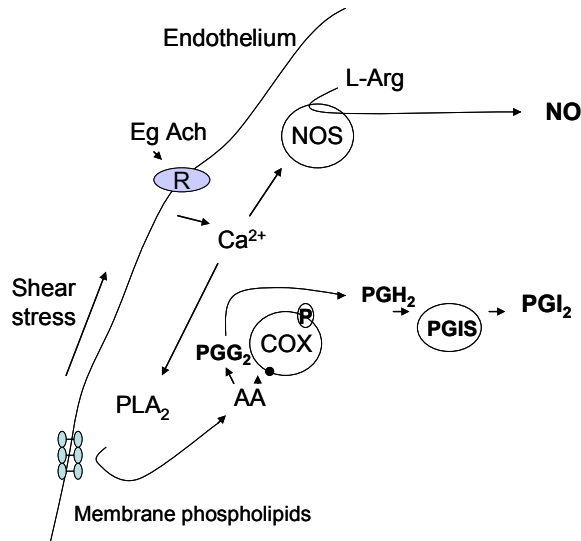
Figure 2.8.1.1. Synthesis of NO and prostacyclin (PGI$_2$, the main vasodilator prostanoid) by endothelial cells. When endothelial cells are activated by physical forces, hormones or autacoids, such as acetylcholine (Ach) via receptors (R), intracellular calcium is elevated. This activates endothelial NOS isoform phospholipase A$_2$ (PLA$_2$). Activated NOS forms NO from the semi-essential amino acid L-arginine (L-Arg). Activated PLA$_2$ liberates arachidonic acid (AA) from membrane-bound phospholipid, which is then available for cyclo-oxygenase (COX), which first forms prostaglandin (PG) G$_2$ and then, via the peroxidase (P) activity of COX, forms PGH$_2$. Prostaglandin H$_2$ is then further metabolized by prostacyclin synthase (PGIS) to form PGI$_2$. Modified from Mitchell et al. 2008 (Mitchell *et al.*, 2008).
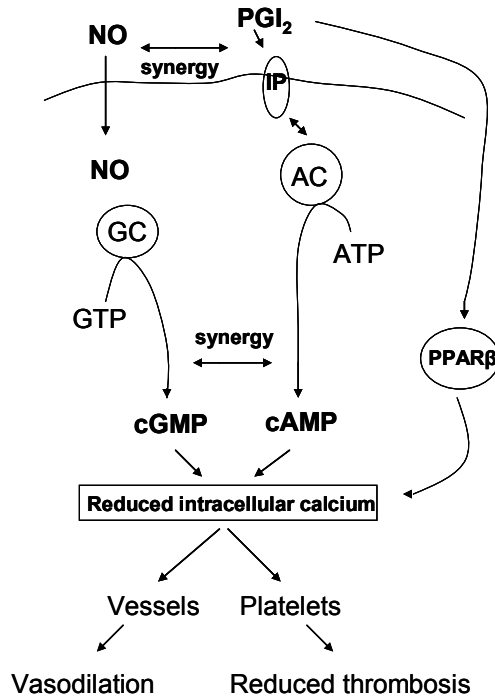
Figure 2.8.1.2 Cellular sensing pathways and effects of nitric oxide (NO) and prostacyclin (PGI$_2$). NO freely enters cells, where it activates soluble guanylyl cyclase (GC), which converts GTP to cGMP. PGI$_2$ activates cell surface IP receptors linked to activation of adenylate cyclase (AC), leading to the conversion of ATP to cAMP. PGI$_2$ may also enter the cell cytosol, where it binds to PPARβ. Activation of GC and AC and the subsequent increase in cGMP and cAMP, respectively, results in inhibition of platelet activation and vasodilation. Modified from Mitchell et al. 2008 (Mitchell *et al.*, 2008).

2.8.2 *The role of nitric oxide in the regulation of cardiac muscle blood flow*

The earliest findings suggesting the important role of NO in coronary circulation were performed by Moncada's group in rabbit heart (Amezcua *et al.*, 1989). Subsequently, it was shown that NO could also be important in human coronary circulation since it was largely responsible for microvascular vasodilation and almost entirely for epicardial vasodilation in response to cardiac pacing induced metabolic stress (Quyyumi *et al.*, 1995). However, as recently reviewed by Duncker and Bache (2008), the majority of animal and human studies indicate that NO plays at best only a modest role controlling coronary blood flow at rest or during exercise. Studies have however shown that flow-mediated dilation in coronaries is largely NO-mediated and NO plays an important role together with prostanoids in cardiovascular diseases (Duffy *et al.*, 1999). Alone, or even in close interaction with NO, prostanoids however do not contribute importantly to the control of coronary blood flow at rest or during exercise in the healthy heart (Duncker & Bache, 2008).

As in peripheral limbs, it has been recently demonstrated that basal coronary arterial tone is largely dependent on neuronal NO, but endothelial NO is responsible for

stimulus-derived blood flow increase (Seddon *et al.*, 2009). Interestingly, it has been suggested the most important function of adenosine may actually not be vasodilation, but rather its role as an inhibitor of neurotrophilic superoxide anion production (Schrader, 1990). Superoxide anion has been shown to substantially attenuate the half-life of NO (Gryglewski *et al.*, 1986) and thereby by inhibiting superoxide anion production it may indirectly contribute to coronary artery vasodilation by increasing NO half-life. There is also evidence from a human cardiac pacing study that adenosine production may compensate when NO production is reduced (Minamino *et al.*, 1998). In subjects without coronary risk factors NO levels but not adenosine increases in cardiac pacing, while in subjects with coronary risk factors adenosine, but not NO levels, increased (Minamino *et al.*, 1998). Relevant for clinical settings is also the knowledge that part of the adenosine-induced blood flow increase may stem from direct or indirect NO release (Buus *et al.*, 2001), although there are also observations that NO may actually impair myocardial blood flow in adenosine stress test (Kaufmann *et al.*, 2004).

2.8.3   *Does nitric oxide affect cellular respiration and thus oxygen consumption?*

In 1994, three groups independently demonstrated that, with respect to oxygen, the primary effect of exogenous NO on mitochondrial activity was reversible and competitive inhibition of cytochrome oxidase activity *in vitro* (Brown & Cooper, 1994;Cleeter *et al.*, 1994;Schweizer & Richter, 1994). Although some animal studies have subsequently found evidence that NO would indeed tonically inhibit mitochondrial respiration also in vivo (Shen *et al.*, 1994;Altman *et al.*, 1994;Shen *et al.*, 1995;Bernstein *et al.*, 1996;Ishibashi *et al.*, 1998;Shen *et al.*, 2000) there has not been any evidence for this in human studies that have also addressed limb blood flow (Radegran & Saltin, 1999;Frandsenn *et al.*, 2001). In animal studies, species differences seem to be important since increased oxygen consumption has only been found in dogs, which is a very aerobic animal, and not for instance in pigs (Duncker *et al.*, 2000). Some human evidence, however, also exists for enhanced aerobic metabolism during NO blockade in humans. Inhibition of skeletal muscle NO production has been found to enhance the acceleration of oxidative metabolism following the onset of vigorous near maximal whole body cycling exercise(Jones *et al.*, 2003;Wilkerson *et al.*, 2004;Jones *et al.*, 2004). Moreover, when systemic NO levels have been raised by dietary nitrate, oxygen consumption during the steady-state submaximal whole body cycling exercise has been lower compared to control (Larsen *et al.*, 2007;Bailey *et al.*, 2009). The proposition has also been advanced during recent years that NO facilitates O_2 distribution within the tissue (Victor *et al.*, 2009).

2.9   Methods to measure tissue blood flow in humans

There are many methods that can be used to measure limb or muscle blood flow. Microspheres are the gold standard in animal studies, which can also detect blood flow heterogeneities in tissues. It is, however, necessary to kill a subject in microsphere measurements, which naturally limits this method only to animal studies.

Electromagnetic flow meters can also be applied to measure blood flow, but they are too invasive to be used in healthy human subjects and in general are restricted to species other than man (Radegran, 1999). The common methods to measure blood flow in humans are plethysmography, dilutions techniques (thermodilution in particular), ultrasound, near-infrared spectroscopy, magnetic resonance imaging, and positron emission tomography.

Plethysmography. Venous occlusion plethysmography is one of the oldest methods to measure limb blood flow in man at rest. According to Wilkinson & Webb, it was first introduced by Hewlett & Zwaluwenburg in the early twentieth century. The underlying principle of venous occlusion plethysmography is simple: when venous drainage from the arm is briefly interrupted, arterial inflow is unaltered and blood can enter the forearm but cannot escape. This results in a linear increase in forearm volume over time, which is proportional to arterial blood flow, until venous pressure rises towards the occluding pressure. Under resulting resting conditions, ~70 % of the total forearm is thought to perfuse through skeletal muscle, with skin blood flow accounting for most of the remainder (Wilkinson & Webb, 2001). However, since the hand contains a high proportion of arterio-venous shunts and because skin blood flow is highly dependent on temperature, it is standard practice to exclude the hands with suprasystolic cuff pressure from the circulation during measurement of forearm blood flow (Wilkinson & Webb, 2001). The method has been used extensively to study human vascular physiology in vivo, and it is most powerful when combined with intra-arterial drug administration. Indeed, forearm venous occlusion plethysmography with local brachial artery infusions is still widely used in the assessment of vascular function in health and disease. The major advances of this approach are that drugs and hormones can be administered at subsystemic doses minimizing disturbances to systemic physiology, but only bulk blood flow is measured, thus, it cannot differentiate blood flow in different tissues of the limb.

Thermodilution. The thermodilution technique to measure limb blood flow was developed by Pavek et al. in the 1960s (Pavek *et al.*, 1964). When, for instance, (whole) leg blood flow is being measured with thermodilution, constant flow of cold saline (near to zero degrees centigrades) is infused into the femoral vein, and blood flow is calculated from the changes in the temperature of femoral blood during infusion. In steady state conditions femoral vein blood flow represents femoral artery blood flow with a reasonable accuracy, but blood flow in different tissues of limb cannot be differentiated. Coronary blood flow can also be measured with thermodilution if a catheter is put into coronary sinus (Pernow *et al.*, 1996;Pernow *et al.*, 1997). Most of the heart's venous blood drains into the coronary sinus, which empties into the right atrium (Rowell, 1986c). This draining of blood also means that sampling of blood oxygen levels from the coronary sinus represent the value of the whole myocardium, which may explain why oxygen extraction values in humans tend to be slightly lower than in animals, where more specific (in respect to left ventricle) sampling procedures can be applied. The thermodilution technique is not anymore the method of choice at least in cardiac studies since it is invasive and beating heart causes movements artefacts and affects temperature analysis and thus, results. Nitrous oxide

saturation method has also been widely used to measure coronary blood flow in humans, also during exercise (Jorgensen *et al.*, 1971).

Ultrasound. Ultrasound Doppler can be applied to measure both coronary and pheripheral limb blood flow. An ultrasound probe is put above a vessel from which blood flow is measured and diameter of the vessel as well as blood velocity is measured (Radegran, 1999). Blood flow is then obtained by multiplying these two parameters. One of the key benefits of ultrasound is that it can measure blood flow pulsations, which is the natural way blood flows. These pulsations but cannot be addressed with methods that need steady-state conditions. However, as with thermodilution, ultrasound cannot differentiate blood flow in different tissues, but only whole blood flow going to the limb.

Near-infrared spectroscopy (NIRS). NIRS has been used to quantify blood flow at rest and during exercise in humans using indocyanine green (ICG) dye as a tracer (Boushel *et al.*, 2000). Blood flow is measured based on the ratio of the tissue (NIRS) to the arterial (ICG) and increases in tissue blood flow are defined by both a greater magnitude and rate of ICG accumulation in tissue for a given volume injected into the circulation. This method also makes it possible to detect perfusion heterogeneity between different tissue regions, which can also be coupled with assessment of the O_2 saturation status of the same tissue region. However, since the NIRS probe is attached on the skin and light penetrates 1-2 cm, blood flow values are always as a result of skin, adipose, and muscle tissue blood flow and deeper muscle blood flow cannot be determined.

Magnetic resonance imaging (MRI). Myocardial but especially true skeletal muscle blood flow during exercise can be measured with MRI using arterial spin labelling (Frank *et al.*, 1999;Richardson *et al.*, 2001). With this technique the distribution of muscle blood flow can also be detected, which since the earliest MRI investigations has been known to vary between limb muscles and within one muscle (Frank *et al.*, 1999;Richardson *et al.*, 2001). MRI also allows studying the matching of muscle blood flow and metabolism, which seems to be poorly coupled in healthy subjects (Richardson *et al.*, 2001) and especially in peripheral artery disease (Anderson *et al.*, 2009).

Positron emission tomography (PET). PET-radiowater ($[^{15}O]$-H_2O), by which flow is measured locally and *directly* within the human muscle, is generally regarded as the gold standard method to measure myocardial and skeletal muscle blood flow in humans (Camici & Crea, 2007;Bengel *et al.*, 2009). The methods (single-tissue-compartment model) have been validated both in heart (Bol *et al.*, 1993) and autogradiography in skeletal muscle (Fischman *et al.*, 2002) against radiolabeled microspheres, the gold standard in animal experiments, showing excellent agreement between methods over wide variations of size of regions and flow values. PET also offers advantages for elucidating blood flow distribution within muscle and between nutritive and non-nutritive compartments since with the PET radiowater technique only muscle capillary blood flow that actually reaches muscle cells is measured

(Lammertsma & Jones, 1983). Thus, PET-radiowater represents the most accurate means to measure true muscle capillary blood flow in human microcirculation.

Due to the short half life of the ^{15}O (123 s), repeated measurements are also possible. The technique, however, requires an online deuterium cyclotron for production of ^{15}O on-site, limiting the applicability of the technique in general and even among PET Centres around the world. However, when applicable, $[^{15}O]$-H_2O is an ideal tracer for blood flow measurements since it is metabolically inert and readily diffuses across the capillary and cellular membranes and thus rapidly equilibrates between the vascular and extravascular spaces, approaching unity independently of blood flow. In particular, the ability of $[^{15}O]$-H_2O to freely diffuse into muscle cells also means that physiological blood flow heterogeneity can be also determined instead of any tracer artefacts, and independent of flow level. Heterogeneity of blood flow with PET can be measured at the voxel level of 16 mm^3, which means that on average approximately 160 microvascular units are included in the voxel (Segal, 2005). Thus the precision is not optimal, but maybe the best that presently can be obtained in humans.

3   OBJECTIVES OF THE STUDY

In light of the undefined role of adenosine, nitric oxide and acute exercise on skeletal muscle blood flow, and adenosine receptor subtypes and exercise training on myocardial blood flow, the purposes of the present study were to investigate

1) the effects of adenosine and incremental exercise on skeletal muscle blood flow and its heterogeneity (I),

2) the effect of long-term vigorous endurance training on myocardial blood flow and its reserve, adenosine A_{2A} receptor density, and its relation to adenosine-induced blood flow (II),

3) the influence of pharmacologically induced blood flow (adenosine) and volitional moderate exercise on muscle perfusion and its heterogeneoty (III),

4) whether endogenous adenosine affects exercising muscle perfusion during moderate systemic hypoxia, (IV) and

5) the effect of inhibition of nitric oxide alone and in combination with prostanoids on skeletal muscle blood flow and oxygen consumption at rest and during exercise (IV)

4   SUBJECTS AND STUDY DESIGN

4.1   Subjects and their recruitment

Subjects for this study consisted of 45 non-obese, non-smoking, young men and women (Table 4.1.1). In regard to female subjects, the possibility of pregnancy was excluded by a pregnancy test before participation. The subjects were not taking any medication other than possible oral contraceptives. Subjects were recruited by local advertisements, though some of the athletes were personally asked by the investigators to participate. The main inclusion criteria were that subjects had to be healthy. Exclusion criteria were physical restrictions, a history of anorexia nervosa or bulimia, obesity (BMI > 25 kg $\cdot$ m^{-2}), any chronic disease, rest- or exercise-induced asthma, previous use of anabolic steroids, additives or any other substances and use of marihuana or other illicit drugs. Before starting any procedures, written informed consent was obtained after the purpose, nature, and potential risks were carefully explained to the subjects. The subjects were requested to abstain from caffeine-containing beverages such as coffee, tea, and cola drinks for at least 24 h before the experiments as well as from exhaustive exercise within 48 h prior to the study. The Joint Commission of Ethics of the University of Turku and Turku University Central Hospital, and National Drug Agency approved the study protocol.

Table 4.1.1. S = sedentary, EA = endurance athlete, W= women, M= men, BMI = body mass index.

	I Mean±SD	II Mean±SD		III and IV	IV
Physical activity level	S	EA	S	S	S
Sex	W	M	M	M	M
Number of subjects	6	10	10	9	8
Age (years)	24 ± 3	25 ± 4	26 ± 4	25 ± 5	26 ± 2
Body mass (kg)	62.1 ± 4.6	78 ± 7	78 ± 7	76 ± 9	82 ± 8
Height (cm)	171.5 ± 2.3	182 ± 8	179 ± 7	184 ± 6	184 ± 4
BMI (kg $\cdot$ m^{-2})	21.0 ± 1.1	23.4 ± 0.9	24.4 ± 2.9	22.1 ± 1.9	26 ± 2
Body fat (%)	-	9.7 ± 2.4	19.0 ± 3.9	14 ± 5.3	-
VO2max (L/min)	2.4 ± 0.2	4.7 ± 0.3	3.5 ± 0.3	3.7 ± 0.7	-

4.2   Study design

All the studies started in the morning at approximately 8 am. Subjects fasted for approximately 3 hours before the measurements. Alcohol, caffeine, and any kind of strenuous physical activity were prohibited for the preceding 48 hours before the study.

Subjects completed the physical activity questionnaire modified from Baecke and co-workers (1982) as well as a health questionare. Thereafter, the study continued with blood samples (III, IV, V), resting ECG, and resting ECHO (III), after which the subject was positioned into the PET scanner. In leg studies, the middle thigh region, and in cardiac experiments the heart, was positioned to imaging area. Heart rate as well as blood pressure was also followed during the study and measured in every different study condition (Omron, M5-1, Omron Healthcare, Europe B.V. Hoofddorf, The Netherlands).

   The detailed study design of substudy I is shown in Figure 4.2.1. The experiment day started with a magnetic resonance imaging (MRI) study to obtain anatomical references from the femoral region (in studies III-V MRI was performed approximately one week before PET). Time control experiments were not performed (in any sub-study).
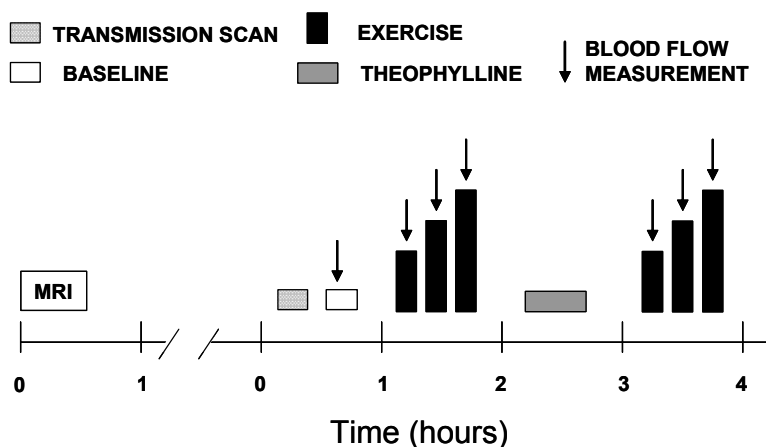
Figure 4.2.1. Study design. Before the PET studies, magnetic resonance imaging (MRI) for the femoral region was performed. PET measurements were started after a transmission scan at rest and blood flow was measured (arrows) first at baseline. Thereafter, blood flow was measured during one-legged intermittent isometric exercise with increasing work-loads (50, 100 and 150 N, respectively) with 15 min resting periods between the bouts of exercise. The same exercise protocol and blood flow measurements were repeated after 90 min rest and theophylline infusion.

   The protocol of sub-study II is shown in Figure 4.2.2. After the blood flow measurements with ^{15}O-H_2O, $A_{2A}R$ density was measured with a recently developed tracer – [7-methyl-(11C)-(E)-8-(3,4,5-trimethoxystyryl)-1,3,7-trimethylxanthine (^{11}C-TMSX) and PET (Mizuno *et al.*, 2005). In substudy III, skeletal muscle blood flow was measured first at resting baseline, then during femoral arterial infusion of high dose adenosine and finally during one-leg exercise with moderate-to high exercise intensity. In substudy IV, muscle blood flow was measured first under normal resting conditions and then during systemic hypoxia (14 % inspired O_2 in N_2; equivalent to altitude of ~3400 m). After these measurements at rest, blood flow was measured during one-leg dynamic exercise in a counterbalanced setting without and with locally administered adenosine receptor antagonism by aminophylline with the subject

breathing either normal room air or hypoxic gas. During systemic hypoxia, breathing of 14 % oxygen gas began five minutes before imaging or exercise. In substudy V PET measurements were first performed at baseline and thereafter during exercise without any drug infusions. 30 minutes later resting and exercising measurements were performed during NO blockade with *N*G-monomethyl-l-arginine (L-NMMA), and finally after 30 min recovery period, both resting and exercising measurements were again repeated under synergistic inhibition of L-NMMA and indomethacin, which blocks COX enzymes.
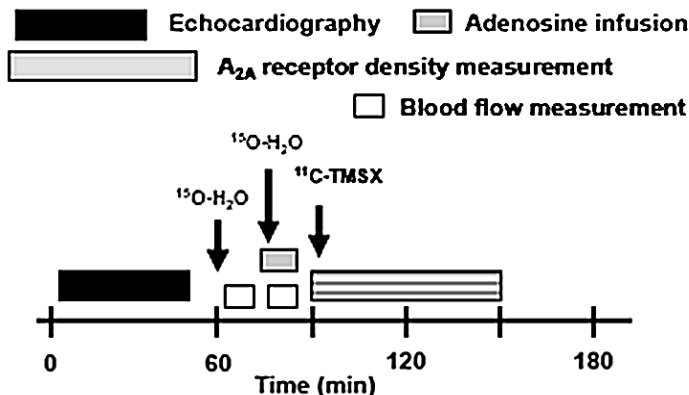
Figure 4.2.2. Study protocol in substudy II. After the blood flow measurements with ^{15}O-H$_2$O, A$_{2A}$R density was measured with a recently developed tracer – [7-methyl-(11)C]-(E)-8-(3,4,5-trimethoxystyryl)-1,3,7-trimethylxanthine (^{11}C-TMSX) and PET in highly trained endurance athletes and healthy untrained men.

4.3   Cannulations and blood sampling

The antecubital vein was cannulated for tracer administration in all studies before the PET experiments. A radial artery cannula was placed in the contralateral arm for blood sampling to quantify radioactivity. In sub-studies III-V, cannulas were also placed into the femoral artery under local anesthesia with standard hospital practices (below inguinal ligament, advanced 15 cm proximally) and into vein for local drug infusion (adenosine) and blood sampling, respectively. Subjects were then moved to the PET scanner in the position with the femoral region in the gantry. Blood samples (brachial artery and femoral vein) were drawn in each condition in a steady state situation in the middle of measurement and analysed for oxygen according to standardized hospital practizes (Radiometer ABL 835 blood gas analyzer). In substudy V, plasma glucose was determined with the glucose oxidase method (Analox GM9 Analyzer, Analox Instruments Ltd., London, U.K.). Serum FFA and plasma lactate were both measured by the enzymatic colorimetric methods (ACS-ACOD method, Wako Chemicals USA, Inc. VA, USA for FFA and Roche Diagnostics GmbH, Mannheim, Germany for lactate, respectively). Both methods were analyzed with the Roche Modular P800 automatic analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

4.4 Study drugs and infusions

In substudy I, theophylline was infused into the antecubital vein and continued for 30 min. The infused dose was 6. 9 mg/kg body weight, which has been previously shown to decrease limb blood flow during exercise and thus, effectively block adenosine receptors (Radegran & Calbet, 2001). After the cessation of theophylline infusion, the subjects were allowed to rest for another 30 minutes. Thereafter, the subjects were positioned back into the PET scanner and similar blood flow measurements during three exercise intensities as without theophylline were performed.

In substudy II, standard dose adenosine (140 µg $\cdot$ min$^{-1}\cdot$ kg body weight $^{-1}$) was infused intravenously. In substudy III, high dose adenosine (1 mg $\cdot$ min^{-1} $\cdot$ litre thigh volume^{-1}) was infused into the femoral artery for six minutes and based on the study by Rådegran & Calbet (2001) at rest and Barden *et al*. (2007) during maximal exercise (Barden *et al.*, 2007). This concentration has been shown to induce maximal femoral artery blood flow as measured with ultrasound Doppler. To elucidate the role of adenosine on exercise muscle blood flow, similar procedures for adenosine receptor blockade with aminophylline were used in substudy IV as previously described (Martin *et al.*, 2006b). In the study by Martin and colleagues, a 15 % reduction in blood flow was observed in mild handgrip exercise. Aminophylline (2 mg/min/per litre thigh volume) was infused into femoral artery three minutes before exercise and continued until the end of the imaging to induce local competitive inhibition of endogenous adenosine receptor binding and to minimize any confounding systemic influence of the drug.

In sub-study V, two drugs were used intra-arterially (femoral artery); to inhibit NO synthases, L-NMMA (Clinalfa, Laufelfingen, Switzerland) was infused intra-arterially five minutes before starting the exercise or other measurements (rest) and continued until the end of imaging with a concentration of 1.0 mg min^{-1} kg leg mass^{-1} and to inhibit cyclooxygenases, indomethacin (Confortid, Alphapharma, Denmark) with a concentration of 50 μg min^{-1} kg leg mass^{-1} (Mortensen *et al.*, 2007).

4.5 One-leg knee extensor exercises during PET imaging

In substudy I, subjects were moved to the PET scanner with the femoral region in the gantry and the left leg was fastened to a dynamometer (Diter Petkin, Oy Diter-Elektroniikka Ab, Turku, Finland) at a knee angle of 40 degrees. After a transmission scan, basal blood flow was measured while the subject was lying at rest. Thereafter, the subject was allowed to briefly become familiarized with the one-leg intermittent isometric knee-extension exercise model. The exercise model consisted of a 1 sec isometric contraction of the knee extensors followed by a 2 sec pause interval. The subject performed exercise at three different workloads (50, 100 and 150 N) and each load lasted 10 minutes with 5-min breaks in between. At all three intensities, blood flow was measured after five minutes of exercise. Instructions about maintaining the exercise intensity, rest and exercise periods were provided to the subject by LED lights and also cued by specific sounds from the dynamometer. After the first section of the

study, the subjects were removed from the PET scanner and a 90-min rest period followed.

In the substudies III-V exercise consisted of dynamic one-leg exercise in a supine position at 40 rpm (metronome) at an individually chosen workload with a knee extension range of motion of almost 90 degrees. Dynamic muscular work for m. quadriceps femoris consisted of a lifting phase and a subsequent braking phase back to the starting position. Exercise commenced three minutes before scanning to achieve a steady-state metabolic situation and continued until the end of imaging (for 2.5 min). In studies III-V subjects were also allowed to familiarize themselves with the dynamic exercise model used approximately one week before the PET day when MRI scanning was performed. During this pre-testing, appropriate exercise load was also chosen for each subject so that they could exercise for ~10 min without fatigue or discomfort.

5 METHODS

5.1 Positron emission tomography

PET is a nuclear imaging technique that enables quantitative analysis of biochemical and physiological processes non-invasively in humans in vivo. The technique is based on measuring the concentrations of short-lived positron-emitting radioisotopes within living tissues. Radioisotopes are normal physiological compunds or their analogs labelled with positron-emitting radionuclides, such as carbon-11, nitrogen-13, oxygen-15 or fluoride-18. Basically, any biological molecule can be labelled with these radionuclides, although radiochemistry may be challenging. When starting a PET study a transmission scan with external positron-emitting source is first peformed in order to correct the tissue attenuation. Thereafter, a positron-emitting radioisotope is injected (or inhaled) into the study subject and the concentration of the radioisotope in tissues is measured using the PET scanner. Radioisotopes used in PET have an excess of protons in the nucleus. These radioisotopes are thus unstable and the stable state is reached by the emission of a positron. The emitted positron travels a short distance until it encounters a tissue electron. The two particles compound and annihilate each other and the masses of the positron and electron are converted into energy in the form of two photons travelling in opposite directions. The energy of these two photons is in the form of gamma radiation. The PET scanner registers this event as a coincidence pair if both photons are detected at opposite detectors within a certain time limit. Detection of a coindicence pair indicates that the annihilation occurred along the line between these detectors. The PET scanner consists of a ring of detectors that surrounds a studied subject. The reconstruction software of the scanner reconstructs an image from all coincidence events measured at all angular and linear positions. This image depicts the localization and concentration of the radioisotope as a function of time within a plane of the region that was scanned.

5.1.1 Production of positron emitting tracers

$[^{15}O]H_2O$ and $[^{15}O]O_2$. $[^{15}O]$ ($t_{1/2}$ = 123 s) were produced with a low-energy deuteron accelerator Cyclone 3 (Ion Beam Application Inc., Louvain-la-Neuve, Belgium). $[^{15}O]$ was produced by $[^{14}N](d,n)[^{15}O]$ reaction using a target gas containing 99 % nitrogen and 1 % oxygen. H_2O was produced using the dialysis technique in a continuously working water module (Sipilä *et al.*, 2001). Sterility and pyrogenity tests were performed to verify the purity of the product. The radiochemical purity of the $[^{15}O]$ was approximately 97 %.

$[^{11}C]TMSX$. $[^{11}C]$acetate ($t_{1/2}$ = 20.4 min) was synthesized from $[^{11}C]$carbon dioxide and methyl magnesium bromide. The radiochemical purity of the final product exceeded 98 %. Distribution volume (DV) of $A_{2A}Rs$ in the cardiac left ventriculum (LV), thus the density of $A_{2A}R$ in LV, was determined according to the established principles of Mizuno et al. (Mizuno *et al.*, 2005) by intravenous bolus injection of ^{11}C-TMSX tracer and measured uptake of the tracer with dynamic PET imaging for 60 min

in two-dimensional mode. Plasma radioactivity was measured from the arterial blood and unaltered ^{11}C-TMSX in the plasma was analyzed by HPLC (Mizuno *et al.*, 2005).

5.1.2 Image acquisition

The PET scans were performed with two very similar scanners: the ECAT EXACT HR+ PET scanner (I-V) (Siemens/CTI, Knoxville, TN, USA) and GE Advance PET scanner (II) (General Electric Medical System, Milwaukee, WI, USA). In order to correct photon attenuation in the body, a five-minute transmission scan of the tissue in question was always performed with a removable ring source of [68]Ge before emission scans.

Myocardial perfusion at rest and during adenosine-infusion. $[^{15}O]H_2O$ was infused into the vein as a 10s bolus and dynamic 6-min PET scanning (6 x 5 s, 6 x 15 s, and 8 x 30 s time frames) was started simultaneously. After radioactivity decay, a 7-min intravenous adenosine infusion (140 µg · min^{-1} · kg^{-1}) was started. After one minute of adenosine infusion, $[^{15}O]H_2O$ was infused into the vein and another perfusion study was repeated in the same manner as in the resting measurement.

Skeletal muscle perfusion. $[^{15}O]H_2O$ was intravenously injected and a dynamic PET scanning lasted six minutes (6 x 5 s, 6 x 15 s, and 8 x 30 s time frames) and 2.5 minutes during exercise. The input function (blood activity curve) was obtained from arterial blood, which was continuously withdrawn with a pump. The radioactivity concentration in arterial blood was measured using a two-channel on-line detector system (Scanditronix, Uppsala, Sweden) that was cross-calibrated with an automatic gamma counter (Wizard 1480 3'', Wallac, Turku, Finland) and the PET scanner.

Skeletal muscle oxygen uptake. Skeletal muscle oxygen consumption was measured by the bolus inhalation technique (Nuutila *et al.*, 2000). Subjects inhaled a single bolus of the mixture of $[^{15}O]O_2$ and room air which was pumped into a rubber bladder. Dynamic skeletal muscle oxygen uptake scanning started simultaneously with the inhalation and lasted for seven minutes (6 x 5 s, 6 x 15 s, 6 x 30 s, and 2 x 60 s time frames). The input function (blood activity curve) was obtained from arterial blood, which was continuously withdrawn with a pump. The radioactivity concentration in arterial blood was measured using a two-channel on-line detector system (Scanditronix, Uppsala, Sweden) that was cross-calibrated with an automatic gamma counter (Wizard 1480 3'', Wallac, Turku, Finland) and the PET scanner.

5.1.3 Image processing

Reconstruction. All PET data were corrected for deadtime, decay, and measured photon attenuation. PET images were processed using 2D-Ordered Subsets Expectation Maximisation and Median Root Prior (2D OSEM-MRP) reconstruction with 150 iterations and the Bayesian coefficient 0.3.

Regions of interest (ROIs). Skeletal muscle ROIs covering the whole quadriceps femoris (QF) muscle group (study III-V) and the individual muscle regions in QF

(study I) were drawn into five subsequent cross-sectional planes in both thighs as previously described (Kalliokoski *et al.*, 2000). When drawing ROIs, great vessels were avoided. The muscle areas were defined as m. rectus femoris (RF), vastus intermedius (VI), vastus lateralis (VL), and vastus medialis (VM). Localisation of the different muscle compartments of the QF was done based on the MRI of corresponding femoral regions of the subjects in question. The ROIs in perfusion images were copied to images in oxygen studies (V). Myocardial ROIs (II) were drawn on four subsequent mid-heart cross-sectional planes covering the anterior, lateral, and septal walls of the left ventricle.

5.1.4 Calculation of myocardial perfusion (II)

MBF was calculated using the previously introduced methods employing the single-compartment model (Iida *et al.*, 1995). This method makes corrections for the limited recovery of the left ventricular (LV) ROI and spillover from the myocardial signals. Measured MBF reserve was defined as the ratio of the perfusion during adenosine administration to the flow at rest. Total MBF in the whole LV was determined as the product of the measured MBF and the LV mass measured with ECHO. In addition, for normalizing the effect of different LV work loads on MBF observed between the groups, individual measured baseline MBF was corrected by multiplying it with LV work obtained from the whole subject population and divided by the individual LV work. Total LV work was calculated as a product of MAP and cardiac output, and to obtain relative LV work per unit mass, total LV work was divided by LV mass. Coronary resistance was calculated by dividing the mean arterial pressure (MAP) by the respective perfusion value, and coronary vascular resistance index was defined as vascular resistance during adenosine infusion divided by resistance at baseline.

5.1.5 Calculation of skeletal muscle perfusion (I, III-V)

Parametric skeletal muscle perfusion image sets were calculated using the autoradiographic method (Ruotsalainen *et al.*, 1997) and at rest a 200 sec and during exercise a 90 sec optimized tissue integration time. The autoradiographic method has been widely used to numerically deduce the flow (f, ml/min/ 100 grams) from the following equation:

$$\int_0^T C_t(t)\,dt = \int_0^T f C_a(t) * \exp\left(-\frac{f}{pt}\right) dt.$$

The right side of the equation can be solved using the measured arterial time-activity curve ($Ca(t)$, nCi/gram) and an assumed fixed value for the distribution constant of water (p) of 0.99 in muscle tissue. The left side is the integrated tissue time activity concentration ($Ct(t)$, nCi/ gram) obtained from dynamic images. The symbol * denotes the convolution integral. A table look-up procedure from the left side to the right side finally gives a unique rate constant f within the integration time scale T. Blood flow was calculated pixel by pixel into parametric flow images, which were

produced by the autoradiographic technique described above. Based on the earlier unpublished data from Turku PET Centre, it can be calculated that the reproducibility (coefficient of variation) of exercise blood flow measurements is 8-10%.

5.1.6   Calculation of skeletal muscle oxygen uptake (V)

Skeletal muscle oxygen uptake was quantified with a non-linear fitting from the $[^{15}O]O_2$ data using a model which includes separate compartments for oxygen bound to myoglobin and hemoglobin (Nuutila *et al.*, 2000). Oxygen uptake was calculated using the equation $rMRO_2 = [O_2]a \cdot rOEF \cdot rMBF$, where MRO_2 is the regional muscle oxygen uptake, $[O_2]a$ is arterial oxygen concentration, rOEF is regional oxygen extraction fraction, and rMBF is regional muscle perfusion (Nuutila *et al.*, 2000).

5.1.7   Calculation of skeletal muscle perfusion heterogeneity (I, III-V)

For blood flow heterogeneity analysis, the voxel by voxel data (of corresponding analysis) from five planes were pooled and mean and standard deviation (SD) were calculated. The relative dispersion (RD) was calculated as the SD divided by the mean (Duling & Damon, 1987). By this analysis, the within-muscle blood flow heterogeneity was obtained from m. quadriceps femoris and individually from its four heads, as well as from the whole thigh and posterior muscle parts (ROI analysis). Heterogeneity among QF muscles (between-muscle heterogeneity) was calculated as above, but instead of voxel values, from the four mean blood flow values.

5.1.8   Magnetic resonance imaging (I, III-V)

Magnetic Resonance Imaging (MRI) was performed one week before the PET study in order to obtain an accurate volume of the thigh for calculation of the infused drug dose. MR images, displaying a high degree of soft tissue contrast, were also used as an anatomical reference during PET-image analysis. MR imaging was performed with a 1.5 Tesla MR-system (Gyroscan Intera Nova Dual, Philips Medical Systems, Best, The Netherlands) using the quadrature body coil. High resolution T1-weighted axial fast field (dual) echo images were obtained (slice thickness 5mm without slice gap) of the entire area of the right thigh. These images were processed using volume rendering software on a standard GE workstation (AW 4.4, GE Medical Systems, Milwaukee, WI, USA). Reconstruction of the axial images in the coronal and sagittal directions allowed for highly accurate marking of the muscle tissue borders and final muscle volume calculation.

5.1.9   Whole body maximal oxygen uptake (VO$_{2max}$)

Maximal oxygen uptake (VO$_{2max}$) was determined within three weeks from the PET measurements using an electrically braked cycle ergometer (Ergoline 800 S, Bitz, Germany) with direct respiratory measurements (Medikro 202; Medikro Oy, Kuopio, Finland) in a continuous incremental protocol. Initial exercise intensity was 50 W and was increased by 30 W every two minutes until exhaustion. A fingertip blood sample

was taken immediately and one minute after the exercise to analyze blood lactate (YSI 2300 STAT, YSI, Yellow Springs, USA). The highest value of oxygen uptake during the test (1-min collection) represents the VO_{2max}. The highest exercise load during the VO_{2max} test represents maximal power (W).

5.1.10 Echocardiography (II)

A comprehensive Doppler ECHO heart study was performed in sub-study II by an experienced cardiologist, who was blinded to the physical activity and the fitness status of the subjects. All measurements were made using Acuson Sequoia C512 ultrasound equipment (Siemens, California, USA). M-mode measurements of the LV were obtained using guidance by two-dimensional ECHO at end-systole and end-diastole, as recommended by the American Society of Echocardiography (Sahn *et al.*, 1978). LV mass was calculated as previously described (Devereux *et al.*, 1986). Left ventricular mass index was calculated as LV mass in grams divided by body surface area in square meters. The LV ejection fraction (LVEF) was calculated as the ratio of SV to end-diastolic volume. From the Doppler scan peak early (E) and peak atrial flow velocity (A) were measured and E/A -ratio was calculated by dividing E with A. The results of ECHO are expressed as the mean of the two independent measurements.

5.1.11 Other measurements

Subjects' height and weight were measured by standard procedures. The waist circumference was measured at the level of the umbilicus in the late exhalation phase and the hip circumference at the level of the greater trochanter in standing position. Four skin folds (subscapular, triceps, biceps, and suprailiac) were measured on the left side of the body with a caliper (The Harpenden Skinfold Caliper Model: HSK-BI.) and the percentage of body fat content was estimated according to Durnin & Womersly (Durnin & Rahaman, 1967). Finally, before any measurements, subjects completed a short physical activity and health questionnaire.

5.1.12 Statistical analysis

Statistical analyses were performed using the SAS/STAT statistical analysis program package, version 8.2 (SAS Institute Inc., Cary, NC, USA). Two-way or three-way ANOVA for repeated measurements was used for the analysis of statistical differences. If a significant main effect(s) was found, pair wise differences were identified using the Tukey-Kramer pos hoc procedure. In substudy II two-tailed Student's t-test was used for comparisons of group differences in body composition, fitness test parameters and echocardiographic measurements. Correlation values were calculated using Pearson's correlation coefficient. The significance level was set at $P \le 0.05$. Results are given as means±SD.

6   RESULTS

6.1   The effect of increasing exercise intensities and endogenous adenosine on skeletal muscle blood flow (I)

Blood flow and flow heterogeneity within and among QF muscles. At baseline, mean blood flow in the whole QF was 4.4 ± 1.8 ml /100g/min **(Fig. 6.1.1 A)**. Mean blood flow at rest was at the same level in VI (5.3 ± 2.1 ml $\cdot$ 100g muscle^{-1} $\cdot$ min^{-1}), VM (4.3 ± 1.2 ml $\cdot$ 100g muscle^{-1} $\cdot$ min^{-1}) and VL (4.2 ± 2.0 ml $\cdot$ 100g muscle^{-1} $\cdot$ min^{-1}). In RF (3.5 ± 1.6 ml $\cdot$ 100g muscle^{-1} $\cdot$ min^{-1}, $p = 0.02$) blood flow was significantly lower than in VI, but not compared to the other two muscles.
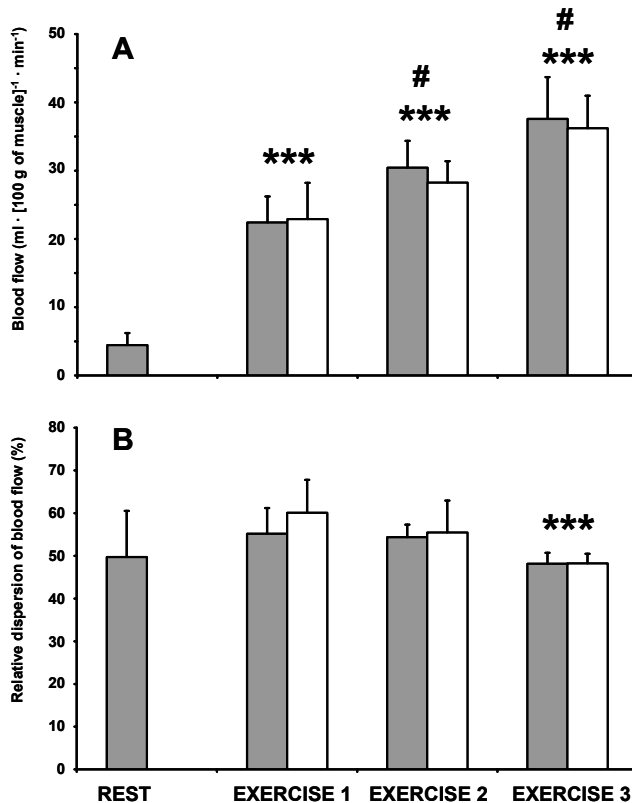
Figure 6.1.1. Mean blood flow (A) and flow heterogeneity (B) in the whole quadriceps femoris muscle group at rest and during exercise without (grey bars) and with theophylline (white bars). # $p < 0.001$ compared to preceding exercise intensity and *** $p < 0.001$ compared to rest (A) and exercise 1 and 2 (B). Blood flow heterogeneity in QF at rest (B) did not differ statistically from the values during exercise ($p = 0.06$) and theophylline did not affect mean blood flow or its heterogeneity at the level of whole QF. Figure is from original communication I.

Mean blood flow in the whole QF **(Fig. 6.1.1 A)** and its four compartments **(Fig. 6.1.2 B)** increased with workload similarly both without and with theophylline. Adenosine receptor blockade with theophylline did not have any significant effect on mean bulk blood flow in the whole QF ($p = 0.5$) or its compartments ($p = 0.2$).
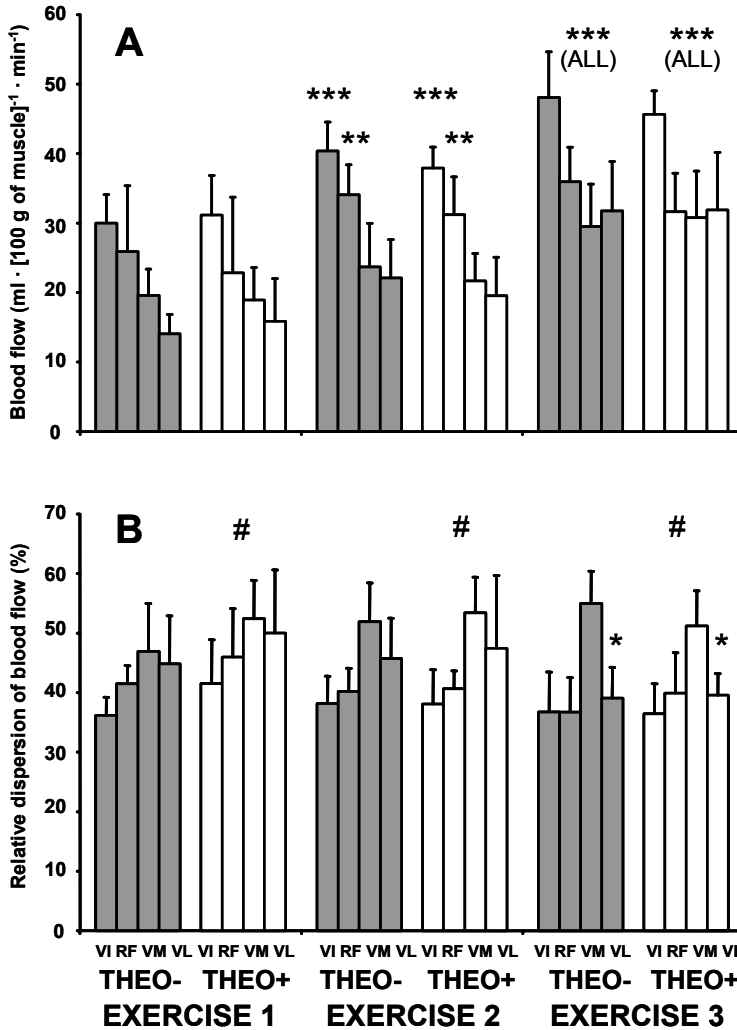
Figure 6.1.2. Mean blood flow (A) and flow heterogeneity (B) in different muscle compartments of quadriceps femoris muscle during exercise without (grey bars) and with theophylline (white bars). The order of the muscles from left to right in every measurement is vastus intermedius (VI), rectus femoris (RF), vastus medialis (VM) and vastus lateralis (VL) and THEO- exercise without and THEO+ exercise with theophylline during different exercise intensities. Blood flow increased with increasing exercise intensity in different muscles, *** $p < 0.001$ and ** $p < 0.01$ compared to the lowest exercise intensity. Theophylline increased blood flow heterogeneity in different muscle compartments of quadriceps femoris during exercise, # $p < 0.05$ compared to the exercise without theophylline, although increased heterogeneity seemed to confined mostly to the lowest exercise intensity ($p = 0.08$ between exercise intensities). Flow heterogeneity decreased with increasing exercise intensity in different muscles only in VL, * $p < 0.05$ compared to the lowest exercise intensity. Figure is from original communication I.

There were differences in blood flow increases between the four muscles with respect to the specific intensities of exercise (**Fig. 6.1.2 A**). In VI, blood flow increased significantly both from the lowest to the modest ($p < 0.001$) and from the modest to the highest ($p < 0.001$) exercise intensity. On the other hand, in RF, blood flow increased

significantly only from the lowest to the modest ($p < 0.01$), but not from the modest to the highest ($p = 1.0$) exercise intensity. In contrast to this, in VM and VL, blood flow did not increase significantly from the lowest to the modest ($p = 0.73$ and $p = 0.08$, respectively), but increased significantly from the modest to the highest ($p < 0.001$ in both) exercise intensity. In all muscles, blood flow increased significantly from the lowest to the highest exercise intensity ($p<0.001$).
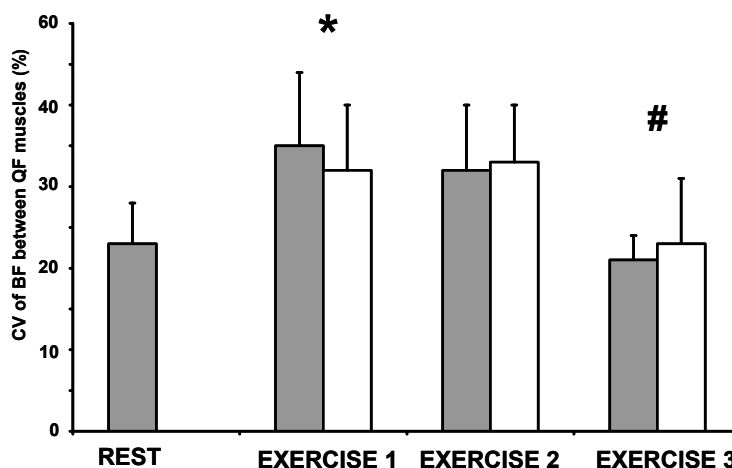

Figure 6.1.3. Coefficient of variation (CV) of blood flow (BF) among the quadriceps femoris (QF) muscles at rest and during exercise without (grey bars) and with theophylline (white bars). BF at rest was fairly uniformly distributed among the QF muscles, but acute low intensity exercise increased mean BF variability among the muscles, * $p = 0.05$. However, when the exercise intensity was increased, mean BF variability was reduced (# $p = 0.05$ compared to preceding exercise intensities) and despite totally different BF levels in QF, BF variability among the four muscles during the highest exercise intensity was similar compared to rest ($p = 0.96$). Figure is from original communication I.

At baseline, blood flow heterogeneity *within* (across) the whole QF was 50 ± 11 % **(Fig. 6.1.1 B)**. At the individual muscle level, there were no differences in blood flow heterogeneity between the four QF muscles at rest, except between VI and VM (CV in VI 36 ± 7 % and in VM 54 ± 15 %, $p = 0.02$) (CV in RF 41 ± 11 % and in VL 45 ± 15 %). In control exercise across all intensities, blood flow heterogeneity within the whole QF decreased **(Fig. 6.1.1 B)**, but out of the four individual parts of QF, *within-muscle* heterogeneity decreased only in the VL **(Fig. 6.1.2 B)**. This differentiation of responses was due to a dominant effect in VL. During exercise with adenosine receptor blockade, blood flow heterogeneity was unchanged from control exercise in the whole QF ($p = 0.35$, **Fig. 6.1.1 B**), but in the four individual parts of QF, heterogeneity increased slightly, but significantly ($p=0.03$, **Fig. 6.1.2 B**). Most of the increase in within-muscle heterogeneity seemed to be confined to the lowest exercise intensity ($p=0.08$).

Acute low intensity exercise increased *between-muscle* region heterogeneity compared to rest ($p = 0.05$, **Fig. 6.1.3**). However, mean blood flow heterogeneity among the four muscles decreased (blood flow became more uniform among the four muscles) with increasing (higher) exercise intensity **(Fig. 6.1.3)**. This was mainly due to a larger increase in mean blood flow in VL (124 % from the lowest to highest

exercise intensity) than in the other three muscles (VI 55 %, p = 0.02; RF 66 %, p = 0.06; VM 58 %, p = 0.03). Theophylline did not affect blood flow heterogeneity among the four QF muscles when exercise intensity was increased (p = 0.8).

At baseline, HR was 68 ± 10 bpm, BPs 121 ± 11, BPd 80 ± 12 and MAP 94 ± 10 mmHg. Values during exercise are shown in Table 6.1.

Table 6.1.1. Heart rate, blood pressure and related calculations during exercise. HR = heart rate, MAP = mean arterial pressure, BPs = systolic blood pressure, BPd = diastolic blood pressure, * $p < 0.05$ compared to previous exercise intensity, # $p < 0.05$ compared to EXE 1, ** $p < 0.01$ compared to EXE 1 and EXE 2.

	Without theophylline			With theophylline		
	EXE1	**EXE2**	**EXE3**	**EXE1**	**EXE2**	**EXE3**
HR (bpm)	75 ± 12	81 ± 12*	90 ± 15*	78 ± 18	84 ± 19*	98 ± 26*
MAP (mmHg)	94 ± 6	95 ± 7	97 ± 3#	91 ± 8	94 ± 9	100 ± 12#
BPs (mmHg)	121 ± 8	123 ± 12	126 ± 12#	120 ± 14	124 ± 15	130 ± 15#
BPd (mmHg)	81 ± 5	81 ± 6	82 ± 7#	77 ± 6	80 ± 7	85 ± 13#

6.2 Myocardial blood flow and adenosine receptor A_{2A} density in endurance athletes and untrained men (II)

Characteristics of the subjects. The groups were closely matched in age, height, weight, and BMI **(Table 6.2.1),** but differed in body fat percentage, fitness level, and the LV structural parameters as determined by ECHO **(Table 6.2.2)**. On average, both the total LV mass and the BSA-normalized LV mass were 71 % higher in ET compared to UT subjects.

Table 6.2.1. Characteristics of the subjects. BMI = Body mass index, BSA = body surface area and Power$_{max}$ = highest workload in VO$_{2max}$ test. ET = endurance trained, UT = untrained.

	ET	**UT**	**P value**
Age (years)	24.6 ± 3.6	25.7 ± 4.2	0.54
Height (cm)	182.3 ± 7.5	179.3 ± 6.6	0.35
Weight (kg)	78.1 ± 6.6	78.1 ± 7.0	1
BMI (kg/m^2)	23.4 ± 0.9	24.4 ± 2.9	0.33
BSA (m^2)	1.99 ± 0.13	1.97 ± 0.09	0.60
Body fat (%)	9.7 ± 2.4	19.0 ± 3.9	<0.001
Power$_{max}$ (W/kg)	4.7 ± 0.3	3.5 ± 0.4	<0.001
Power$_{max}$ (W)	367 ± 26	271 ± 35	<0.001
VO$_{2max}$ (ml/kg/min)	61.7 ± 5.0	46.2 ± 3.2	<0.001
VO$_{2max}$ (l/min)	4.7 ± 0.3	3.5 ± 0.3	<0.001

Table 6.2.2. Echocardiography parameters of the study subjects. EDD-l = longitudinal end diastolic diameter, EDD = end diastolic diameter, SWd = septal wall thickness in diastole, PWd = posterior wall thickness in diastole, AWd = anterior wall thickness in diastole, SV = stroke volume, CO = cardiac output, EF = ejection fraction, FS = fractional shortening, E/A ratio = ratio between early and late diastolic filling rate. ET = endurance trained, UT = untrained.

	ET	UT	P value
EDD-l (mm)	106.6 ± 7.1	96.9 ± 4.2	< 0.001
EDD (mm)	58.1 ± 2.5	53.2 ± 3.1	< 0.01
SWd (mm)	12.4 ± 0.8	9.0 ± 0.9	< 0.001
PWd (mm)	12.3 ± 0.7	9.1 ± 0.9	< 0.001
AWd (mm)	12.3 ± 0.7	9.0 ± 0.9	< 0.001
LV mass (g)	385 ± 43	225 ± 34	< 0.001
LV mass index (g/m^2)	193 ± 18	114 ± 13	< 0.001
SV (ml)	110 ± 8	91 ± 10	< 0.001
CO (l/min)	5.8 ± 1.1	5.6 ± 0.9	0.60
EF (%)	66.0 ± 4.5	66.3 ± 3.9	0.87
FS (%)	37.1 ± 2.9	37.3 ± 3.6	0.89
E/A ratio	1.8 ± 0.2	2.2 ± 0.6	0.07

Hemodynamic parameters during imaging. Heart rate, blood pressure, and LV work values during the PET measurements are shown in **Table 6.2.3**. Adenosine infusion increased heart rate, but had no effect on blood pressure parameters. All hemodynamic parameters except systolic blood pressure were similar between the groups during the imaging procedures.

Myocardial blood flow (Table 6.2.3). Adenosine infusion increased MBF ~5-fold both in the whole LV **(Fig. 6.2.3)** as well as in all three regions of it **(Fig. 6.2.2)** as compared to the resting values. ET had lower measured MBF per gram of tissue both at rest and during adenosine infusion **(Fig. 6.2.1 and 6.2.3)**, but MBF reserve was similar between the groups **(Fig. 6.2.3)**. When calculated for whole LV, total LV MBF was substantially higher in ET than UT both at baseline (217 ± 61 vs 155 ± 28 ml/min, respectively, $p < 0.001$) and during adenosine (1056 ± 272 vs 777 ± 223 ml/min, $p < 0.001$). When MBF was normalized for myocardial work, baseline MBF was significantly higher in ET than in UT ($p < 0.01$) **(Fig. 6.2.1)**. Coronary resistance was higher in ET than UT at baseline (156 ± 32 vs 122 ± 15 mmHg $\cdot$ ml $\cdot$ min^{-1} $\cdot$ g^{-1}, respectively, $p < 0.04$), but the difference was not statistically significant during adenosine-induced hyperemia (34 ± 11 and 25 ± 7 mmHg $\cdot$ ml $\cdot$ min^{-1} $\cdot$ g^{-1} in ET and UT, respectively, $p = 0.17$) and the coronary vascular resistance index was similar in both groups (0.23 ± 0.08 in ET and 0.21 ± 0.06 in UT, $p = 0.65$).

Table 6.2.3. Hemodynamic variables during the PET measurements. REST, ADO and ^{11}C-TMSX = values at baseline, during adenosine and ^{11}C-TMSX infusions, respectively, HR = heart rate, BPs = systolic blood pressure, BPd = diastolic blood pressure, MAP = mean arterial blood pressure, and LV work = left ventricular myocardial work, MBF = myocardial blood flow. Adenosine increased HR as well as relative and total LV work significantly from rest to ADO in both groups, †p < 0.001. Per cent increase in these parameters was similar between the groups (~75%). However, adenosine induced no changes in BPs, BPd and MAP, but ET had higher BPs, and lower HR and LV work in every measurement, *** p < 0.001, ** p < 0.01, * p < 0.05 compared to UT.

	REST		ADO		^{11}C-TMSX	
	ET	UT	ET	UT	ET	UT
HR (bpm)	47 ± 7***	59 ± 8	80 ± 9†***	117 ± 10†	47 ± 6***	61 ± 8
BPs (mmHg)	123 ± 7*	115 ± 11	127 ± 8*	117 ± 10	126 ± 12*	115 ± 11
BPd (mmHg)	64 ± 5	68 ± 7	66 ± 5	67 ± 8	70 ± 9	69 ± 8
MAP (mmHg)	84 ± 4	84 ± 8	86 ± 5	84 ± 9	89 ± 9	84 ± 8
LV work ($mmHg \cdot L\ min^{-1}\ g^{-1}$)	1.1 ± 0.2***	2.0 ± 0.4	2.0 ± 0.2†***	3.5 ± 0.6†	1.2 ± 0.2***	2.1 ± 0.5
Total LV work ($mmHg \cdot L\ min^{-1}$)	433 ± 79	448 ± 82	759 ± 89†	765†	474 ± 103	456 ± 85
MBF ($ml \cdot min^{-1} \cdot g^{-1}$)	0.56 ± 0.13***	0.69 ± 0.09	2.75 ± 0.75***	3.48 ± 0.94	-	-
Normalised MBF ($ml \cdot min^{-1} \cdot g^{-1}$)	0.79 ± 0.16**	0.56 ± 0.13	-	-	-	-
Total MBF ($ml \cdot min^{-1}$)	217 ± 61***	155 ± 28	1056 ± 272***	777 ± 223	-	-
Coronary resistance ($mmHg \cdot ml \cdot min^{-1} \cdot g^{-1}$)	156 ± 32*	122 ± 15	34 ± 11	25 ± 7	-	-

Adenosine $A_{2A}R$ density. The total volume of cardiac $A_{2A}R$ in LV was significantly higher in ET compared to UT **(Fig. 6.2.4A)**, but the DV of $A_{2A}R$ (receptor density) did not differ between the groups **(Fig. 6.2.4B)**. Spillover and partial volume corrected DV values did not differ from the reported uncorrected values (p = 0.7). A relatively small fraction of ^{11}C-TMSX was metabolised during the scan duration (< 20 % at the 60 min).

Relations of measured parameters. In the whole subject population, LV mass and LV mass index correlated negatively with MBF at rest (r = -0.46, p = 0.04 and r = -0.49, p = 0.03, respectively) and almost significantly during adenosine infusion (r = -0.43, p = 0.057 and r = -0.42, p = 0.067, respectively), but not with MBF reserve. In addition, a negative correlation between MBF at rest and VO_{2max} (r = -0.59, p < 0.01) was observed. However, significance levels of these correlations were not reached when parameters were correlated separately in the two groups.

Measured MBF at rest or during adenosine administration in the LV did not correlate significantly with $A_{2A}R$ DV values in the whole study population or within the groups (p in all = NS). In all subjects, DV in the whole LV tended to however be negatively related to measured MBF reserve (r -0.46, p = 0.07). In addition, there was a tendency for a negative relation between whole LV ^{11}C-TMSX DV values and LV mass in ET (r = -0.63, p = 0.07) but not in UT (r = -0.38, p = 0.27). Finally, the fitter the athlete was, the lower MBF was during adenosine induced hyperemia **(Figure 6.2.5)**.
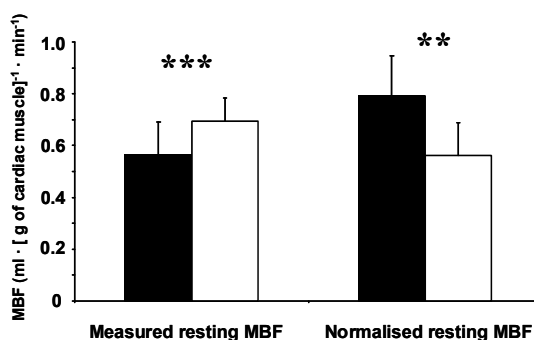

Figure 6.2.1. Measured and normalised myocardial blood flow (MBF) at rest. Black bars = ET, white bars = UT. ** p < 0.001 and *** p < 0.001. After considering the significantly lower myocardial work load of ET, ET showed significantly higher MBF per gram of cardiac tissue at rest. Figure is from original communication II.
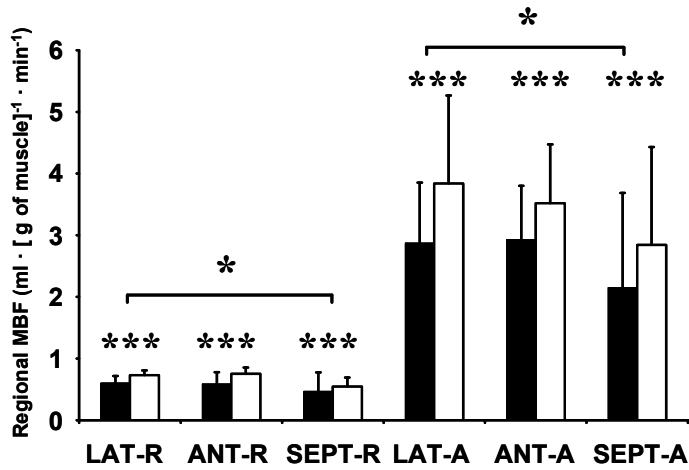
Figure 6.2.2. Regional MBF. ET (black boxes) had lower MBF in all three region of LV compared to UT men (white boxes) both at rest and during ADO, *** $p < 0.001$. LAT-R = MBF in lateral part of LV at rest and during ADO (LAT-A), ANT-R = MBF in anterior part of LV at rest and during ADO (ANT-A) and SEPT-R = MBF in septal part of LV at rest and during ADO (SEPT-A). Among all subjects, MBF in lateral region of LV was higher compared to septum, * $p < 0.05$ both at rest and during ADO, but not compared to the anterior region ($p = 0.94$). MBF between the anterior and septal regions did not differ statistically significantly ($p = 0.06$). Figure is from original communication II.
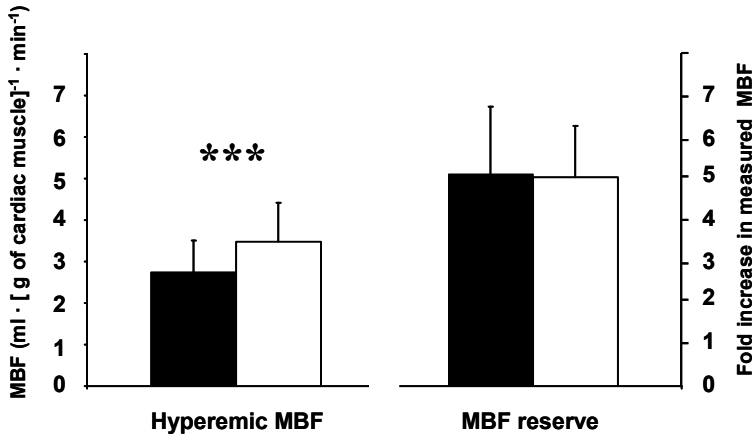
Figure 6.2.3. Adenosine induced hyperemic myocardial bood flow (MBF) and measured MBF reserve. Black bars = ET, white bars = UT. Adenosine increased MBF significantly compared to resting MBF (Fig. 1) in both groups, $p < 0.001$. Endurance athletes had significantly lower hyperemic MBF, but their MBF reserve calculated from measured MBF values was similar compared to untrained men. ***, $p < 0.001$. Figure is from original communication II.
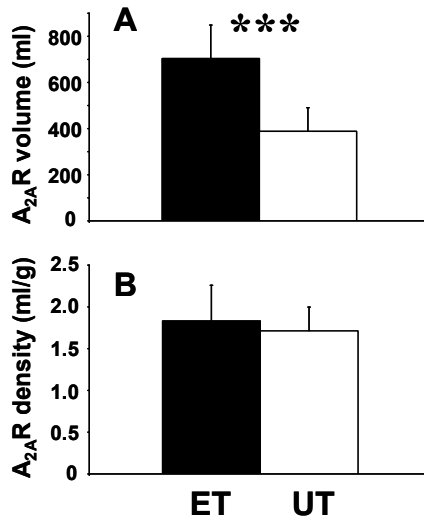
Figure 6.2.4. Total volume (A) and density (B) of myocardial adenosine $A_{2A}R$. No difference in $A_{2A}R$ density was found, but ET had higher total volume of $A_{2A}Rs$. Figure is from original communication II.
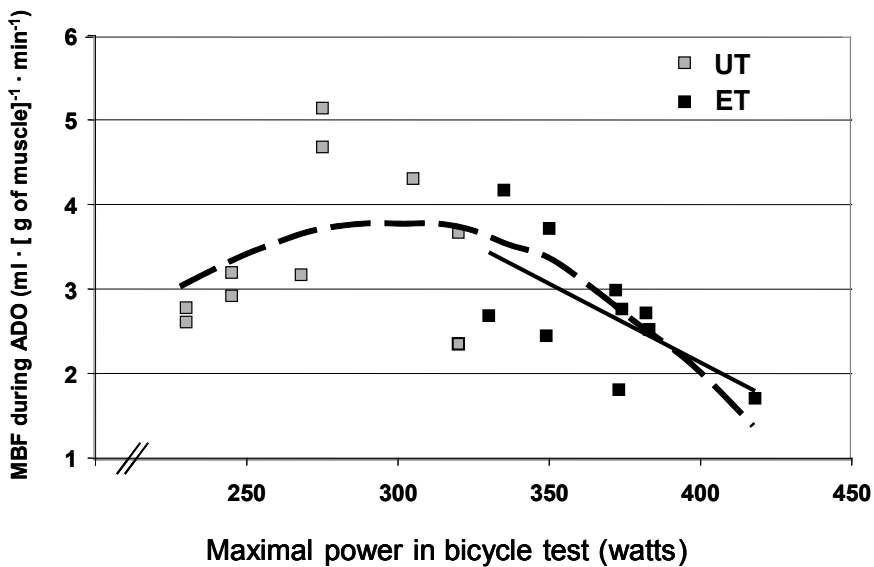
Figure 6.2.5. Relationship between maximal sustained power obtained during VO_{2max} bicycle test (W_{max}) and myocardial blood flow during adenosine infusion (MBF during ADO). In UT, W_{max} was not related to MBF during ADO (r = 0.28, p = 0.44), but in ET these parameters were inversely related (black boxes and line, r = 0.64, p = 0.04). Interestingly, if the subjects are considered showing a continuum of inborn and acquired fitness, a curvilinear relation between fitness level and MBF during adenosine-induced hyperemia in the whole subject population is observed (black dashed line, $y = 0.0002x^2 + 0.0981x - 10.824$, $r^2 = 0.42$, p = 0.002). Figure is from original communication II.

6.3 The effects of exogenous adenosine and voluntary exercise on skeletal muscle perfusion and perfusion heterogeneity (III)

The characteristics of the subjects are shown in **Table 6.3.1**. **Table 6.3.2** summarizes the effects of adenosine infusion on heart rate, blood pressure and blood oxygen. The infusion of adenosine into the femoral artery increased heart rate moderately compared to rest but blood pressure was unaffected. Adenosine increased muscle blood flow significantly, on average 14-fold from 2.9 ± 1.6 ml $\cdot$ 100^{-1}g $\cdot$ min^{-1} at baseline to 40.0 ± 7.2 ml $\cdot$ 100^{-1}g $\cdot$ min^{-1} during adenosine **(Figure 6.3.3A)**. The blood flow increase from baseline to adenosine varied from 5 to 30-fold individually **(Figure 6.3.1)**. With almost every subject capillary blood flow values of over 400 ml $\cdot$ 100^{-1}g $\cdot$ min^{-1} were seen in the deepest regions of the thigh musculature **(Figure 6.3.2)**. Calculated *total thigh muscle* blood flow was 0.16 ± 0.06 L $\cdot$ min^{-1} at rest and 2.3 ± 0.6 L $\cdot$ min^{-1} during adenosine infusion. Vascular resistance decreased and conductance increased significantly during adenosine infusion **(Table 6.3.2)**.

Table 6.3.1. Characteristics of the subjects.

Age (yrs)	25 ± 5
Height (cm)	184 ± 6
Weight (kg)	76 ± 9
BMI (kg/m^2)	22.1 ± 1.9
Body fat percent (%)	14.4 ± 5.3
VO_{2max} (L/min)	3.7 ± 0.7
VO_{2max} (ml/min/kg)	49 ± 9
Peak power output (W)	290 ± 50
Whole thigh volume (L)	7.3 ± 1.2
Thigh muscle volume (L)	5.5 ± 1.0
Thigh muscle Weight (kg)	5.8 ± 1.0
Thigh muscle proportion (%)	76 ± 14

There were large inter- and intraindividual differences in blood flow distribution and heterogeneity in response to adenosine as illustrated in **Figures 6.3.1 and 6.3.2.** Blood flow heterogeneity increased in subjects who had relatively low resting heterogeneity, while subjects who had relatively high resting heterogeneity showed an opposite response **(Figure 6.3.4B)**. Arterial oxygen content remained similar to baseline during adenosine infusion **(Table 6.3.2)**, but venous oxygen content significantly increased, almost reaching the level of arterial oxygen content ($p = 0.07$ for the difference between arterial and venous). Oxygen consumption of the leg remained unchanged ($p = 0.26$) and, thus, there was an oxygen extraction of just 11 ± 14 millilitres per litre (6 ± 7 %) during adenosine infusion.

Blood flow in the resting contralateral leg during adenosine infusion was reduced to approximately one third from a baseline value of 3.1 ± 1.9 to 1.3 ± 1.3 ml $\cdot$ 100^{-1}g $\cdot$

min^{-1} (p < 0.001), while blood flow heterogeneity increased substantially at the same time (from 56 ± 12 % to 109 ± 32 %, p = 0.02, **Figure 6.3.2)**.

Table 6.3.2. The effect of adenosine (ADO) and exercise (EXE) on heart rate, blood pressure, and blood oxygen. HR = heart rate, MAP = mean arterial pressure, BPs = systolic blood pressure, BPd = diastolic blood pressure, VR = vascular resistance (QF), VC = vascular conductance (QF), Oxygen saturation and content-A = arterial oxygen saturation and content, Oxygen saturation and content-V = venous oxygen saturation and content, OEF = Oxygen extraction fraction, VO2 = oxygen consumption, * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to rest, † p < 0.05 compared to adenosine, § p < 0.05 compared to rest and exercise, # p < 0.001 compared to both rest and adenosine.

	BASELINE	ADO	EXE
HR (bpm)	61 ± 10	78 ± 9***	92 ± 12*** †
MAP (mmHg)	91 ± 7	95 ± 8	108 ± 6*** †
BPs (mmHg)	125 ± 9	133 ± 11	146 ± 7**
BPd (mmHg)	74 ± 6	76 ± 7	90 ± 9*** †
VR (mmHg • [ml/100g/min]$^{-1}$)	39 ± 19	2 ± 1§	3± 1**
VC ([ml/100g/min] • mmHg^{-1})	0.03 ± 0.02	0.5 ± 0.1§	0.3 ± 0.1***
Oxygen saturation-A (%)	98 ± 1	98 ± 1	98 ± 1
Oxygen content-A (ml/L)	199 ± 9	202 ± 7	205 ± 9
Oxygen saturation-V (%)	76 ± 9	93 ± 7§	43 ± 16#
Oxygen content-V (ml/L)	152 ± 23	191 ± 14§	88 ± 32#
Oxygen extraction (ml)	48 ± 16	11 ± 14§	117 ± 33#
OEF (%)	24 ± 9	6 ± 7§	57 ± 16#
VO$_2$ (ml/100g/min)	0.1 ± 0.1	0.4 ± 0.5	4.1 ± 1.0#

The effects of exercise. Blood pressure and heart rate increased during the knee-extension exercise **(Table 6.3.2)**. Muscle blood flow in m. quadriceps femoris during exercise (36 ± 9 ml$^{.}$min^{-1} per 100g^{-1}) was higher than at baseline, and blood flow did not differ statistically significantly between adenosine infusion and exercise **(Figure 6.3.3A)**, although a tendency for increased flow during adenosine was found (p = 0.07)**.** Blood flow heterogeneity in m. quadriceps femoris was however significantly higher during adenosine infusion compared to exercise, but similar to rest **(Figure 6.3.3B)**. Vascular resistance decreased and conductance increased from baseline to exercise and resistance was lower and conductance higher with adenosine than during exercise **(Table 6.3.2)**.

Relationships between parameters. Blood flow during adenosine infusion correlated significantly to systemic VO$_{2max}$ **(Figure 6.3.4A)**, but blood flow and oxygen extraction fraction at rest, and heterogeneity at rest or during adenosine infusion were all unrelated to VO$_{2max}$. It was of note that oxygen extraction fraction correlated positively with blood flow heterogeneity at rest **(Figure 6.3.4C)**, but not during

adenosine or exercise. The change in heterogeneity from baseline to adenosine infusion tended to be inversely related to the change in magnitude of blood flow increase (r = 0.62, p = 0.09).
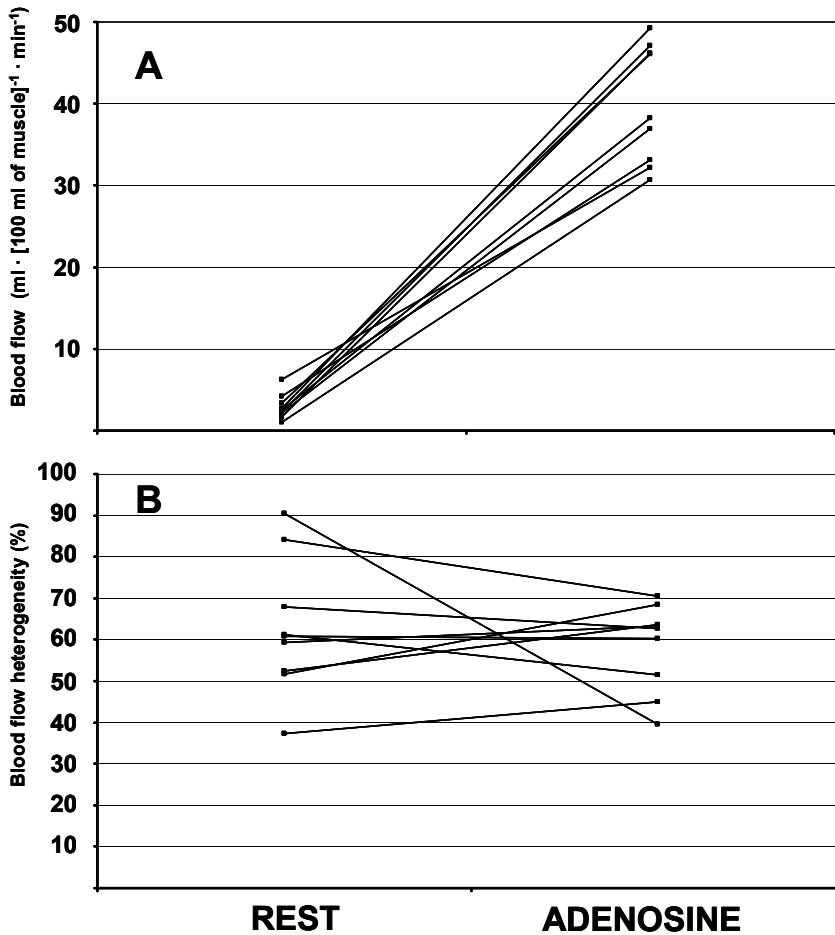

Figure 6.3.1. The individual blood flow (A) and flow heterogeneity (B) responses to adenosine infusion. Although variation was increased from rest, blood flow was enhanced significantly in every subject with adenosine infusion. Despite the increased mean blood flow, flow heterogeneity did not, however, change during adenosine infusion. Figure is from original communication III.
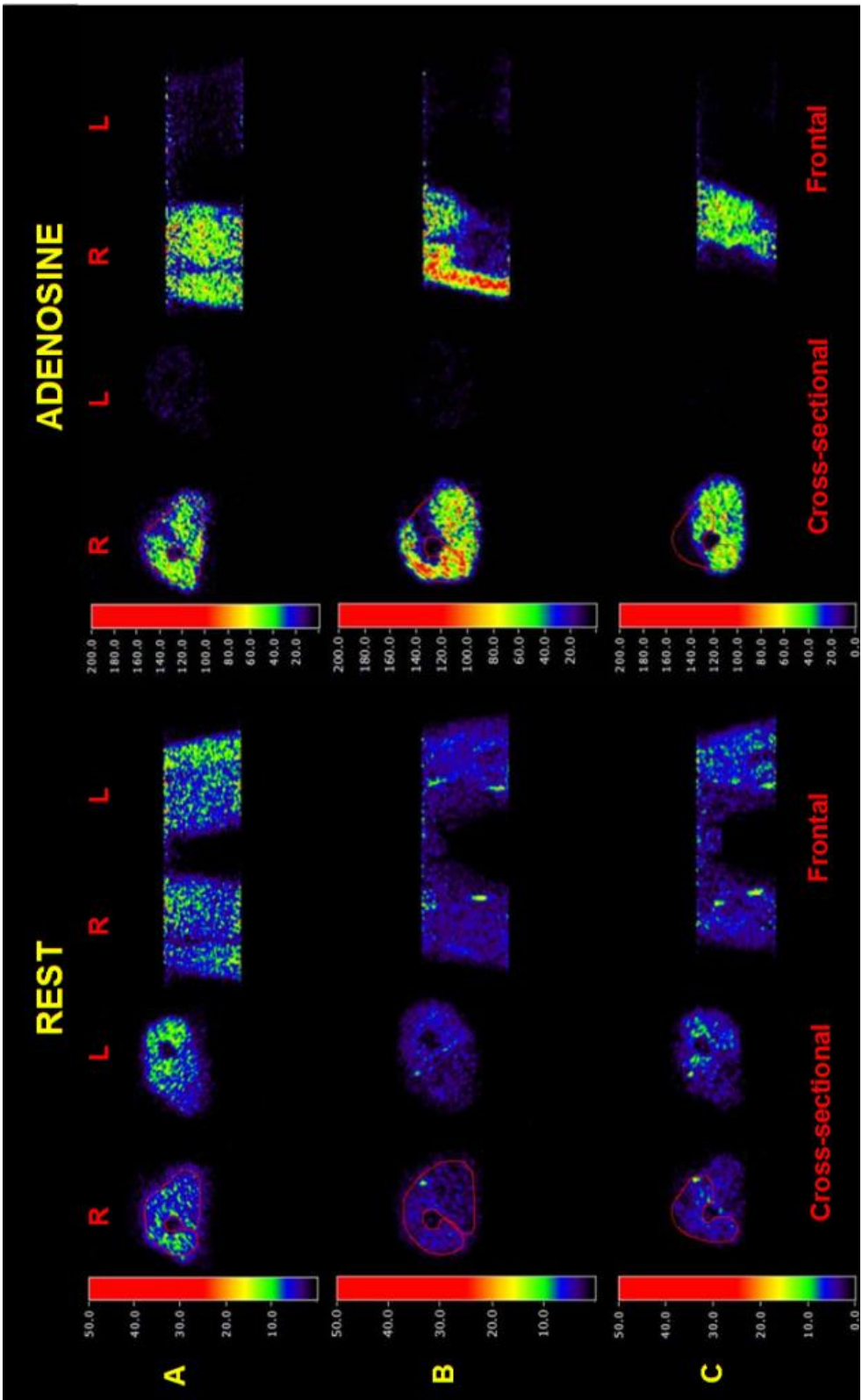
Figure 6.3.2 (Previous page). Three representative individual responses to femoral arterial infusion of adenosine in the middle thigh region. A. A subject who showed the most uniform distribution response to adenosine. **B.** A subject showing a 'horse shoe' response to adenosine. Despite the lack of clearly increased perfusion near the bone, a maximal pixel blood flow value of 457 ml·100g^{-1}·min^{-1} was documented in the highest blood flow areas while averaged thigh muscle blood flow was 49 ml·100g^{-1}·min^{-1} in this subject. **C.** Differing from other non-uniform responses (B) one subject showed a response where the upper part of the QF muscle (one third of the QF, *m. rectus femoris* in particular) was almost totally unaffected by adenosine. The blood flow was doubled from rest in the black upper areas, but it was clearly less than in other parts. This subject also had a distinct oxygen extraction response since venous oxygen saturation remained at 78 % while in all others it was increased > 94 % during adenosine. This could otherwise be interpreted as an increase in oxygen consumption if this individual response has not been elucidated (Fick principle). In general, decreased perfusion and increased pixel-by-pixel heterogeneity is a universal response in the left (L) leg when adenosine is infused in the right (R) leg. Figure is from original communication III.
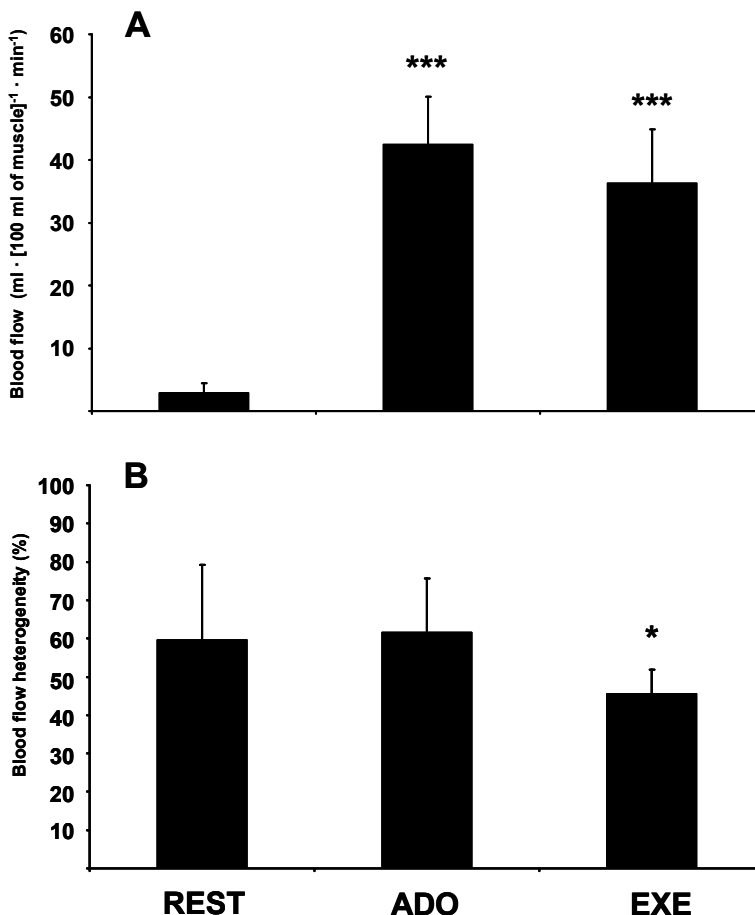
Figure 6.3.3. The effect of adenosine (ADO) and exercise (EXE) on m. quadriceps femoris blood flow (A) and its heterogeneity (B). Adenosine increased mean blood flow to a similar level to exercise, but blood flow heterogeneity was significantly higher during infusion than during exercise. *** compared to REST, * p < 0.05 between ADO and EXE.
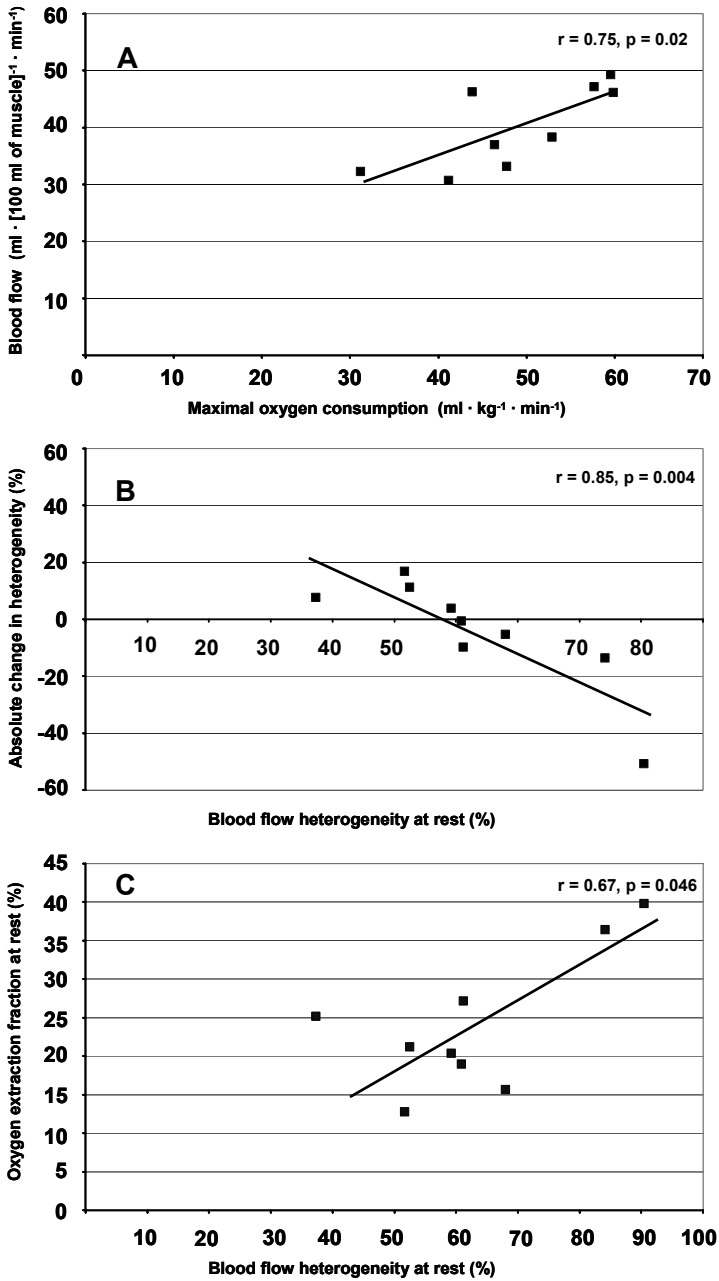
Figure 6.3.4. The relationship between thigh muscle blood flow during adenosine and systemic maximal oxygen consumption (A), absolute change in heterogeneity during adenosine in relation to heterogeneity at rest (B), and the relationship of oxygen extraction fraction and blood flow heterogeneity at rest (C). Adenosine-induced thigh muscle blood flow correlated significantly with maximal systemic aerobic power (A) suggesting increased peripheral vascular capacity in fit subjects. Blood flow heterogeneity at rest was inversely related to adenosine-induced change in heterogeneity (B). Blood flow heterogeneity was related to the oxygen extraction fraction irrespective of blood flow levels at rest (C) but not during adenosine or exercise. Figure is from original communication III.

6.4 The role of adenosine in regulating exercising muscle blood flow during systemic moderate hypoxia (IV)

Heart rates and blood pressures at rest and during exercise are shown in **Tables 6.4.1 and 6.4.2**, respectively. Hypoxia did not change mean thigh muscle blood flow at rest $(2.9 \pm 1.6 \text{ ml/100g/min}$ vs $3.3 \pm 1.4 \text{ ml/100g/min}$, $p = 0.62$, normoxia vs hypoxia) and did not have an effect on vascular resistance ($p = 0.86$) or conductance ($p = 0.71$). Hypoxia decreased arterial oxygen content **(Table 6.4.1)**, which was followed by a concomitant decrease in venous oxygen content while oxygen extraction remained unchanged ($p = 0.6$). Hypoxia did not affect blood flow heterogeneity at rest (63 ± 16 % in normoxia and 60 ± 13 % in hypoxia, $p = 0.72$).

Table 6.4.1. The effect of hypoxia (HYPO) at rest on heart rate, blood pressure, vascular resistance and conductance, and blood oxygen. HR = heart rate, MAP = mean arterial pressure, BPs = systolic blood pressure, BPd = diastolic blood pressure, VR = vascular resistance, VC = vascular conductance, Oxygen saturation and content-A = arterial oxygen saturation and content, Oxygen saturation and content-V = venous oxygen saturation and content, OEF = Oxygen extraction fraction, VO2 = oxygen consumption, * $p < 0.05$, ** $p < 0.01$ compared to rest.

	BASELINE	HYPO
HR (bpm)	61 ± 10	69 ± 10
MAP (mmHg)	91 ± 7	98 ± 12
BPs (mmHg)	125 ± 9	137 ± 18
BPd (mmHg)	74 ± 6	79 ± 10
VR (mmHg • [ml/100g/min]$^{-1}$)	39 ± 19	37 ± 22
VC ([ml/100g/min] • mmHg^{-1})	0.03 ± 0.02	0.04 ± 0.02
Oxygen saturation-A (%)	98 ± 1	91 ± 5**
Oxygen content-A (ml/L)	199 ± 9	186 ± 13**
Oxygen saturation-V (%)	76 ± 9	71 ± 10*
Oxygen content-V (ml/L)	152 ± 23	141 ± 18*
Oxygen extraction (ml/L)	48 ± 16	45 ± 14
OEF (%)	24 ± 9	24 ± 8
VO$_2$ (ml/100g/min)	0.1 ± 0.1	0.1 ± 0.1

Table 6.4.2. Heart rate, blood pressure, vascular resistance and conductance, and blood oxygen during exercise in normoxia (NORMO) and hypoxia (HYPO) without and with intra-arterial aminophylline infusion. HR = heart rate, MAP = mean arterial pressure, BPs = systolic blood pressure, BPd = diastolic blood pressure, VR = vascular resistance, VC = vascular conductance, Oxygen saturation and content-A = arterial oxygen saturation and content, Oxygen saturation and content-V = venous oxygen saturation and content, OEF = Oxygen extraction fraction, VO2 = oxygen consumption, * $p < 0.05$, ** $p < 0.01$ compared to NORMO.

	Without aminophylline		*With aminophylline*	
	NORMO	**HYPO**	**NORMO**	**HYPO**
HR (bpm)	92 ± 12	102 ± 10*	97 ± 20	110 ± 13*
MAP (mmHg)	108 ± 6	112 ± 10	105 ± 7	107 ± 8
BPs (mmHg)	146 ± 7	152 ± 11	147 ± 12	149 ± 13
BPd (mmHg)	90 ± 9	92 ± 12	83 ± 7	86 ± 11
VR (mmHg • [ml/100g/min]$^{-1}$)	3.2 ± 1.0	2.8 ± 0.4	2.8 ± 0.4	2.9 ± 0.9
VC ([ml/100g/min] • mmHg^{-1})	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Oxygen saturation-A (%)	98 ± 1	88 ± 5**	99 ± 0.4	89 ± 4**
Oxygen content-A (ml/L)	205 ± 9	182 ± 11***	208 ± 24	182 ± 8***
Oxygen saturation-V (%)	43 ± 16	37 ± 13*	48 ± 17	41 ± 18*
Oxygen content-V (ml/L)	88 ± 32	78 ± 26*	96 ± 31	83 ± 35*
Oxygen extraction (mL/L)	117 ± 33	101 ± 29*	114 ± 44	96 ± 37*
OEF (%)	57 ± 16	56 ± 16	53 ± 18	53 ± 20
VO$_2$ (ml/100g/min)	4.1 ± 1.0	4.0 ± 1.3	4.4 ± 1.9	3.9 ± 1.9

Mean blood flow and oxygen consumption in working QF muscle was higher during exercise than at rest ($p < 0.001$). Oxygen saturation (~88 %) and oxygen content of arterial blood was significantly lower during one-leg exercise in hypoxia compared to normoxia **(Table 6.4.2)**. Arterial-venous oxygen extraction was reduced under hypoxic exercise ($p = 0.02$), but aminophylline had no effect on extraction ($p = 0.92$). Blood flow was higher in QF in hypoxic than normoxic exercise ($p = 0.02$), but aminophylline did not affect mean blood flow in exercising muscle either in normoxia or hypoxia ($p = 0.5$) **(Figure 6.4.2A)**. Muscle oxygen consumption was similar in all four exercise interventions (Table 6.4.2, $p > 0.15$). Blood flow heterogeneity in working QF muscle tended to be significantly reduced compared to rest (60 ± 19 % normoxia at rest and 46 ± 6 % normoxia during exercise $p = 0.055$), but neither hypoxia nor aminophylline changed this heterogeneity during exercise ($p > 0.61$, **Figure 6.4.2B**). There were no differences in vascular resistance or conductance between different exercise interventions **(Table 6.4.2)**, but vascular resistance decreased and conductance increased from rest. It was of importance that the hypoxia-induced increase in blood flow confined only to working QF muscle since blood flow in the posterior part of thigh muscles was unchanged between different exercises studied ($p > 0.4$, **Figure 6.4.3A**).
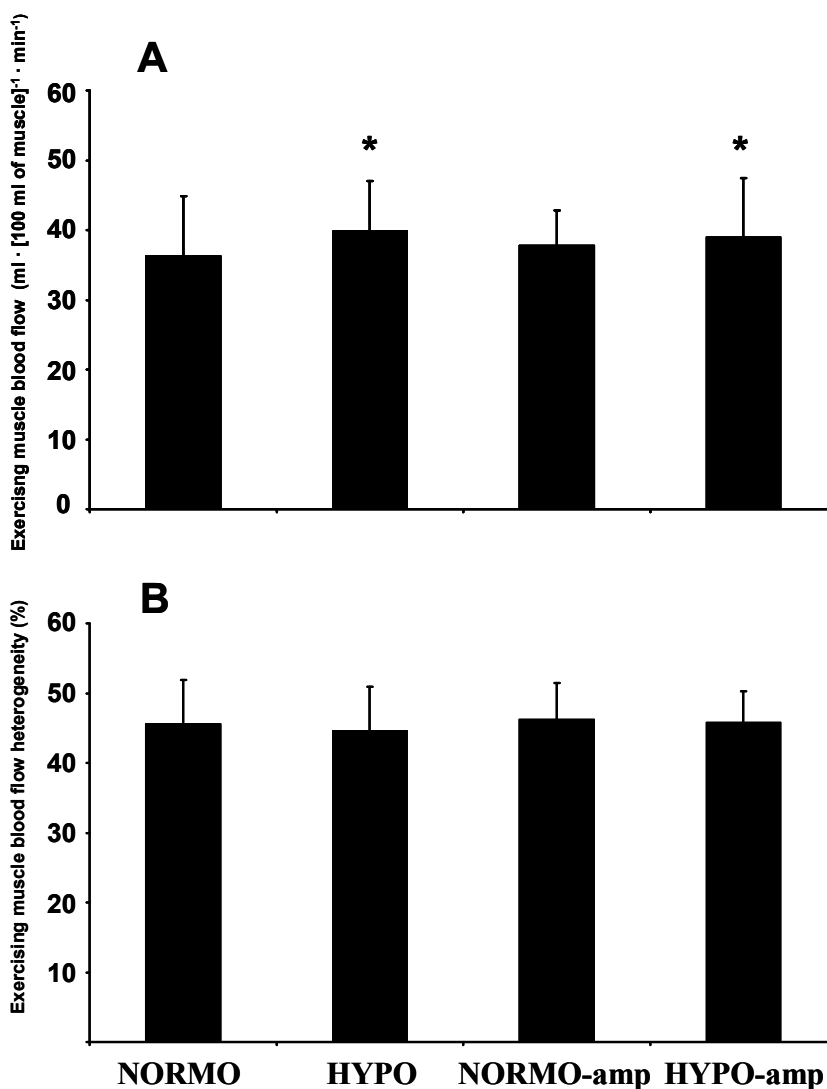
Figure 6.4.2. The effect of hypoxia (HYPO) and aminophylline (amp) on m. quadriceps femoris blood flow (A) and its heterogeneity (B) during one-legged exercise. Hypoxia increased blood flow in exercising m. quadriceps femoris, but aminophylline did not affect it either in normoxia or hypoxia. Despite increased blood flow in hypoxia, exercising muscle flow heterogeneity was similar to normoxic exercise. NORMO = normoxia, -amp = under aminophylline blockade.

Posterior hamstring muscle blood flow during the normoxic control exercise was comparable to flow at rest **(Figure 6.4.3A)**. Blood flow heterogeneity was however increased significantly from rest to exercise in the posterior muscles of the thigh, but there were no changes in posterior muscle blood flow heterogeneity between the different exercise interventions **(Figure 6.4.3B)**. Vascular conductance or resistance did not change significantly in the posterior muscles in any condition studied **(Table**

6.4.3). Mean blood flow, or vascular conductance or resistance in the whole thigh musculature of the resting contralateral leg was not changed significantly from rest to one-leg normoxic control exercise **(Table 6.4.3)**, but heterogeneity of blood flow was markedly increased **(Table 6.4.3)**. There were, however, no changes between different exercise conditions in blood flow or its heterogeneity during exercise **(Table 6.4.3)**.
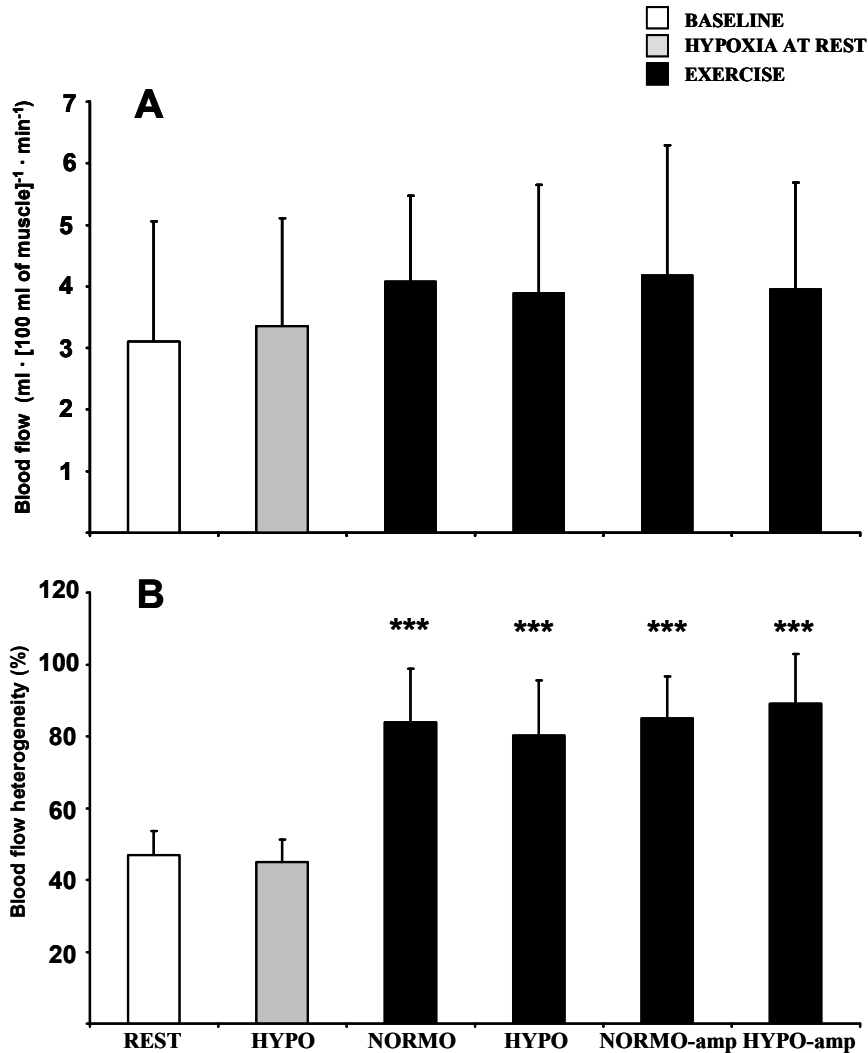

Figure 6.4.3. Mean blood flow (A) and its heterogeneity (B) in non-active posterior hamstring muscles of the working leg at resting baseline, in hypoxia at rest and during exercise in four different study conditions. While posterior muscle blood flow did not change significantly between any of the conditions studied (p > 0.3), flow heterogeneity in posterior muscles was increased from rest when exercise was performed by m. quadriceps femoris. Similar but more pronouncedly enhanced flow heterogeneity was also observed in resting contralateral musculature (See the end of the result section for details). *** p < 0.001 compared to resting baseline and hypoxia at rest. NORMO = normoxia, HYPO= hypoxia, -amp = under aminophylline blockade.

Table 6.4.3. Vascular conductance, resistance as well as mean blood flow and flow heterogeneity in non-contracting muscles at resting baseline, at rest in hypoxia, and during exercise in different conditions studied. VR = vascular resistance, VC = vascular conductance, Post = posterior muscles, CL = (resting) contralateral leg, BF = blood flow, HYPO = hypoxia, NORMO = normoxia, *** $p < 0.001$ compared to resting baseline and hypoxia. Blood flow and its heterogeneity is reported here only from resting contralateral whole thigh muscles since data from the posterior muscles of the working leg is illustrated in Figure 3.

	Rest		*Exe without aminophylline*		*Exe with aminophylline*	
	Baseline	**Hypoxia**	**NORMO**	**HYPO**	**NORMO**	**HYPO**
VC- Post ([ml/100g/min] • mmHg^{-1})	0.03 ± 0.02	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02
VR- Post (mmHg • [ml/100g/min]$^{-1}$)	40 ± 22	39 ± 23	31 ± 13	35 ± 18	32 ± 17	30 ± 9
VC- CL ([ml/100g/min] • mmHg^{-1})	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.02	0.04 ± 0.03	0.04 ± 0.04	0.04 ± 0.03
VR- CL (mmHg • [ml/100g/min]$^{-1}$)	38 ± 19	39 ± 22	47 ± 39	37 ± 33	44 ± 32	53 ± 41
Muscle BF- CL (ml/100g/min)$^{-1}$	3.1 ± 1.9	3.4 ± 1.9	3.7 ± 2.6	4.0 ± 4.0	4.5 ± 4.2	4.1 ± 3.9
Muscle BF heterogeneity- CL (%)	56 ± 12	52 ± 10	113 ± 14***	112 ± 28***	107 ± 21***	107 ± 9***

6.5 The effects of nitric oxide alone and in combination with prostanoids in regulating skeletal muscle blood flow and oxygen consumption at rest and during exercise (V)

At rest thigh muscle blood flow was reduced significantly from baseline during both NO inhibition and combined NO and COX inhibition **(Figure 6.5.1A)**. The limb oxygen extraction fraction (OEF) was increased significantly in both of these conditions **(Figure 6.5.1B)**, but only during NO inhibition was OEF increased more than muscle blood flow was reduced, and consequently, muscle oxygen consumption was 20 % higher compared to baseline **(Figure 6.5.1C)**.

Figure 6.5.1. The effects of NOS inhibition alone (L-NMMA) and in combination with indomethacin (L-NMMA+INDO) on resting thigh muscle blood flow (A), oxygen extraction fraction of the limb (B), and leg muscle oxygen consumption (C). Although muscle blood flow was reduced significantly during blockade of NOS, oxygen extraction was exaggerated compared to blood flow and oxygen consumption was higher during L-NMMA infusion compared to baseline. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Regarding the relative dispersion of blood flow in muscle, that is blood flow heterogeneity, NOS inhibition alone tended to, and double inhibition significantly augmented, the heterogeneity of resting muscle blood flow **(Figure 6.5.2)**. Energy substrate analysis at resting state did not show any other statistically significant differences except that arterial glucose and free fatty acid levels increased during combined NO and COX inhibition (thus at the end of the experimental day) and the baseline release of free fatty acids was turned to limb uptake during NOS inhibition **(Table 6.5.1)**. At the systemic level, however, despite local femoral arterial infusions of the drugs, diastolic and mean arterial blood pressure was increased especially during combined NOS and COX inhibition and as a consequence most likely due to the baroreceptor loading, heart rate decreased **(Table 6.5.1)**.

During exercise NOS blockade did not affect working QF muscle blood flow **(Figure 6.5.3A)**, but limb OEF was increased **(Figure 6.5.4)**. Double inhibition reduced QF blood flow and increased OEF, but muscle oxygen consumption (VO_2) determined by Fick's principle was not statistically different from control conditions during NOS or NOS + COX inhibition. Direct oxygen-15 determined muscle OEF and VO_2 analysis, however, revealed that working QF muscle OEF was increased from control conditions during NOS inhibition and double inhibition increased it further **(Figure 6.5.4),** approaching very high OEF values despite moderate working intensity. As a consequence, working QF muscle VO_2 tended to be increased during NOS inhibition ($p = 0.07$) and was significantly enhanced when both NOS and COX were inhibited **(Figure 6.5.4)**. Compared to overall limb OEF values, direct muscle OEF was not different during control or NOS inhibition, but was significantly higher during double inhibition ($p = 0.001$) **(Figure 6.5.4)**. There were no statistical differences between the VO_2 determination methods regarding muscle VO_2 ($p = 0.2$) and overall the muscle VO_2 values determined by the Fick's principle correlated well with the direct VO_2 measurements from the muscle **(Figure 6.5.4)**.

There were no significant changes in blood flow heterogeneity in exercising QF between measurements during exercise **(Figure 6.5.3C)**, but posterior muscle blood flow almost doubled (2.0 ± 0.7 ml/100g/min vs $3.9 \pm$ ml/100g/min) and flow heterogeneity in the same muscles increased (42 ± 3 % vs 65 ± 5 %) from rest to control exercise ($p < 0.001$ in both). However, NO inhibition or NO + COX inhibition did not affect posterior muscle blood flow **(Figure 6.5.3B**, $p = 0.59$) or flow heterogeneity **(Figure 6.5.3D**, $p = 0.39$). There were no other changes in hemodynamical or energy substrate parameters during exercise except that arterial glucose and free fatty acid levels were increased during the double inhibition as was the case at resting state **(Table 6.5.2)**. Heart rate was also lower under the combined NOS and COX enzyme inhibition and the A-V difference of free fatty acids as well as free fatty acid uptake tended to be increased towards the end of the experiments ($p = 0.06$ and $p = 0.09$ for measurements, respectively) **(Table 6.5.2)**.

Both NO inhibition alone and double inhibition reduced subcutaneous adipose tissue blood flow of the leg at rest, but during exercise adipose tissue blood flow was not changed significantly during inhibition, although biologically the reduction in blood flow was at least maintained or even increased in absolute terms. (**Figure 6.5.6**).
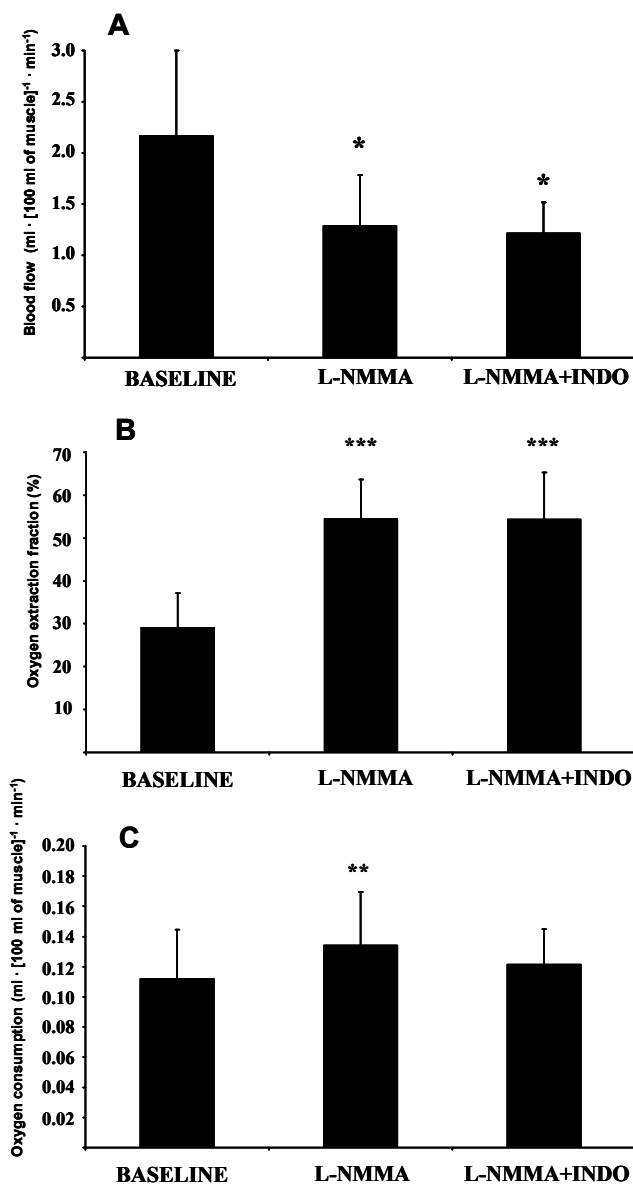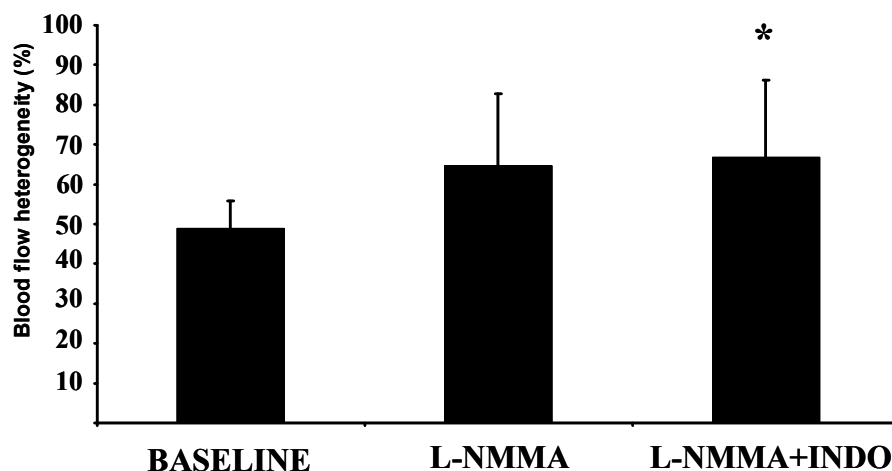
Figure 6.5.2. The effects of NOS inhibition alone (L-NMMA) and in combination with indomethacin (L-NMMA+INDO) on resting thigh muscle blood flow heterogeneity. ** p < 0.01. Combined blockade of NOS and COX enzymes clearly enhanced muscle blood flow heterogeneity, but also L-NMMA alone tended to increase it (p = 0.06).

Table 6.5.1. Hear rate, blood pressure and blood characteristics at resting state. HR = heart rate, MAP = mean arterial pressure, BPs = systolic blood pressure, BPd = diastolic blood pressure, Oxygen saturation and content-A = arterial oxygen saturation and content, Oxygen saturation and content-V = venous oxygen saturation and content, OEF = Oxygen extraction fraction, * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to BASELINE, † p = 0.01 compared L-NMMA, to glucose (G), lactate (LA) and free fatty acids (FFA) Diff = arterial-venous (A-V), L-NMMA = L-NMMA+INDO = # p < 0.001 compared to baseline and L-NMMA £ p < 0.05 compared to L-NMMA.

	BASELINE	L-NMMA	L-NMMA+INDO
HR (bpm)	56 ± 6	53 ± 8	44 ± 3***†
BPs (mmHg)	137 ± 15	139 ± 13	137 ± 16
BPd (mmHg)	78 ± 10	85 ± 8*	89 ± 7**
MAP (mmHg)	98 ± 11	103 ± 9	105 ± 9*
Oxygen saturation-A (%)	99 ± 1	99 ± 1	98 ± 1
Oxygen content-A (ml/L)	196 ± 14	196 ± 15	192 ± 17
Oxygen saturation-V (%)	70 ± 7	46 ± 9***	44 ± 10***
Oxygen content-V (ml/L)	138 ± 16	89 ± 17***	88 ± 24**
Oxygen extraction (ml/L)	58 ± 18	108 ± 22***	104 ± 20***
G artery (mmol/L)	5.6 ± 0.2	5.6 ± 0.2	6.1 ± 0.3#
G diff (mmol/L)	0.1 ± 0.2	0.2 ± 0.1	0.4 ± 0.2
G uptake (μmol/100g/min)	0.2 ± 0.4	0.2 ± 0.2	0.4 ± 0.2
FFA artery (mmol/L)	0.327 ± 0.141	0.412 ± 0.180	0.573 ± 0.232*
FFA diff (mmol/L)	-0.039 ± 0.074	0.035 ± 0.035*	0.029 ± 0.108
FFA release/uptake (μmol/100g/min)	-0.05 ± 0.09	0.04 ± 0.05**	0.01 ± 0.14
LA artery (mmol/L)	0.7 ± 0.1	0.7 ± 0.2	0.7 ± 0.2
LA diff (mmol/L)	-0.1 ± 0.1	-0.1 ± 0.1	-0.1 ± 0.1
LA uptake (μmol/100g/min)	-0.1 ± 0.1	-0.1 ± 0.2	-0.1 ± 0.1

Table 6.5.2. Hear rate, blood pressure and blood characteristics during exercise. HR = heart rate, MAP = mean arterial pressure, BPs = systolic blood pressure, BPd = diastolic blood pressure, Oxygen saturation and content-A = arterial oxygen saturation and content, Oxygen saturation and content-V = venous oxygen saturation and content, OEF = Oxygen extraction fraction, * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to BASELINE, † p = 0.01 compared L-NMMA, to glucose (G), lactate (LA) and free fatty acids (FFA) Diff = arterial-venous (A-V), L-NMMA = L-NMMA+INDO = # p < 0.001 compared to baseline and L-NMMA £ p < 0.05 compared to L-NMMA.

|	CONTROL	L-NMMA	L-NMMA+INDO
HR (bpm)	80 ± 11	73 ± 10	63 ± 7#
BPs (mmHg)	158 ± 23	160 ± 19	158 ± 18
BPd (mmHg)	90 ± 12	95 ± 12	96 ± 12
MAP (mmHg)	113 ± 15	117 ± 13	116 ± 13
Oxygen saturation-A (%)	98 ± 0	98 ± 0	98 ± 1
Oxygen content-A (ml/L)	201 ± 15	195 ± 15	196 ± 18
Oxygen saturation-V (%)	37 ± 3	32 ± 4**	21 ± 3#
Oxygen content-V (ml/L)	76 ± 5	63 ± 9***	42 ± 5#
Oxygen extraction (ml/L)	125 ± 13	132 ± 16	154 ± 18***†
G artery (mmol/L)	5.6 ± 0.2	5.6 ± 0.3	6.3 ± 0.5#
G diff (mmol/L)	0.1 ± 0.1	0 ± 0	0.1 ± 0.1
G uptake (µmol/100g/min)	2.6 ± 3.2	0.7 ± 1.5	3.2 ± 3.0
FFA artery (mmol/L)	0.319 ± 0.120	0.382 ± 0.150*	0.616 ± 0.237***£
FFA diff (mmol/L)	0.026 ± 0.032	0.041 ± 0.054	0.074 ± 0.046
FFA uptake (µmol/100g/min)	0.88 ± 1.07	1.24 ± 1.47	2.28 ± 1.44
LA artery (mmol/L)	1.1 ± 0.4	1.0 ± 0.3	1.0 ± 0.3
LA diff (mmol/L)	-0.4 ±0.5	- 0.1 ± 0.2	- 0.1 ± 0.2
LA release (µmol/100g/min)	- 17.0 ± 20.4	- 3.7 ± 7.0	- 3.2 ± 6.3
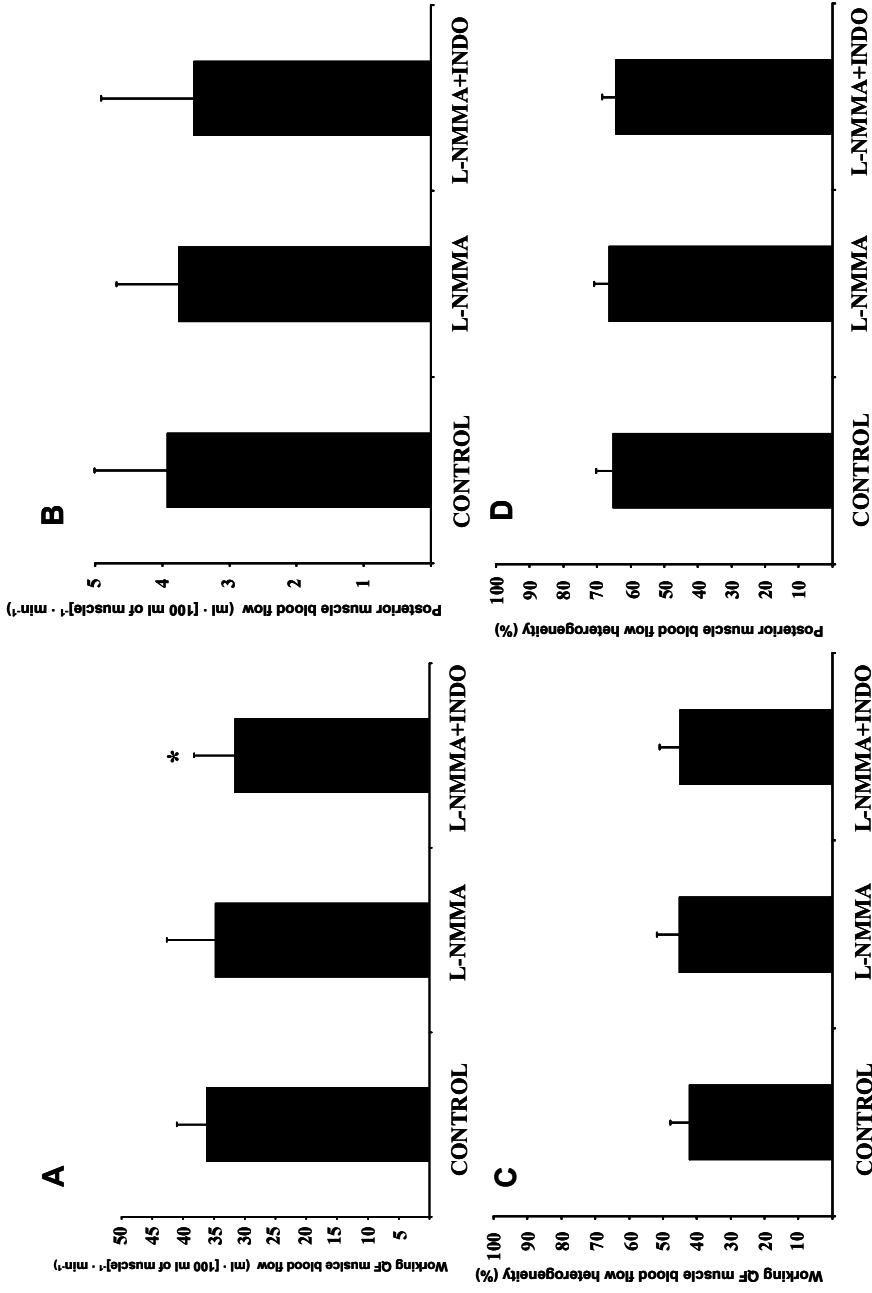
Figure 6.5.3. The effects of NOS inhibition alone (L–NMMA) and in combination with indomethacin (L–NMMA+INDO) on working QF muscle (A) and resting posterior muscle (B) blood flow and their flow heterogeneity (C and D, respectively). Combined NOS and COX inhibition reduced working QF muscle blood flow during exercise, but blood flow during single NOS blockade or in resting posterior hamstring muscles was unaffected. * p < 0.05.
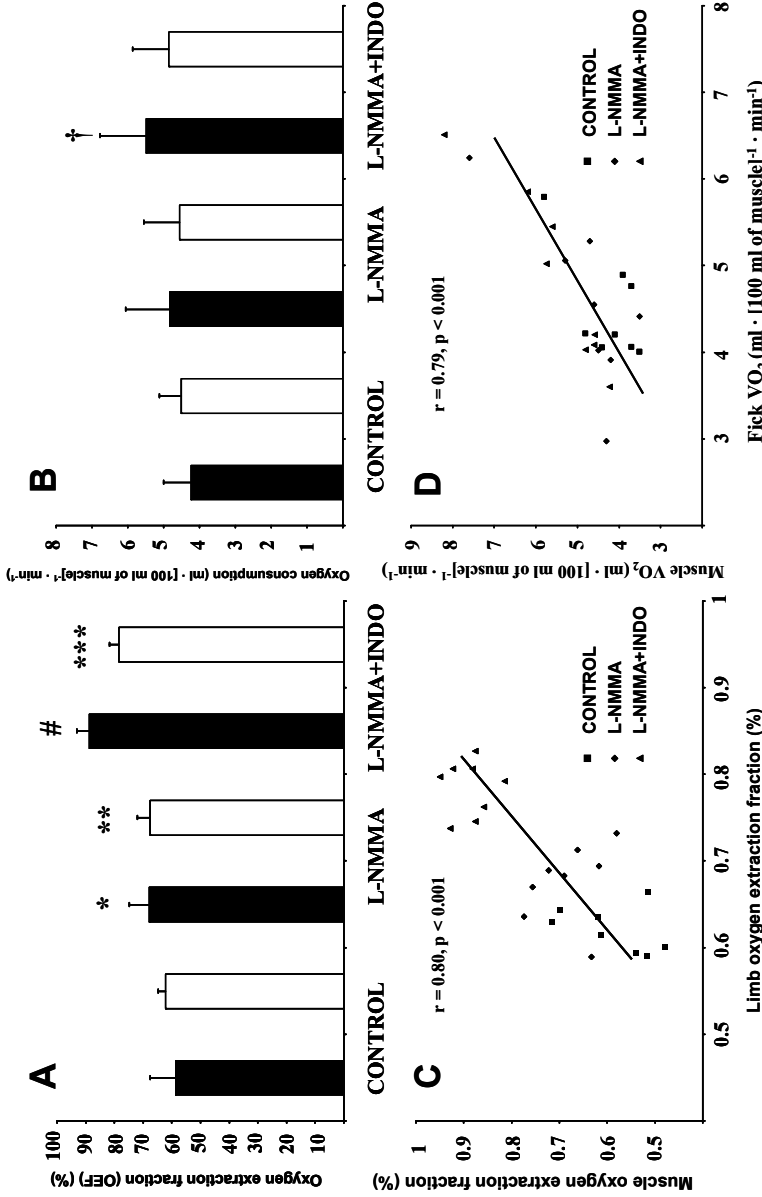
Figure 6.5.4. The effects of NOS inhibition alone (L-NMMA) and in combination with indomethacin (L-NMMA+INDO) on working QF muscle and exercising limb oxygen extraction fraction (A) and oxygen consumption (B). In Figures C and D are shown the correlation of directly with oxygen-15 determined muscle oxygen extraction fraction (OEF) and oxygen consumption (VO_2) compared to limb-based OEF and VO_2 during exercise. Both NOS blockade alone and in combination with indomethacin increased muscle and limb OEF, but muscle VO_2 was significantly increased only during double blockade. Additionally, muscle OEF was higher than whole limb OEF only during double blockade. Finally, Fick's principle determined VO_2 correlated well with VO_2 determined directly from muscle.* $p < 0.05$ and ** $p < 0.01$ compared to control, *** $p < 0.001$ compared to control and L-NMMA, # $p < 0.001$ compared to control, L-NMMA and Fick-OEF. † $p < 0.01$ compared to control. Black bars = muscle, white bars = whole limb.
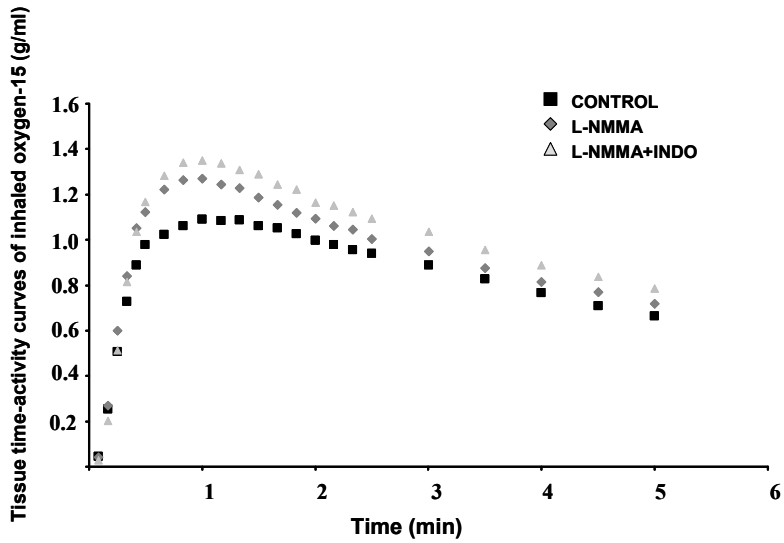
Figure 6.5.5. Muscle tissue time activity curves (TACs) of inhaled oxygen-15 during exercise (standardized uptake values, SUV, normalized for radioactivity dose and weight of the subject). Increased consumption of oxygen was evident already during NOS inhibition, but especially when both NOS and COX enzymes were blocked. Standard deviations are not shown for the sake of clarity.

A

B
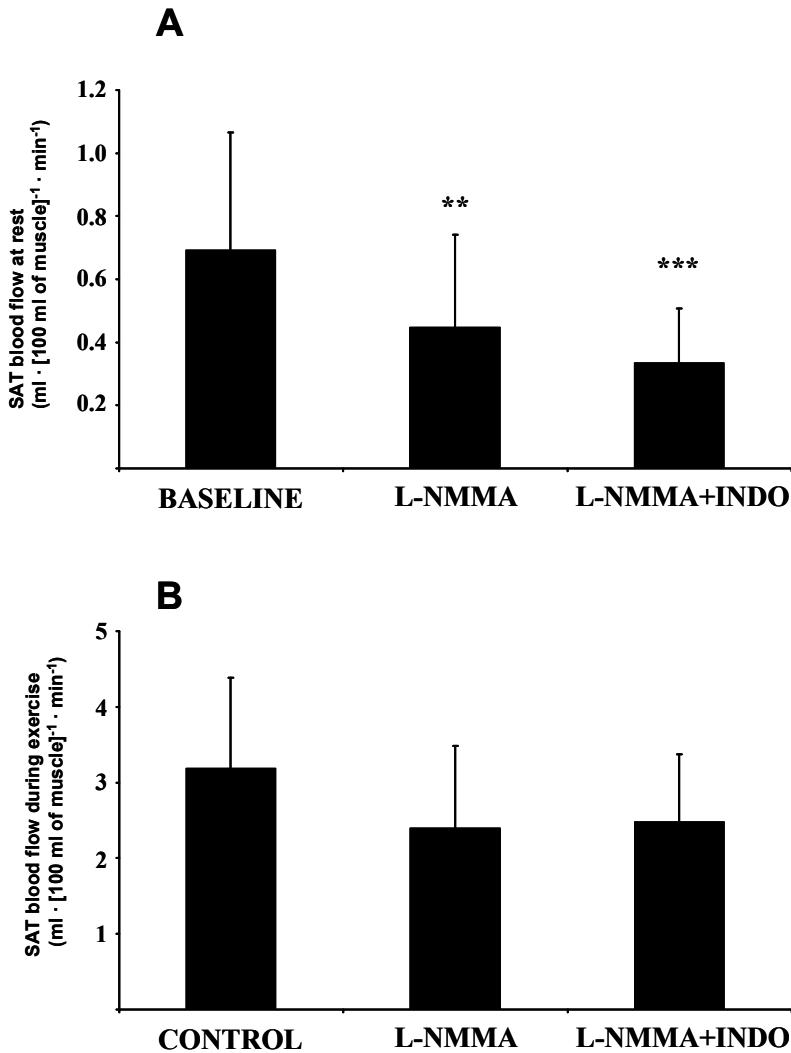
Figure 6.5.6. Subcutaneous adipose tissue (SAT) blood flow at rest (A) and during exercise (B) under NOS inhibition alone (L-NMMA) and in combination with indomethacin (L-NMMA+INDO). At rest SAT blood flow was reduced significantly during these pharmacological inhibitions, but during exercise statistically significant differences were not observed ($p = 0.11$), although biologically the reduction in blood flow was at least maintained or even increased in absolute terms. ** $p < 0.01$ and *** $p < 0.001$ compared to BASELINE.

7   DISCUSSION

7.1   Does muscle fibre and vascular unit recruitment account for changes in muscle blood flow heterogeneity (I and III)?

It was found in the present study that muscle blood flow heterogeneity may actually increase from rest to low intensity exercise, but then starts to decrease with increasing exercise intensities. The main reason for this appears to be that although there are some diffences between different muscle parts, blood flow is fairly uniformly distributed between muscles at rest in humans, but the variation between muscles increases at low exercise intensity since work is first performed by slow-type muscle fibres. When more force needs to be produced, more muscle fibres are called into action and between-muscle blood flow variation also decreases. In the isometric knee extension exercise, m. vastus lateralis was activated the most as judged from relative blood flow increases. When more muscle fibres are engaged in action, blood flow becomes more uniform (Ray & Dudley, 1998), which most likely also means that more capillary units are recruited with increasing exercise intensity (Laughlin *et al.*, 1996). This was not directly tested in the present study, but the result of no change in blood flow heterogeneity during pharmacological adenosine infusion, which raised the mean blood flow to a comparable level with exercise, supports the many animal studies (Duling & Damon, 1987;Laughlin *et al.*, 1996) that show that flow heterogeneity does not become more uniform just by increasing blood flow per se. This was not the first observation when the effect of pharmacological vasodilators on human muscle blood flow heterogneity was studied, but in previous studies no comparisons were performed in relation to exercise. However, when all these studies are taken together, it seems to be established using various vasodilators such as (Laine *et al.*, 1998;Pitkanen *et al.*, 1999) inducing various mean blood flow levels, and now adenosine, that flow heterogeneity is not changed from rest although mean blood flow increases substantially. Thus, decrease in muscle blood flow heterogeneity during exercise most likely stems from muscle fibre and vascular unit recruitment.

Interestingly, it was found in the present study that arterial-to-venous oxygen extraction correlated positively with muscle blood flow heterogeneity (III). This may be the first attempt to elucidate the coupling of blood flow distribution to aerobic metabolism in humans, and suggests that there is a matching of at least some degree at rest. This finding also supports the suggestions of Laughlin and collegues (1996) that if the exchange surface area is heterogeneously distributed, a matched, heterogenous distribution of blood flow is most efficient for oxygen transport since the largest amount of O_2 can be transported with the smallest amount of blood (Laughlin *et al.*, 1996). Thus, the presence of microvascular blood flow heterogeneity is not nessesarily negative, or a sign of poor vascular function (Laughlin *et al.*, 1996). However, the heterogeneity of muscle blood flow did not correlate with oxygen extraction during exercise, which may mean that in this situation it is important for effective oxygen utilization to maximize the capillary surface area via it can diffuse, as the decreased muscle blood flow heterogeneity from rest suggests. On the other hand, this may also

be suggestive of rather luxurious perfusion during one-leg knee extension with relatively small muscle mass.

The concept of luxurious perfusion during one-leg knee extension exercise is supported by the fact that microvascular units are not precisely matched to motor units (Fuglevand & Segal, 1997;Emerson & Segal, 1997) and vascular units are perfused rather abundantly compared to heterogeneously recruited muscle fibres during submaximal exercise. Moreover, during maximal exercise of a small muscle mass there always appears to be some reserve in oxygen extraction fraction which is not explained by a too short red blood cell transit time (Richardson *et al.*, 1993). Moreover, during submaximal knee-extension exercise pharmacologically reduced bulk blood flow can be compensated for by increased oxygen extraction (Radegran & Calbet, 2001;Mortensen *et al.*, 2007) meaning that the oxygen reserve is not completely exploited. This overperfusion concept is also supported by the finding that the *muscle* oxygen extraction fraction analysed by direct oxygen-15 tracer is not higher compared to whole limb A-V difference determinations, although one would expect to see lower oxygen extraction in the whole limb due to the blood flow through tissues not extracting much oxygen (e.g. skin) (substudy V). Only when luxurious muscle perfusion is limited for instance by inhibiting NO and COX products, does muscle oxygen extraction increase above limb values and reach almost maximal extraction. Additionally, even during maximal one-leg knee extension exercise femoral venous oxygen content is always somewhat higher (5-7 ml/100 ml) than during conventional maximal bicycle exercise (~2 ml/100 ml) (Rowell, 1986b), although not even during maximal leg work does venous oxygen drop below critical levels (Pirnay *et al.*, 1972a;Pirnay *et al.*, 1972b). The fact that femoral venous oxygen pressure never falls to zero has been interpreted to mean that there is diffusion limited O_2-supply (Hogan *et al.*, 1988;Richardson *et al.*, 1995). This general finding can however be perhaps equally well explained by the fact that some muscle areas are not well perfused, thus flow heterogeneity affects O_2 extraction, or simply that there is also blood flow through tissues that extract less oxygen, such as inactive muscles (see the end of discussion in section 7.2) or skin, if its flow is increased to eliminate thermal stress that increases towards maximal effort.

7.2 The effect of moderate systemic hypoxia and possible nervous constraints on skeletal muscle blood flow (IV)

In the present study, moderate acute systemic hypoxia corresponding to 3000 meters of altitude caused a modest (10 %) increase in exercising muscle blood flow. The fact that hypoxia was only moderate in nature is also supported by the fact that resting muscle blood flow or its heterogeneity did not change in the present study. Since heart rate tended to be increased by 8 beats per minute and MAP also tended to increase in hypoxia, this suggests increased chemoreflex-evoked sympathetic nervous system activation (Heistad & Abboud, 1980;Somers *et al.*, 1989;Halliwill & Minson, 2002), which most likely restrained local muscle vasodilation. On the other hand, the observation of unchanged muscle blood flow is not that suprising since several previous studies have already made a similar observation in respect to whole thigh

blood flow (Rowell *et al.*, 1986;Brooks *et al.*, 1998), although this may be the first observation directly from muscle. In the present study, dimished arterial oxygen content was followed by a concomitant reduction in venous oxygen content and thus oxygen extraction remained similar to resting value. However, during exercise this was not enough for proper tissue oxygenation and muscle blood flow was therefore also increased moderately (~10%).

Despite increased mean blood flow in exercising muscle, flow heterogeneity remained similar to normoxic control condition. This suggests that capillary recruitment was already maximal during the exercise, or hypoxia per se simply does not induce greater capillary recruitment within the contracting muscle. This pattern may be altered by more pronounced hypoxia and/or intensive exercise (Bourdillon *et al.*, 2008). However, put into perspective by a recent study of Richardson's group (Barden *et al.*, 2007) even the combination of more severe hypoxic exposure (12 % O_2) and exercise intensity (maximal one-legged exercise) does not decrease arterial oxygen saturation lower than 88%, the value that was also observed in the present study. This also suggests that pulmonary gas exchange and O_2 delivery are well preserved in hypoxia with small muscle mass exercise (Calbet *et al.*, 2009). In addition, oxygen extraction fraction values (~57 % in control exercise) in general suggest that this one-leg exercise indeed was of moderate to high intensity since they are close to the well-established maximal value of 70 % in comparable (exercise duration etc.) one-leg exercise (Andersen & Saltin, 1985). Thus, severity of hypoxia or exercise intensity is unlikely to explain similar blood flow heterogeneity during hypoxic and normoxic exercise.

Since it is known that hypoxia potentiates the exercise-induced muscle symphatetic nervous activity (Seals *et al.*, 1991) it was expected in the present study that this would lead to a decrease in inactive muscle blood flow. This would effectively direct flow just to active muscles and increased blood flow heterogeneity in non-contracting muscels would be seen. This was, however, not the case and it is highly likely that both exercise (Laughlin *et al.*, 1996) and moderate hypoxia (Seals *et al.*, 1991) were of 'borderline' stimulus for potentiation of symphatetic nervous activation. In general, the dynamic one-leg knee extensor exercise increased the heterogeneity of inactive muscle blood flow, which was interpreted as nervous constraints to blunt blood flow increase elsewhere than in the working muscles. Mean blood flow seemed to be preserved to resting levels, although in substudy V inactive posterior muscle blood flow was doubled from baseline. It is likely that an increase in inactive muscle blood flow cannot be avoided especially when exercise intensity increases even if it is partially restricted by the nervous system. Increased perfusion pressure may drive inactive muscle blood flow to increase and inactive muscle oxygen extraction may actually decrease since metabolism is not increased. The possibility however exists that the importance of nervous constraints increases with increasing exercise intensity and/or muscle mass. Thus, the issue of increased inactive muscle blood flow may not be so critical as when large muscle mass is being activated such as during cycling, running or especially cross-country skiing when sympathetic activation is also much higher. It, however,

may affect the per 100g VO$_2$ analysis of active muscle if the Fick principle is being used.

7.3 The effect of adenosine on skeletal muscle blood flow (I, III and IV)

7.3.1 Exegenous adenosine (III)

This study gives the first illustration of the effects of intra-arterially infused adenosine on blood flow distribution and heterogeneity *within* a human muscle. In the present study only one dose of adenosine was chosen to be infused to limit the radiation exposure caused by the many PET measurements. The concentration was chosen to match with the dose previously shown to induce peak blood flow measured by ultrasound Doppler in the femoral artery (Radegran & Calbet, 2001). The direct infusion of adenosine leads to a somewhat puzzling finding when total thigh muscle blood flow is considered. It was not possible to perform ultrasound Doppler blood flow measurements simultaneously when performing PET imaging. However, the estimated whole thigh total muscle blood flow was based on the measured flow per mass (PET) and measured thigh muscle volume (MRI) to enable comparison to previously published data (Radegran & Calbet, 2001). Rådegran & Calbet *(2001)* reported a thigh (pressure cuff isolation of the calf) blood flow of ~8 litres during infusion of adenosine of 1 mg·min^{-1} L^{-1} thigh, representing a 33-fold increase from baseline. The total thigh *muscle* capillary blood flow value observed in the present study was approximately 2.3 litres (a 14 fold-increase), which is surprisingly low compared to that of the previous study and does not support the preliminary hypothesis that muscle would account for 80-90 % of whole thigh vasodilation during adenosine infusion. There are some possible factors that can affect this discrepancy. First, the subjects in the previous ultrasound study (Radegran & Calbet, 2001) had a greater body mass, thus their thigh volumes were also greater. This difference, does not seem however, to play a significant role since if these results are extrapolated to represent the highest thigh volumes of 10 litres (as was the case in Rådegran & Calbet's study), muscle would account for just ~3 litres of total thigh flow (~76 % of total thigh volume was muscle in the present study). It is noteworthy that when blood flow values from PET and ultrasound Doppler methodologies are compared at rest, total blood flows are almost exactly similar (0.22 l·min^{-1} and 0.23 l·min^{-1}, respectively), which corroborates previous work showing the close connection between the resting limb and muscle blood flow (Raitakari *et al.*, 1996).

Blood flow diversion to regions other than muscle cannot account for the difference in the calculated bulk blood flow to the whole limb compared to the study of Calbet & Rådegran. Therefore, non-nutritive routes (Clark *et al.*, 2000a;Clark *et al.*, 2000b) within the muscle tissue remain the one rational explanation for the large total blood flow differences. Thus, the physiological non-nutritive shunt, which in various species has been shown to account for over 50 % of muscle bulk blood flow at rest (Harrison *et al.*, 1990;Newman *et al.*, 2007), could well explain the differences between these studies. Non-nutritive vessels in muscle are mostly capillary-like rather than direct arteriovenous shunts (Clark *et al.*, 2000b), but when dilated, are capable of carrying 16

times the flow of a typical muscle capillary (Poiseulle's law) since they are twice the diameter of muscle nutritive capillaries (Grant & Wright, 1970). In addition to these non-nutritive shunt routes within the muscle mostly supplying connective tissue (see aforementioned references), many of the vasa vasorum vessels ('vessels of the vessels') have long been known to constitute short direct arteriovenous shunts (Lowenberg & Schumacher, 1948). Contrary to bulk blood flow analysis, which combines both nutritive and non-nutritive flow, blood flow through these non-nutritive routes cannot be tracked with PET since there is no capillary level exchange of water molecules in non-nutritive vessels (Lammertsma & Jones, 1983).

From a methodological point of view it cannot be totally excluded that during very high blood flow conditions, such as during adenosine infusion, water molecules may not have time to reach equilibrium with the muscle cells, which would underestimate the blood flow measured by PET. Nevertheless, this does not seem likely as very high capillary blood flow rates were mapped in high flow areas near the bone which are known to be very rich in capillaries, reaching values of over 400 ml$^.$100g^{-1} $^.$min^{-1} in almost all subjects which are well in accordance with maximal exercise blood flow values (Richardson *et al.*, 1993). Moreover, in cardiac PET measurements radiowater has been used to measure even higher blood flow rates ($\sim$500 ml$^.$100g^{-1} $^.$min^{-1}) as also reported in a substudy II. These findings are actually very reasonable in the light of leg blood flow during maximal one-leg knee extension, which is usually in the range of 6-8 L$^.$min^{-1} and is confined to the quadriceps muscles comprising $\sim$2.5 kg muscle. By extrapolation to quadriceps volume this gives maximal flow values of 250-350 ml$^.$100g^{-1}min^{-1} muscle (Andersen & Saltin, 1985;Richardson *et al.*, 1993). Adenosine infusion has been reported to elicit similar limb flow values, but this flow is distributed to the whole leg which comprises $\sim$7-8 kg of muscle alone, meaning that by a general estimate, maximal unit muscle blood flow evoked by adenosine is only around $\sim$100 ml$^.$100g^{-1}min^{-1} or less. These flow values of 40 ml$^.$100g^{-1} muscle with adenosine infusion are lower than the rough estimates provided from the other studies, but nonetheless are direct measurements at the capillary level within the muscle. Taken together, it is obvious that adenosine does not account for the patterns of blood flow observed during voluntary exercise. Moreover, other vasodilator candidates and factors such as perfusion pressure (driving force), the mechanical effects of contraction and thus effective venous return, and work efficiency are of importance for reaching the high blood flows observed during exercise (Laughlin, 1987).

Considering the findings of exogenously infused adenosine it is important to discuss the physiological importance of adenosine. First of all, the results strongly suggest, although indirectly, that adenosine is a potent vasodilator not only for muscle but also for skin and adipose tissue, and potentially bone. Secondly, the suggestion that adenosine also seems to induce increases in non-nutritive shunt blood flow in muscle raises some concerns about vasodilator studies in general with regard to infusions planned to mimic exercise hyperemia, if precise matching of metabolism and blood supply is 'detached' and bulk blood flow is indeed enhanced, but it flows through non-nutritive routes. In this respect a body of evidence indicates that several vasodilators, adenosine included, when infused exogenously exclusively increase non-nutritive

blood flow, which results in a higher total bulk blood flow (Clark *et al.*, 2006). However, it must also be emphasized that adenosine also increased muscle nutritive blood flow substantially in the present study, especially in the deep muscle regions. Additionally, these findings do not necessarily indicate that non-nutritive vascular pathways are not of importance in the regulation of vascular function during exercise (e.g. blood pressure regulation). Finally, there is no reason to have non-nutritive vessels open during exercise since this would impair effective tissue oxygenation. It is therefore speculated here whether increased sympathetic activation is actually acting to close these pathways during exercise and cause flow redirection rather than SNA being blunted to a large extent. To test this hypothesis vascular innervation of nerve endings should be clarified and functional investigations should be performed to compare total limb blood flow in relation to muscle capillary blood flow. Thus, Doppler ultrasound measures of blood flow needs to be compared to PET radiowater flow.

Despite the observation that large dose adenosine infusion may lead to a substantial increase in non-nutritive (shunt) blood flow, it was also observed that there was also substantial increase in nutritive flow. The present study also strongly supports previous findings that adenosine infusion induces very heterogeneous blood flow responses (Sollevi, 1986;Martin *et al.*, 2006a). This was evident with regard to the magnitude of blood flow increase between individuals as previously found, but especially for local distribution of blood flow in the muscle. Interestingly, the change in heterogeneity from baseline to adenosine infusion tended to be inversely related to the change in magnitude of blood flow increase (r = 0.62, p = 0.09) and could well explain some of the individual variation of the effects of adenosine (Sollevi, 1986;Martin *et al.*, 2006a). Moreover, PET-measured capillary blood flow also correlated positively with bicycle-determined systemic maximal oxygen consumption suggesting that blood flow capacity may play a role in achieving high maximal oxygen consumption since the abililty to vasodilate muscle microvasculature can affect utilization of oxygen by affecting the surface area for oxygen diffusion during exercise. It is also likely that better vasodiltory response also depicts higher capillary densities in the muscle.

Although very high blood flow values were observed in the deepest intermedius musce near the bone, in general mean blood flow during this pharmacological infusion was not even close to reported maximal values during exercise. This strongly suggests that for instance musle pump and effective perfusion pressure are of great importance achieving very high blood flows (Laughlin, 1987). It is likely that clear if not maximal vasodilation was achieved in the present investigation (venous oxygen saturation 94 % etc.), but as pointed out by Laughlin, after maximal vasodilation has been reached, blood flow values are a result of perfusion pressure (Laughlin, 1987). This may be the main explanation for the relatively 'low' (in relation to established values from maximal exercise) capillary blood flows measured in the present investigation during high dose local adenosine infusion.

The finding that blood flow heterogeneity at rest was inversely related to the adenosine-induced change in heterogeneity suggests that in subjects with low initial resting heterogeneity blood flow is increased mainly through vessels and capillaries

that are already open while in subjects with relatively high resting heterogeneity new vessels are opened by adenosine receptor activation and blood flow becomes more uniform. This finding can be suggested to stem from the differences in adenosine receptor densities in muscles (Lynge & Hellsten, 2000). Dense capillarity and resistance vessels are related to higher densities of adenosine receptors in endothelial cells and differences in muscle fibre type composition. This is also in agreement with the fact that very high blood flow values (~400 ml $\cdot$ 100^{-1}g $\cdot$ min^{-1}) were seen in the deepest regions of the thigh musculature, which are generally known to consist almost solely of red slow type fibres and to be rich in capillary supply. Interestingly, in relation to flow heterogeneity response in general (unchanged from rest) and persons with low resting flow heterogeneity, Rowell et al. also has previously observed and suggested that when muscles are dilated by cholinergic stimulation, blood flow bypasses some capillaries and escapes tissue exchange (Rowell, 1986d). This means that despite the increase in blood flow more capillaries are not open and more blood simply surges through already open capillaries (Rowell, 1986d). Similar observations have been made in animal studies (Duling & Damon, 1987). In some subjects flow heterogeneity however decreased from rest, which suggest that they may have higher muscle capillary and/or adenosine receptor density.

7.3.2 Endogenous adenosine (I and IV)

Based on the data from substudies I and IV it is concluded that adenosine is not obligatory for the increase in muscle capillary blood flow in exercising muscle in normoxia or hypoxia. This does not however exclude the possibility that adenosine is one of the many tonic regulators of skeletal muscle blood flow as shown by Duncker and colleagues in cardiac tissue (Duncker *et al.*, 1998), rather than acting as a metabolic distress signal as originally proposed (Berne, 1963). It is known that normally adenosine stems mainly from the extracellular pathways and intracellular adenosine formation and its subsequent release to the muscle interstitial space in the vicinity of the smooth muscle cells of resistance vessels is triggered only when muscle metabolic demands exceed oxygen delivery (Duncker & Bache, 2008). Thus, the physiological contribution of adenosine signalling to muscle hyperemia does not seem to be activated in steady-state exercising muscle even in moderate systemic hypoxia, but requires severe ischemic conditions. There were no signs of this in the present study since, for instance, blood lactate remained essentially similar during all conditions (data not shown). Therefore, hyperaemic mediation of adenosine needs to be further tested in maximal exercise where clear mismatch between metabolism and oxygen delivery can be assumed to exist.

Some animal studies suggest that adenosine can account for up to 40 % of exercise hyperemia, but results are very controversial (Marshall, 2007). Moreover, some (Radegran & Calbet, 2001;Mortensen *et al.*, 2007), but not all (Casey *et al.*, 2009) human studies suggest that adenosine plays a role in exercise hyperemia. In the present study, there was no support for adenosine in the regulation of muscle capillary blood flow during exercise. Clearly, methodological reasons are the most obvious factors accounting for these different outcomes. While Doppler ultrasound or thermodilution

measures bulk limb blood flow, PET technique allows determination of nutritive capillary blood flow within the muscles. Thus it seems that capillary blood flow is not as easily disturbed by blockade as bulk blood flow. Supporting this, it is known from the coronary circulation that when stenosis limits blood flow in large coronary arteries, there is a substantial compensatory downstream vasodilation (Duncker & Bache, 2008). Since it is obvious that quadriceps femoris is at least slightly overperfused during one-leg knee extension (see discussion chapter 7.1 for details) it may well appear that blockade indeed affects proximal resistance vessels and overall conduit blood flow may be slightly reduced (Radegran & Calbet, 2001;Mortensen *et al.*, 2007). Simultaneously there is a compensatory vasodilation in more distal resistance vessels close to the capillaries preserving more critical working muscle capillary nutritive blood flow. This has been shown to be the case regarding NO, but the concept needs to be clarified when adenosine is considered.

Adenosine receptor inhibition by methylxanthine caffeine may reduce leg vascular conductance by sympathetic nervous activation, which is elevated during blockade, but does not necessarily reduce leg blood flow (Graham *et al.*, 2000). Finally, in addition to inactive muscle vascular beds and non-nutritive pathways, other leg tissues must also be considered. Interestingly, subsequent data analysis of sub-study I has shown that there is atually some 30 % reduction in adipose tissue blood flow, which however is confined only to the highest exercise intensity and in the vicinity of exercising muscle. Due to the much lower per unit adipose blood flow both at rest (1ml/100g/min) and during exercise (~6 ml/100/min) and its lower total tissue volume in the leg, this reduction clearly does not explain all the discrepancy with the previous human studies, although it suggests that adenosine may play a role controlling adipose blood flow during exercise. In support of this, it has previously found that as in skeletal muscle (Hellsten *et al.*, 1998), adenosine concentration also increases in adipose tissue during exercise (Carey *et al.*, 2004).

The findings that adenosine plays no role in controlling physiological muscle blood flow increase in a response to increased metabolic demand are well in accord with cardiac studies showing no indication for adenosine mediation in normal coronary exercise hyperemia in dogs, swine, or humans (Duncker & Bache, 2008). Only when stenosis or severe ischemia affects cardiac perfusion, adenosine contributes to coronary vasodilation (Duncker & Bache, 2008) and this concept also seems to apply to human skeletal muscle capillary blood flow. In general, it is highly unlikely that working skeletal muscle is that different to beating cardiac muscle, which is not dependent on adenosine mediation during exercise. Almost exactly similar regulative factors have been proposed in both muscles, although there indeed may be qualitative differences in different factors. Although it is well established that adenosine indeed contributes to vasodilation in severe hypoxic and/or ischemic conditions, it is questionable whether it could solely explain 40-60% exercise hyperemia as some studies suggest (Radegran & Calbet, 2001;Mortensen *et al.*, 2007) (assuming that part of the adenosine mediation is masked by blocker-induced vasodilation). All the previous data has also pointed to the conclusion that no single factor can account for a large part of this flow increase (Clifford & Hellsten, 2004). This notion is also well supported by the present

investigation. The view that adenosine could account for such a large part of flow increase during exercise also largely neglects the many other endogenous vasodilator factors, such as K^+, H^+, CO_2, PO_2, H_2O_2, CO, reduction of nitrite to NO by myoglobin and haemoglobin that may be hard or impossible to block, as well as more common players as synergistic actions of NO and prostanoids and β-adrenergic vasodilation among others. The idea of redundancy, which means that other factors can appear and compensate if one regulatory factor is inhibited, seems no longer to be anymore valid if results accumulate favoring just one important factor.

Although it is appreciated that many compensatory factors other than adenosine, such as increased ATP release from red blood cells, β-adrenergic vasodilation and endothelium derived factors may be involved in explaining the increase in exercising muscle blood flow during systemic hypoxia, one highly fascinating aspect is also that reduced oxygen content per se could directly cause vasodilation in resistance arteries. After Hilton and Eichholtz first proposed in 1925 that reductions in (coronary) vascular resistance produced by hypoxemia are mediated by the direct effects of low arterial partial pressure of oxygen on the microvasculature (Hilton & Eichholtz, 1925), it has been shown in several isolated vessel studies that reductions in O_2 lead to decrease in vessel tone (Busse *et al.*, 1983;Busse *et al.*, 1984;Busse & Bassenge, 1984;Jackson, 1987). These data indicate that there are local O_2 sensing pathways in vessels that regulate their vasomotor tone in response to hypoxia. These sensors seem to be located in terminal arterioles, capillaries, and venules and that they detect changes in oxygen and initiate a conducted vasodilation from the sensors to distal arterioles (Jackson & Duling, 1983;Jackson, 1987). Finally, especially K^+_{ATP} channels seem to be of ultimate importance in this direct oxygen-dependent vasodilation by reduced partial pressure of oxygen (Tune, 2007).

Despite mean bulk blood flow level, it has been suggested that metabolic (by)products such as adenosine could also affect the matching of oxygen supply to cellular metabolism, thus blood flow heterogeneity in muscle (Clark *et al.*, 1995). Some evidence for this was observed in sub-study I since blood flow heterogeneity seemed to be somewhat higher during adenosine receptor inhibition, but when the hypothesis was tested by direct adenosine infusion (III), heterogeneity did not change from rest, although it must be acknowleged that increased sympathetic nervous activation and subsequent vasoconstriction may have counfounded these findings. However, when one carefully considers the proposed theory it appears that there are several problems. It is known that capillary (endothelial) cells are capable of initiating the dilation cascade that travels upstream to arterioles and tries to precisely match blood flow to muscle fibre recruitment (Pittman, 2000;Murrant & Sarelius, 2000). Metabolic vasodilators, such as adenosine, have however generally known to dilate (resistance) arterioles, which would lead to increased perfusion of all downstream capillary networks, regardless whether those capillaries are associated with working muscle fibres or not (Murrant & Sarelius, 2000). Adenosine could also work at the capillary level and initiate upstream conductive vasodilation. However, this neither seems to be the case. Inhibition of A_1 and A_2 adenosine receptors at the capillary level does not affect remote upstream dilation (Fredholm *et al.*, 1994). In this case arteriolar

responses to adenosine were inhibited, but the role of K_{ATP} channels in this capillary-to-arteriole vasodilation was strongly implicated since glibenclamide blocked 75 % of the dilation (Murrant & Sarelius, 2000). In this regard it is of note that adenosine is indeed capaple of inducing upstream vasodilation when injected directly to arterioles (Thengchaisri et al., 2009). This suggests that adenosine may not directly couple muscle fibre recruitment to capillary perfusion. Thus, when all this information is taken into account, it can be concluded that the adenosine hypothesis fails in many parts and leads us to suggest that adenosine, as well as possibly acethylcoline, are distress signals that should not normally circulate in blood. If present, they are an unspecific signal to restore the situation back to normal on a large scale and, therefore, they are capable of inducing powerful vasodilation, which has led them to be useful in many cardiovascular investigations. It must also considered that since adenosine concentration increases in both cardiac and skeletal muscle interstitium when their contractile activity increases, adenosine may serve to regulate functions other than blood perfusion. These may be slowing heart rate by affecting electric conductivity in cardiac muscle especially at maximum intensities to avoid heart exhaustion and development of ischemia, or regulate glucose and other energy subsrates in skeletal muscle (Vergauwen et al., 1994). Adenosine may also play a role in regulating blood flow in recovery from exercise (Bangsbo & Hellsten, 1998). Thus, it concluded based on the results presented in the present thesis and that of others that adenosine does not seem to affect skeletal muscle blood flow at the capillary level in humans, but may mediate other functions.

7.4 Myocardial blood flow and A_{2A} receptor density in endurance athletes and untrained men (II)

7.4.1 The effect of pronounced athlete's heart on myocardial blood flow at rest and during adenosine stimulation

Structural and functional changes in athlete's heart are considered a favourable physiological phenomenon with no known harmful consequences except for increased incidence of atrial fibrillation at an older age (Karjalainen et al., 1998). However, extreme forms of myocardial hypertrophy due to heredity and vigorous physical conditioning can resemble a structural heart disease such as hypertrophic cardiomyopathy, which is associated with reduced MBF reserve (Camici & Crea, 2007) and substantially increased risk of cardiac event (Maron & Pelliccia, 2006;Thompson et al., 2007). LV cavity dimensions and wall thicknesses of athletes in the present study were in the near upper normal limits observed previously in world-class athletes (Pelliccia et al., 1991;Pelliccia et al., 1999). LV mass normalized to BSA ranged in athletes from 159 g/m^2 to 221 g/m^2, even exceeding the upper normal limits also among athletes (Pelliccia et al., 1991;Rodriguez Reguero et al., 1995;Pluim et al., 2000). Compared to the previous PET study where highly trained rowers were studied (Kjaer et al., 2005), athletes in the present study had on average 51 % higher LV mass index (128 vs 193 g/m^2, respectively). In line with the study by Kjaer and colleagues, uncorrected MBF was significantly lower in ET than UT at rest in the present study. In

other previous human studies with PET, uncorrected MBF has been similar between the trained and untrained groups at rest (Radvan *et al.*, 1997;Toraa *et al.*, 1999;Kalliokoski *et al.*, 2002;Laaksonen *et al.*, 2007) despite the larger LV mass in athletes. Given the known close relationship between LV work output, myocardial oxygen consumption, and myocardial blood flow, it is necessary to relate the measured blood flow values against LV work load. When this was done in the present study, MBF at rest was significantly higher in ET than UT. This leads to two alternative consequences: athletes had either 1) impaired myocardial efficiency of work with comparable myocardial oxygen extraction between the groups or 2) reduced oxygen extraction with unchanged efficiency of work.

Myocardial oxygen extraction and efficiency have been little studied in athletes mainly due to methodological difficulties. Takala and colleagues found no differences in myocardial efficiency and oxygen extraction between ET and UT subjects during insulin-clamp at rest (Takala *et al.*, 1999). A recent report of a study performed with monozygotic twins discordant for physical activity and fitness showed that myocardial oxygen extraction tended to be lower and efficiency higher at rest in the more active and fitter group, despite relatively small difference in fitness level between the groups (Hannukainen *et al.*, 2007). Additionally, in a study by Heiss and colleagues (Heiss *et al.*, 1976), resting oxygen extraction also tended to be lower in endurance athletes than in untrained subjects (calculated from the individual data presented in the article). Thus, although solid evidence on the relationship between flow and oxygen extraction in the present study is lacking, the data from this study point to conclusion that the athletes may have lower myocardial oxygen extraction at rest, which may then, in turn, allow higher increase in oxygen extraction whenever myocardial demand is elevated, such as during strenuous exercise. This implies that the myocardial oxygen extraction reserve (the capacity to increase oxygen extraction) is increased in the trained state and this, together with an unchanged perfusion capacity, leads to higher oxygen transport capacity.

It was stated in the sub-study II that higher vagal tone in athletes could readily explain the higher workload-normalized resting myocardial perfusion since more Ach is released from parasymphatetic nerves, which by increasing NO levels vasodilates coronary arteries (the well known endothelium mediation). This may not however be absolutely true. It is namely well known that parasymphatetic nerves innervate coronary arteries abluminally in between smooth muscle cells and the direct effect of Ach on smooth muscle cells in vasoconstriction. Exegenous Ach causes vasodilation only when endothelium is healthy and intact, but when for instance atherosclerosis has damaged endothelium, there is a net vasoconstriction to Ach (Ludmer *et al.*, 1986), although it is also known that abluminally-derived Ach can diffuse between smooth muscle cells and cause endothelium-dependent vasodilation. The normal vasoconstrictive effect of Ach in vivo in athletes in the present study is however supported by the finding that athletes also had higher myocardial blood flow resistance. This is well in accordance with a previous study, which showed that there are no differences in resting coronary artery diameter between athletes and controls, although the maximal calibre of arteries is much higher in athletes (Haskell *et al.*, 1993).

However, better OEF capacity (reduced resting and increased maximal OEF) would lead to the situation that the cardiac aerobic metabolism is met not only by increasing myocardial blood flow. It also fits to the idea that endurance training does not seem to increase myocardial capillary density and well-documented exercise-induced increased MBF capacity is solely the result of increased diffusion and/or transport capacity (Laughlin & Tomanek, 1987). This would maximaze cardiac performance, although it is seems to be generally accepted that coronary blood flow does not limit maximal cardiac performance in humans at least during normoxia since it can be increased by superimposition of hypoxic air breathing (Grubbstrom et al., 1991;Grubbstrom et al., 1993). However, careful consideration of the paper by Grubström and collegues (Grubbstrom et al., 1993) raises some concerns, although it is appreciated that with invasive chatheterizations studies are not easily conducted. It is however likely that true maximal (cardiac) performance was not achived in this study since maximal watts (198 W) and heart rate (169 bpm) are normally higher (similar to upright max or still very close to maximal of ~300 W and 200 bpm) also during maximal supine bicycle exercise in young healthy men (Heinonen et al., unpublished). Thus, it still remains to be established in humans whether coronary blood flow may limit cardiac performance or whether OEF capacity is increased in athlete's heart. This can only be tested with direct measures of oxygen content and blood flow (thermodilution) from the coronary sinus during a graded exercise test to maximal exhaustion. However, animal studies have already shown that the coronary vascular bed is not exhausted during maximal normoxic exercise since direct intracoronary infusion of adenosine (Duncker & Bache, 2008) and induction of hypervolemia (Norton et al., 1990) can elevate coronary perfusion during exercise. Thus, factors other than coronary perfusion, such as filling capacity of the left ventricle, may limit maximal cardiac output in the healthy heart.

It was found in the present study that highly trained endurance trained athletes excelling in cross-country skiing showed significantly reduced adenosine-induced myocardial blood flow. This may derive from experimental issues rather than being a true physiological response. The possibility exists that due to a very short half-life of adenosine (Moser et al., 1989) caused by its rapid cellular uptake through a high-affinity nucleoside transporters (Loffler et al., 2007) especially in endothelium and erythrocytes abundant in athletes (Heinicke et al., 2001), the efficiency of a standard dose of adenosine inducing hyperemia may not be similar in athletes and untrained men. The adenosine degradation hypothesis is also supported by the finding that the fitter the athlete, the lower the adenosine-induced blood flow. The fittest athletes most likely have higher blood volume and red blood cells (oxygen carrying capacity) and also high a capillary and thus endothelial surface area (oxygen diffusion). To highlight the degradation of adenosine, studies performed by Swedish researchers in the 1980s can be mentioned (Sollevi et al., 1984;Sollevi et al., 1985). They infused adenosine 100 µg/kg/min intravenously (140 µg/kg/min in the present study) and observed variable 10-20 higher than normal adenosine values in infused venous blood. However, the concentration of adenosine on the arterial side that also reaches the coronary arteries was elevated only ~two-fold, from 0.15 mM to 0.30 mM or less (Sollevi et al., 1984;Sollevi et al., 1985). Thus, it is clear that adenosine is degraded to a large extent

during intravenous infusion and it is reasonable to assume that the rate of degradation is even more pronounced in highly fit athletes, which lowers the effective arterial adenosine concentration and may affect adenosine-induced myocardial blood flow. Adenosine infusion is also a marked external stress to the body since for instance arterial stress hormones increases 50-60 % (Edlund *et al.*, 1990) and fit athletes are likely to handle this by less disturbed bodily homeostasis.

As was already mentioned, it is typical for HCM to show reduced adenosine-induced blood flow as well as blood flow reserve. It is also known that this largely results from reduced capillary density in respect to myocardial hypertrophy (Johansson *et al.*, 2008). This raises some concerns that physiologically developed, pronouncedly thick myocardium as in athletes in the present study may show similar characteristics to HCM, although it is generally considered that the coronary vasculature seems to simply increase in parallel with increases in exercise-induced LV mass (Hudlicka 1982), which is also supported by the knowledge of the close connection between the size of epicardial coronary arteries and LV mass (Rodriguez & Robbins 1959; O'Keefe, Jr. et al. 1987; Leung et al. 1991; Dodge, Jr. et al. 1992; Zandrino et al. 2000) together with the tight coupling of coronary arterial vasculature volume within the LV wall and adenosine induced maximal coronary flow (Wusten *et al.*, 1977). Moreover, in this case, it would be reasonable to think that adenosine-induced hyperemia could indeed have been blunted in athletes since according to a recent study, during adenosine infusion capillaries become the bottleneck for myocardial blood flow resistance (Kaul & Jayaweera, 2008). Thus, even when the large epicardial arteries in athletes have twice or more the capacity to dilate (Haskell *et al.*, 1993), capillary bed may constrain flow increase during hyperemia, although arterioles may also play a role. It has also long been known that enlargement of even the healthy coronary arteries may not necessarily keep pace with that of cardiac mass (Roberts & Wearn 1941; Rodriguez & Robbins 1959), a fact that must be considered when discussing the vasculature of athlete's heart. However**,** even if athlete's heart may structurally resemble HCM, in HCM several ECHO parameters are different (impaired), such an A/E lower than 1, but none of the parameters in the present investigation showed abnormalities. Moreover, even if HCM is the leading cause of sudden death in young athletes, the prevalence of HCM in elite athletes is extremely low. In the western world the prevalence is 0.09 % (Basavarajaiah *et al.*, 2008), and thus it is very unlikely that in this investigation there were any HCM or HCM-like cases. It also has to be emphasized that actual blood flow reserve was similar between the groups. It is thus likely that experimental conditions related to intravenous adenosine infusion and its subsequent degradation mainly explain the reduced myocardial blood flow in athletes, although this conclusion dilutes some of the importance of the applied adenosine investigation. This reasoning can also be extended as follows.

The finding discussed above of the inverse relationship between adenosine-induced blood flow and the fitness level of the athlete paradoxical since the fitter the the athlete, the higher cardiac output he/she can reach, and thus set more demand to the requirements for myocardial oxygen consumption and blood flow. Thus, it may be that in normal healthy or diseased subjects intravenous adenosine is capable of inducing

maximal or near maximal myocardial blood flow, which also correlates positively to the fitness level of the subject, but not in athletes. It is unlikely that the fittest athlete could obtain the highest cardiac output with the lowest myocardial blood flow and concern rises whether this intravenous adenosine approach is at all capable of inducing maximal blood flow in athletes, as it has also previously been wondered in more general pharmacological terms (Rowell, 1986c). It is concluded that if true maximal per gram of myocardium perfusion would be obtained in athletes (which might be the case in untrained men also in the present study), it is highly likely that supraphysiological perfusion reserve could be observed. Thus, if these experimental factors are considred, it still remains to conclusively shown whether endurance training indeed leads to better reserve in myocardial blood flow. This could only be investigated with direct coronary arterial vasodilator infusions with increasing drug doses or by measuring coronary blood flow during maximal exercise, when perfusion and metabolism are not 'detached' as during infusions.

7.4.2 *Adenosine receptor A_{2A} receptor density and its relationship to adenosine-induced blood flow (IV)*

In the present study in humans myocardial $A_{2A}R$ density and MBF were not tightly coupled and receptor density was not different between the groups. Athletes are however shown to have higher total volume of $A_{2A}Rs$ due to their significantly larger LV mass despite similar receptor density. Many animal studies suggest that coronary vasodilation caused by adenosine is solely or at least primarily mediated via $A_{2A}Rs$ (Belardinelli *et al.*, 1998;Hein *et al.*, 1999;Hein *et al.*, 2001). Although direct *in vivo* human studies attempting to elucidate the coupling of adenosine-induced vasodilation and $A_{2A}Rs$ are lacking, it has been shown that specific $A_{2A}R$ agonists cause MBF increases comparable to adenosine with less side effects (Udelson *et al.*, 2004;Iskandrian *et al.*, 2007), supporting the essential role of $A_{2A}Rs$ in the vasodilation of adenosine, also in humans. In the present study, however, there was a trend of an inverse relationship between $A_{2A}R$ density and MBF reserve among all the subjects, which may suggest that the amount/density of $A_{2A}Rs$ may not be the most important factor in adenosine-induced myocardial hyperemia in humans. In addition to the likely role of other adenosine receptors modulating (Shryock & Belardinelli, 1997;Tawfik *et al.*, 2006) or directly evoking vasorelaxation like A_{2B} as it has been importantly shown in humans (Kemp & Cocks, 1999) and in animals (Abebe *et al.*, 1994;Olanrewaju & Mustafa, 2000;Morrison *et al.*, 2002;Talukder *et al.*, 2003), the divergent interindividual effects of intravenously infused adenosine (Chan *et al.*, 1992) may obscure a direct comparison in this non-invasive *in vivo* correlation approach of examining the role of $A_{2A}Rs$ as a predominant adenosine receptor responsible for adenosine-induced vasodilation in humans. Moreover, it also has to be considered that A_2Rs are not completely cell membrane-fixed receptors but rather, are upregulated from the cytosol when needed (Milojevic *et al.*, 2006). Thus, it may appear that there are no differences between the groups at rest, but strenuous exercise for instance may induce receptor upregulation that could be variable. On the other hand, continuous stimulus due to extensive infusion of adenosine may also induce $A_{2A}R$ down-regulation or inactivation (stimulus desensitization), and possibly more extensively in ET,

potentially explaining also their reduced hypereamic MBF. Moreover, one possible reason for poor coupling of $A_{2A}R$ density and MBF is that the extent of adenosine-induced hyperemia is not solely due to the amount/density of $A_{2A}Rs$, but also the sensitivity and activity of different signalling cascades downstream of $A_{2A}R$ activation is also important.

Finally, it should also be considered that variabilty of NO release modulates the findings. It has namely been shown that in addition to direct smooth muscle relaxation of adenosine and subsequent NO release (Ali *et al.*, 1997), part of the adenosine-induced vasodilation is also mediated by substantial direct endothelium $A_{2A}R$-linked (Newman *et al.*, 1988;Nees, 1989a;Nees, 1989b;Li *et al.*, 1995;Li *et al.*, 1998;Hein *et al.*, 1999;Hein & Kuo, 1999), or well known increased flow mediated indirect (shear stress) NO release (~comparable to cuff release in FMD) which by flow "accumulation" accounts for adenosine vasodilation. Furthermore, it has been suggested (Buus *et al.*, 2001) that adenosine-induced sympathetic activation (MacLean *et al.*, 1997) might lead to endothelial α-2 –receptor stimulation, which further on induces NO-dependent vasorelaxation by counterbalancing vascular smooth muscle alfa-2 –receptor vasoconstriction (Ishibashi *et al.*, 1997) which may variably affect adenosine vasodilation. Moreover, at least regarding animal skeletal muscle, A_1 receptors have also been shown to play a significant role in adenosine infusion (Marshall, 2007) and may also operate similarly in the heart. Thus, these factors associated with adenosine-induced vasodilation in humans may have precluded a direct correlation between MBF and $A_{2A}R$ density in the present study.

7.5   The effects of inhibitions of nitric oxide alone and in combination with prostanoids on skeletal muscle blood flow and oxygen consumption at rest and during exercise (V)

7.5.1   *The effect of single NO inhibition on muscle blood flow and oxygen consumption*

The present study demonstrates that inhibition of NOS increases muscle oxygen consumption by 20 %. This is in accordance with in vitro studies which suggest that NO competes with oxygen and tonically inhibits mitochondrial respiration by binding to cytochrome c oxidase complex (Brown, 1999;Brown, 2001;Moncada & Erusalimsky, 2002;Erusalimsky & Moncada, 2007;Cooper & Giulivi, 2007). Some animal studies have also previously found increased oxygen consumption when NO formation is blocked (Shen *et al.*, 1994;Shen *et al.*, 1995;Shen *et al.*, 2000). In previous human studies, oxygen consumption of the limb has been determined by multiplying whole limb blood flow by whole limb oxygen extraction while in the present study muscle capillary blood flow that is mainly responsible for oxygen diffusion in muscle was used to calculate oxygen uptake. In all previous human studies oxygen consumption during NOS inhibition was unchanged (Radegran & Saltin, 1999;Frandsenn *et al.*, 2001). These findings could be explained by the fact that relative decrease in blood flow of skin and adipose tissue during NOS inhibition is higher compared to muscle while oxygen is still consumed almost solely in muscles.

In recent years it has been established that NO is involved in the maintenance of resting skin blood flow in humans (Goldsmith *et al.*, 1996) and indeed, a previous study showed more pronounced reduction in skin blood flow compared to total forearm flow (Coffman, 1994). Adipose tissue blood flow is also dependent on NO formation and the relative decrease in adipose tissue blood flow during L-NMMA inhibition also seems to be more pronounced (Ardilouze *et al.*, 2004) compared to the whole limb (Mortensen *et al.*, 2007). In the current study, it was found that NO inhibition decreased adipose tissue blood flow to similar degree as in muscle (34 % in adipose tissue and 38 % in muscle). Unfortunately, skin blood flow can not be reliably addressed with current PET devices. Another possible explanation for the discrepancy between the present and earlier findings is that during NO inhibition blood flow is reduced relatively more in non-nutritive pathways. Importantly, NO-mediated vasoregulation is particularly prominent in the arterial-to-venous anastomoses in the human fingertips (Noon *et al.*, 1996) indicating that the arterial NO component is pronounced in those large scale vessels not directly nutritive to muscle. Thus, previous attempts to elucidate the effects of NO on human muscle oxygen consumption may have been masked by the insensitivity of whole limb oxygen consumption determinations.

The heterogeneity of the oxygen supply clearly tended to be increased when formation of NO was inhibited, and this was even more prominent when synergistic blockade of NO and COX enzyme was tested. This finding is in accordance with the proposition that NO facilitates O_2 distribution within the tissue (Victor *et al.*, 2009). This is also suggestive that impairments in normal function of NO and COX products (prostacyclin specifically) seen for instance in aging (Nicholson *et al.*, 2009) may indeed contribute to impairments in effective tissue perfusion distribution, which is also common in hypertension, obesity, and diabetes (Levy *et al.*, 2008).

It is noteworthy that in vitro studies suggest that tonic inhibition of aerobic metabolism by NO is most readily seen in hypoxic conditions (Brown, 1999;Brown, 2001;Moncada & Erusalimsky, 2002;Erusalimsky & Moncada, 2007;Cooper & Giulivi, 2007). In the present study increased muscle oxygen consumption was apparent already during sea level room air breathing, but as has already been previously proposed (Radegran & Saltin, 1999) it may appear that some parts of muscle may actually face hypoxia during NO inhibition due to markedly reduced limb oxygen supply. This may actually create a vicious circle. Interestingly, formation of reactive oxygen species (ROS) is also known to increase in hypoxia, although this is paradoxical since the presence of at least some O_2 is also needed (Guzy & Schumacker, 2006;Clanton, 2007). Since the formation of ROS is known to derive mostly from mitochondrion, this leads to the speculation whether the physiological action of NO inhibition is to decrease the formation of ROS (Iwase *et al.*, 2007;Cooper & Giulivi, 2007;Korge *et al.*, 2008) by inhibing aerobic respiration from which ROS mainly derive (Alexeyev, 2009). This could explain why in many cardiovascular diseases such as diabetes and hypertension there is simultaneous reduced NO formation/action and increased formation of ROS. This aspect should be studied in more detail. Finally, it has been shown that NO improves the efficiency of oxidative

phosphorylation by reducing the slipping of the proton pumps (Clerc *et al.*, 2007) and this may also explain some of the deficits in aerobic respiration in cardiovascular pathophysiology or why more oxygen is needed even at the resting state in these healthy subjects.

Muscle oxygen consumption was also measured during exercise when NO was inhibited, and it was observed that in contrast to resting condition, there was no significant alteration in blood flow or oxygen uptake, although limb oxygen extraction specified to active muscle was increased. It therefore seems that the inhibitory effect of NO decreases when the aerobic metabolism is increased by natural muscle contractions. Thus, oxygen binds more vigorously to mitochondrial terminal enzyme cytochrome c oxidase and counteracts the inhibitory effects of NO during exercise since its availability (capillary recruitment) and gradient increases. Although previous studies addressing the role of NO in exercise did not find increased oxygen consumption during NO blockade (Radegran & Saltin, 1999;Frandsenn *et al.*, 2001), there is, however, some earlier evidence for enhanced aerobic metabolism during NO blockade in humans. Inhibition of skeletal muscle NO production has been found to enhance the acceleration of the oxidative metabolism following the onset of vigorous near maximal whole body cycling exercise (Jones *et al.*, 2003;Wilkerson *et al.*, 2004;Jones *et al.*, 2004). Moreover, when systemic NO levels have been raised by dietary nitrate, oxygen consumption during steady-state submaximal whole body cycling exercise has been lower compared to control (Larsen *et al.*, 2007;Bailey *et al.*, 2009).

Finally, exercising muscle blood flow was not affected under NOS inhibition suggesting no role for NO controlling exercise-induced muscle hyperemia. It is however unclear why inactive posterior muscle blood flow did not decrease during exercise under inhibitions, although both inhibitions were started at resting state five minutes before exercise and at rest flow was markedly reduced during both inhibitions.

7.5.2 Why does nitric oxide not account for blood flow increase in exercising skeletal muscle?

During recent decades there have been times when everything regarding the control of vascular tone was explained by one single factor such as a substance released from the nerves, or later by prostaglandins, for which researchers received the Nobel Prize in 1982 having discovered this important regulatory pathway some decades earlier. Since the discovery of the importance of NO in vascular regulation and especially since the researchers who made these discoveries were awarded the Nobel Prize in 1998, there has been a tendency to believe that everything is explained almost solely by NO (Personal communication with Heikki Vapaavuori, Professor in Pharmacology, emeritus). Interestinly, blood flow increase in response to exercise if something is an obvious case where one would expect to see NO involved, but this usually 10 or even 20-fold increase in limb blood flow seems to be explained mainly by factors other than NO. In fact, if one considers the significanty reduced resting blood flow during NO

inhibition, exercise-induced hyperemia was actually even slighty higher during NO blockade since it was similar to control. This is unexplained and needs consideration.

One obvious explanation for the failure of NO inhibitions to reduce blood flow during exercise is that NO simply does not play a role in exercise hyperemia. Only tonic baseline vasodilation is affected. Shear stress however increases along with increases in blood flow during exercise, which is considered important for NO release, but still inhibitions show little if any effect. One possibility could be that there is a dilution effect, meaning that even if resting blood flow is effectively inhibited by NO blockade but since blood flow is increased substantially, the effective concentration of NO blocker is much less compared to rest. This however does not seem to be a possibility since NO is blocked by competitive inhibition of L-arginine and it is thus substrate availability rather than receptor binding that affects the inhibition, which is already present before dilution. A similar argument can also be applied to the speculation that NOS activity increases substantially because of muscular contractions (Balon & Nadler, 1994) and even if NOS is being inhibited effectively (Frandsenn *et al.*, 2001), this may not be enough during exercise, which in such is a possibility. However, as already mentioned, if the initial pathway is already blocked, there would be little increase in NOS activity (Personal communication with Ylva Hellsten). Only if the pool of L-argine changes (increases) could this possibility explain the results, which, however, seems unlikely. Moreover, very high concentrations of NO inhibitors have also been tried, but the results have not changed. Finally, substrate availability must be considered, especially oxygen. Oxygen is needed for ATP production, which may be preferential during contractions. Importantly, it is also known that any reduction in tissue PO_2, which naturally occur during exercise, decreases NO production by inhibiting NOS actitivy (Manukhina *et al.*, 2006). Acidity, which is likely to develop at least to some extent with contractions, is generally also known to inhibit NOS activity. Moreover, it may appear that NO indeed plays a more important role regulating the tone of large scale arteries and capillary blood flow is not that easily disturbed during exercise. It may also appear that during exercise NO is derived mainly from nitrate (NO_{2-}) reduction rather than NOS enzymes. The requisite one-electron reduction can be catalysed by xanthine oxyreductase, hemoglobin, myoglobin, and even by endothelial NOS, and also by the mitochondrial electron transfer complexes (van Faassen *et al.*, 2009) and happens in conditions of physical exercise (Lundberg *et al.*, 2008;van Faassen *et al.*, 2009). Thus, it appears that Paul Vanhoutte indeed was correct when he intuitively suggested in Nature in 1987 when NOS enzymes were unknown that NO could be formed from nitrite or nitrate (Vanhoutte, 1987). Finally, NO can also derive NOS-independently from adventitia (Feletou & Vanhoutte, 2006).

In addition to exercise, inhibiting both NOS and COX at rest may create problems when trying to intepret the source of vasoconstriction since it is known that there is an increase in the lipooxygenase pathway producing leucotriens when COX enzyme is blocked, and leucotriens usually constrict smooth muscle cells. Finally, COX inhibition also leads to a significant release of endothelin-1 (in mucosal microcirculation) (Funatsu *et al.*, 2007), which is the most potent vasoconstrictor in the human body. On

the other hand, it is well established that COX blockade also reduces the formation of thromboxane in platelets, which favors the development of vasodilation.

7.5.3 The effects of double inhibitions of NO and cyclooxygenase on skeletal muscle blood flow and oxygen consumption at rest and during exercise

Combined inhibition of NOS and COX enzymes caused a similar reduction in resting blood flow as NOS inhibition alone. The effect of sole COX inhibition on muscle hemodynamic was not addressed in the present study mainly since previous studies have shown that acute COX inhibition alone does not affect blood flow (Mortensen *et al.*, 2007). However, since combined inhibition of NOS and COX enzymes did not change resting muscle oxygen consumption, but single NOS inhibition did, it is plausible that COX products counteract the effects of NO on aerobic respiration (Mortensen *et al.*, 2007) as some in vitro studies actually directly suggest (Jacob *et al.*, 2001;Krause *et al.*, 2003). It however needs to be determined whether this effect only occurs as a result of synergism of NO and prostanoids, or is a direct effect of COX products also in vivo.

The fact that oxygen consumption of the exercising muscle was increased during the combined inhibition of NOS and COX is in contrast with previous observations (Mortensen *et al.*, 2007) and suggests that in a similar manner as the dualism in regards to vascular tone regulation (Vanhoutte & Tang, 2008), some of the COX products triggered by exercise may actually show NO-like inhibitory effects in regard to oxygen consumption. On the other hand, it is likely that there was some 'background' enhancement in oxygen extraction during this double inhibition since NOS inhibition alone also increased muscle oxygen extraction. Furthermore, one explanation for this observation of increased muscle oxygen consumption could be the time sequence of the measurements during the study day since the double inhibition measurements were always the last experiments. The subjects had been fasting for at least 4-5 hours and circulating free fatty acid concentrations were increased towards the end of the experiments. The limb uptake of free fatty acids tended to increase, indicating a shift to fatty acids in energy production, which could contribute to increased oxygen consumption. Secondly, muscle fatigue development in our untrained men may have caused recruitment of more fast-twitch type II muscle fibres. These muscle fibres extract and consume more oxygen and are in this respect more inefficient compared to type I muscle fibres (Coyle *et al.*, 1992;Krustrup *et al.*, 2008) and their activation has been shown to increase oxygen consumption by increasing oxygen extraction rather than changing muscle blood flow(Ferreira *et al.*, 2006). Thus, these factors may have confounded the findings of the detected increased oxygen consumption, which may have been caused by the experimental setting rather than by the direct inhibitory effects of COX enzymes.

There is, however, still another possible mechanism for increased oxygen consumption, which is the direct effect of COX inhibition, which also supports the hypothesis of different COX products triggered during exercise rather than at rest. In vitro studies have namely found that COX inhibition leads to significant uncoupling in

cellular energetics, which means that there is a leak of protons from mitochondrial intermenbranes that do not lead to normal ATP production (Jacob *et al.*, 2001;Krause *et al.*, 2003). Thus, in this situation there is an increased demand to consume oxygen if uncoupling reduces the effectiveness. More oxygen is then needed to restore the normal ATP levels needed for a given level of exercise. The situation appears to be somewhat similar to that in brown adipose tissue. It is well known that due to the uncoupling, there is substantial oxygen consumption in brown adipose tissue and energy is released as heat. The hypothesis that COX inhibition would exaggerate the need for oxygen to produce similar levels of ATP than without blockade needs further experimentation.

8   SUMMARY AND CONCLUSIONS

This study was undertaken to investigate the effects and regulatory aspects of acute exercise on skeletal muscle blood flow, and long-term endurance training on myocardial blood flow in humans. The results are as follows.

Muscle blood flow heterogeneity decreases from rest to exercise only if exercise intensity is strenuous enough. This is because although there are differences in blood flow in the deepest and superficial resting muscles due to differences in fiber-types and capillary densities, they are fairly uniformly perfused. At low exercise intensity the variation in blood flow between different muscles actually increases and starts to reduce again when more force is needed and more (superficial) muscle fibres are enganged in exercise. Activation of muscle fibres also triggers the recruitment of vascular units around the fibres and blood flow in exercising muscle becomes more uniform, although it seems to be perfused relatively luxuriously at least during small muscle mass exercise. The idea of muscle fibre and vascular unit recruitment explaining the reduced blood flow heterogeneity is also supported by the fact that blood flow heterogeneity was not changed from rest with local pharmacological adenosine infusion, although mean blood flow was increased substantially to exercising levels, meaning that increased blood flow as such does not reduce blood flow heterogeneity. Blood flow heterogeneity also differed significantly between exercising and adenosine-induced response indicating that exercise-induced muscle capillary blood flow hyperemia is a fairly complex process that cannot be mimicked by pharmacological infusions.

Physiological moderate systemic hypoxia does not seem to affect exercising or non-active muscle blood flow heterogeneity, although mean blood flow in working muscles is increased to preserve the normal arterial oxygen supply. Increased inactive muscle blood flow heterogeneity during small muscle mass exercise may represent nervous constraints to blunt flow increase elsewhere than in working muscles, but it is not potentiated by hypoxia and nervous restrains are not extremely powerful since during dynamic knee-extensor exercise inactive posterior muscle blood flow may also increase.

Direct infusion of adenosine into the femoral artery with a concentration that has previously been shown to induce maximal thigh blood flow obtainable with pharmacological infusion showed that the vasodilator influence within the thigh musculature is very heterogenous. Moreover, the aggregate total thigh muscle capillary blood flow of ~2-3 litres per min was fairly low compared to a previously established ultrasound Doppler determined whole thigh blood flow of ~8 L/min suggesting that a large part of the pharmacologically induced blood flow may actually represent flow shunting in the muscle. This proposition should be investigated in more detail in humans. However, the finding of close positive coupling of adenosine-induced muscle blood flow to systemic maximal oxygen consumption is in line with the idea that the inherent ablty to increase capillary surface area will effectively maximize oxygen and nutrient exchange during strenuous exercise.

One important aspect of the present study was to investigate whether adenosine plays a role in the regulation of muscle blood flow during exercise. The results clearly suggest that adenosine is not importantly involved in exercising muscle hyperemia in normoxia or in physiological systemic hypoxia. These results strongly support the accummulating view that no single factor can account for a large part of blood flow increase and moreover, that exercising muscle hyperemia is such a fundamental and vital process for the proper continuation of exercise that even if one component is inhibited, others can appear and restore blood flow to normal. Although when using unspecific adenosine receptor inhibitor theophylline in substudy I some evidence was found that endogenous adenosine might play a role in regulating muscle blood flow heterogeneity, this was not confirmed by exogenous adenosine infusion or by using aminophylline as a adenosine receptor blocker in substudy IV. It however seems likely that endogenous adenosine may be involved in regulating the tone of the vessels not directly nutritive to muscle, and in tissues other than muscle such as in subcutaneous adipose tissue. Additionally, it remains to be deterrmined whether adenosine plays a role regulating muscle blood flow during strenuous maximal or near maximal exercise involving possible partial ischemic conditions where adenosine can be expected to be released to the extent having clear physiological effects.

In the myocardium, adenosine-induced blood flow or blood flow reserve did not correlate with adenosine A_{2A} receptor density, although animal studies suggest this receptor subtype is the sole adenosine receptor by which vasodilation is mediated. It may be that other adenosine receptors account for vasodilation. Moreover, adenosine receptor A_{2A} density did not explain the reduced adenosine-induced blood flow in athlete's heart. Structurally athlete's heart closely resembles hypertrophic cardiomyophathy, which is the leading cause of sudden death in athletes where capillary density is reduced, and it thus follows that in some cases capillaries may not keep pace with cardiac hypertrophy, also in healthy athletes, and explain reduced adenosine response. It is however more likely that experimental reasons (degradation of intravenous adenosine in athletes due to a higher amount of red blood and endothelial cells) better explain the reduced adenosine-induced blood flow in atheletes. It is emphasized that myocardial blood flow reserve was similar to normal healthy fit subjects since resting blood flow was also lower in athletes. This lower resting blood flow was, however, not precisely matched to myocardial work, which was also reduced in athletes. This is suggestive of relative overperfusion due to high stroke volume and lower coronary arterial tone in the athletes' myocardium if oxygen extraction is reduced, or impaired efficiency due to pronouncedly increased myocardial mass, if oxygen extraction is preserved.

By inhibiting the formation of nitric oxide (NO) alone and in combination with cyclooxigenases (COX) it was observed for the first time in humans that nitric oxide may indeed tonically inhibit mitochondrial respiration by binding to cytocrhome oxidase and comptete with oxygen in quiescent skeletal muscle as in vitro and some animal studies have suggested. Previous attempts to elucidate this may have failed due to inability of bulk limb blood flow measurements to detect the changes occurring only in the muscle. It is speculated that the physiological significance of NO in this

respect is to limit the production of free radicals increased along with aerobic respiration. Both decreased NO production and/or its bioavailabilty and increased production of free radicals is observed in various pathophysiological diseases such as diabetes and hypertension and also in aging and this connection should be studied in more detail. The products of COX may however oppose the inhibitory effects of NO since double inhibition did not show increased oxygen consumption. Moreover, the inhibitory effects of NO seem to be reduced during exercise possibly since there is a need for oxygen to bind cytochrome c oxidase more vigorously. Additionally, although mean blood flow was reduced substantially mostly due to NO inhibition at rest, exercising muscle blood flow was modestly reduced only during double inhibition. This confirms that NO alone does not play a significant role in the exercising muscle hyperemia, but acts synergistically with prostanoids. Finally, that the heterogeneity of the oxygen supply was increased, especially during double inhibition of NO and COX, suggests that these agents are also important at the microcirculatory level to match blood flow to cellular metabolism and impairments in their normal function may explain some of the impairments in effective perfusion distribution common in diabetes, obesity and hypertension.

In conclusion, this study strongly supports the view that no single factor can account for exercise hyperemia, which is a very complex and fundamental process that can not either be mimicked by exogenous pharmacological vasodilator infusions. However, in the present study it was observed for the first time humans that nitric oxide not only affects muscle blood flow, but also oxygen consumption in quiescent skeletal muscle. Finally, life-long vigorous endurance training induces several pronounced alterations in cardiac muscle, and it needs to be determined whether myocardial overperfusion at rest is a result of impaired efficiency due to pronouncedly enhanced myocardial mass, or it is a result of reduced oxygen extraction in athlete's heart.

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Giving up is not an option.
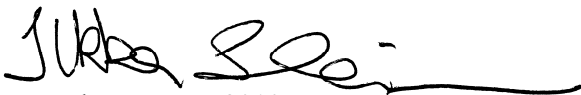
Turku, January 2010

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