

## INTRODUCTION

The human body is a multi-cell organism in which all cells require delivery of oxygen (O<sub>2</sub>) and nutrients as well as removal of byproducts of metabolism. The cardiovascular system facilitates this delivery and removal by ensuring that adequate blood flow is delivered to all organs. The cardiovascular system can be divided into three separate components: the heart that ensures the circulation of blood; the vascular tree that distributes that blood to the cells; and the blood that contains O<sub>2</sub> and nutrients. When the ventricles of the heart contract, blood is pumped into the aorta and distributed into conduit arteries, smaller arteries and arterioles before reaching the capillaries where the majority of exchange of O<sub>2</sub> and nutrients from the blood to the surrounding tissue occurs. A vital part of the cardiovascular system is its ability to distribute the flow of blood to any given tissue and precisely match the O<sub>2</sub> utilization while maintaining a stable arterial pressure. The precise distribution of blood flow is accomplished via a complex interplay between neurogenic and local factors (Saltin, 2007), but much remains to be known regarding the mechanisms behind the matching of blood flow and O<sub>2</sub> utilization. The present thesis focuses on the specific role of cGMP for the regulation of skeletal muscle blood flow in aging and with physical activity.

### Regulation of blood flow to skeletal muscles during exercise

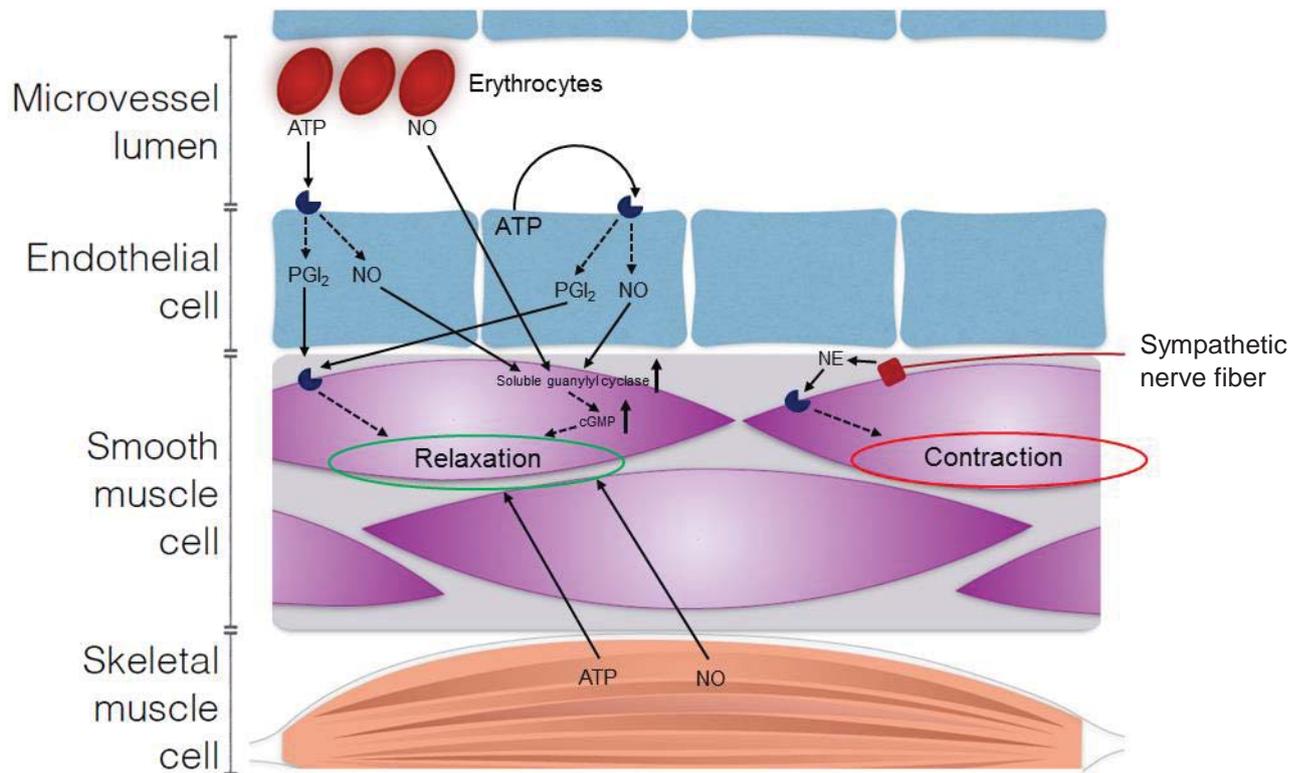
Skeletal muscles have a remarkable vasodilatory capacity and during dynamic contractions engaging a small muscle mass, blood flow can increase nearly 100-fold (Richardson *et al.*, 1993). Furthermore, the skeletal muscle blood flow and O<sub>2</sub> delivery is regulated precisely to match the O<sub>2</sub> demand of the skeletal muscle (Andersen & Saltin, 1985; Gonzalez-Alonso *et al.*, 2002). This ability to precisely match blood flow and metabolism ensures that an increase in muscle work is matched by an increase in O<sub>2</sub> delivery and is, therefore, essential for physical performance. It is generally accepted that regulation of blood flow and O<sub>2</sub> delivery results from the integration of several vasodilator and vasoconstrictor stimuli including mechanical effects of contraction, local metabolic and endothelium-derived substances, vasoactive substances from the erythrocytes, and the sympathetic nervous system (Hearon, Jr. & Dinunno, 2016). From a hierarchical point of view, this integrative control of skeletal muscle blood flow operates in a

way that 1) maintains arterial blood pressure at a minimal “acceptable” level at ~100 mmHg, 2) facilitates perfusion of the active muscles (Joyner & Casey, 2015). An important factor in the redistribution of blood flow and maintenance of arterial blood pressure during exercise is the sympathetic nervous system, as sympathetic vasoconstrictor activity impairs perfusion of inactive muscle and most non-muscular tissues (Rowell, 1997). Increased sympathetic drive also leads to vasoconstriction in the arterioles of the working muscles; however, contracting skeletal muscle has the ability to attenuate sympathetic vasoconstriction in order to ensure adequate O<sub>2</sub> delivery to the working muscle (REMENSNYDER *et al.*, 1962; Dinunno & Joyner, 2003; Rosenmeier *et al.*, 2004; Mortensen *et al.*, 2012b) (see Functional sympatholysis for further details). It is worth noting that during whole-body exercise (running, cycling, cross-country skiing etc.), where the vasodilatory capacity of the working muscle exceeds the cardiac output, skeletal muscle blood flow and vascular conductance is restrained by the sympathetic nervous system to avoid hypotension (Secher *et al.*, 1977; Calbet *et al.*, 2004). Although a central limitation to skeletal muscle blood flow is important for whole-body performance, the focus in this thesis is regulation of skeletal muscle blood flow during exercise with a small muscle mass, where peripheral blood flow is not limited by cardiac output.

Several vasodilatory substances derived from skeletal muscle cells, endothelial cells and erythrocytes have been proposed to be important for blood flow regulation; these include adenosine, adenosine triphosphate (ATP), nitric oxide (NO), prostaglandins and endothelial-derived hyperpolarization factors (Clifford & Hellsten, 2004). These vasodilators are formed in exercising muscle, and studies using pharmacological interventions to either inhibit or promote a vasodilator system have provided evidence for their role in vasodilation. Notably, it seems that none of the vasodilators operate independently, as no more than a ~20% reduction in exercise hyperemia is observed when one single vasodilator system is inhibited in humans (Radegran & Calbet, 2001). Instead, there is a redundancy among the vasodilator systems, where one vasodilator can compensate to ensure adequate blood flow when another vasodilator is impaired (Hillig *et al.*, 2003; Mortensen *et al.*, 2007).

During exercise, there is a marked dilation in the arteriolar tree with the most pronounced dilation observed in the smallest arterioles (Joyner & Casey, 2015). These arterioles (also known as resistance arterioles) are composed of a monolayer of endothelial cells surrounded by

several layers of smooth muscle cells. Contraction or relaxation of the smooth muscle cells control the arteriolar diameter, thus controlling resistance in the vessel and blood flow. The monolayer of endothelial cells is important in the integration of vasoactive mechanical and chemical stimuli (e.g. shear stress and endothelial receptor agonists) into the release of vasodilatory substances like NO and prostaglandins. In turn, smooth muscle cells integrate the vasoconstrictor and vasodilator signals into changes in vessel diameter (Figure 1). In the balance between constrictive and dilatory signals in the vascular smooth muscle cells, the cyclic nucleotide cyclic guanosine monophosphate (cGMP) is considered one of the most important second messengers that mediate vasodilation (Morgado *et al.*, 2012), especially in mediating the effects of NO (Bina *et al.*, 1995). In hypertensive subjects, it has been shown that acute potentiation of cGMP signaling by inhibition of the enzyme phosphodiesterase 5 (PDE5) increased blood flow following forearm contractions, suggesting that alteration in this important second messenger system can influence blood flow (Attina *et al.*, 2008). As the vascular changes associated with hypertension can be considered to be an accelerated form of the changes seen with aging (Taddei *et al.*, 1997; Soltis *et al.*, 1986), the vascular effects of potentiating cGMP in older individuals are intriguing.



**Figure 1. Integration of vasodilator and vasoconstrictor signals in the vascular smooth muscle cells.**

Theoretical illustration of how vasodilator and vasoconstrictor signals of interest in the present thesis affect vascular smooth muscle cell relaxation/contraction and thus blood flow. Contraction is primarily induced by sympathetic nervous activity and release of norepinephrine (NE) that binds to adrenergic receptors on the smooth muscle cells. On the other hand, vasodilators released from erythrocytes, endothelial cells and skeletal muscle cells induce relaxation in smooth muscle cells. ATP is released from erythrocytes, endothelial cells and skeletal muscle cells in response to deoxygenation of the hemoglobin molecule, mechanical stress (deformation and shear, not depicted), temperature and contraction of skeletal muscle cells. ATP acts by binding to purinergic receptors on endothelial cells leading to the formation of the vasodilators NO and prostacyclin (PGI<sub>2</sub>). Furthermore, NO is released from erythrocytes and skeletal muscle cells and acts directly on smooth muscle cells with cGMP as an important second messenger. The interaction between NO and PGI<sub>2</sub> is important in the vasodilatory response; however, this thesis primarily focuses on ATP and NO. The figure is adapted from (Nyberg *et al.*, 2015)

## NO

As a gas, NO acts directly on soluble guanylyl cyclase in smooth muscle cells leading to an increase in cGMP and consequent relaxation of the smooth muscle cells. NO is produced by nitric oxide synthase (NOS) which consists in different isoforms: neuronal (nNOS) in skeletal muscle cells and nerve cells, inducible (iNOS) in immune cells, and endothelial (eNOS) located in the endothelial cells (Ghimire *et al.*, 2017). In addition, there is evidence of NOS in other cell types such as the erythrocytes; erythrocyte NOS (RBC-NOS) (Kleinbongard *et al.*, 2006; Mozar *et al.*, 2016). Moreover, NO can be formed non-enzymatically and there is evidence to suggest that erythrocytes release NO from S-nitrosohemoglobin or via nitrite reduction (Pawloski *et al.*, 2001; Cosby *et al.*, 2003).

NO is important for vascular tone at rest and in recovery from exercise as indicated by an approximate 50% reduction in blood flow with NOS inhibition (Vallance *et al.*, 1989; Dietz *et al.*, 1997; Radegran & Saltin, 1999). However, in the majority of studies where NOS has been inhibited prior to exercise, NO is identified as a non-obligatory substance for exercise hyperemia (Radegran & Saltin, 1999; Frandsen *et al.*, 2001; Schrage *et al.*, 2004; Heinonen *et al.*, 2011). In contrast, when NOS inhibition is performed during steady-state exercise, it has been demonstrated that NO contributes to exercising blood flow in the forearm (Schrage *et al.*, 2004). Although it remains controversial if NO has an independent role in blood flow regulation during exercise, skeletal muscle blood flow and vascular conductance can be attenuated by 15-30% when the production of NO and prostaglandins is inhibited in combination (Boushel *et al.*, 2002; Mortensen *et al.*, 2007; Mortensen *et al.*, 2009b; Heinonen *et al.*, 2011). This suggests that different vasodilatory compounds can act in a redundant manner to ensure adequate blood flow if one vasodilator is experimentally removed.

## ATP

The erythrocytes are considered the primary source of ATP in the blood vessels. Evidence has been provided for release of ATP from the erythrocytes in response to deoxygenation, mechanical deformation, elevated shear stress and increased temperature (Gonzalez-Alonso, 2012). ATP has also been shown to be released from endothelial cells in response to mechanical stimulus and hypoxia (Burnstock, 2017). ATP is also present in skeletal muscle interstitial fluid

(Mortensen *et al.*, 2009a) and as the endothelium presents a barrier for passage of ATP (Ballard *et al.*, 1987), likely sources for the interstitial ATP are skeletal muscle cells, nerve cells and the abluminal side of endothelial cells (Hellsten *et al.*, 2012). In the intravascular space, ATP binds to P2Y<sub>2</sub> purinergic receptors on the endothelial cells, thereby stimulating the production of endothelial NO, and prostaglandins which act on the surrounding vascular smooth muscle cells to cause vasodilation (Gonzalez-Alonso, 2012). The binding of ATP to purinergic receptors also initiates a conducted vasodilator response in which the vasodilatory signals starts in the smallest vessels and capillaries and leads to upstream vasodilation in larger vessels (McCullough *et al.*, 1997; Collins *et al.*, 1998; Sprague *et al.*, 2011).

The main arguments for ATP being an important vasodilator during exercise are that, when given exogenously, ATP induces similar increases in blood flow as during heavy exercise (Gonzalez-Alonso *et al.*, 2002; Rosenmeier *et al.*, 2004; Gonzalez-Alonso *et al.*, 2008) and also has sympatholytic properties and attenuates sympathetic vasoconstriction (Rosenmeier *et al.*, 2004; Kirby *et al.*, 2008). It has been demonstrated that venous plasma ATP concentration ([ATP]) increases progressively with exercise intensity (Mortensen *et al.*, 2011; Kirby *et al.*, 2012) and is coupled to the offloading of O<sub>2</sub> from the erythrocytes (Gonzalez-Alonso *et al.*, 2002). Furthermore, a correlation has been observed between plasma [ATP] and vascular conductance in the exercising leg (Mortensen *et al.*, 2011). Collectively, these data suggest that ATP is coupled to blood flow regulation during exercise; however, the absence of specific antagonists for vasodilatory P2Y<sub>2</sub> purinergic receptors is lacking for firm conclusions regarding the involvement of ATP in exercise hyperemia (Clifford & Hellsten, 2004).

### **Impaired regulation of skeletal muscle blood flow during exercise with aging**

Since Wahren and colleagues in 1974 (Wahren *et al.*, 1974) reported an age-associated impairment in blood flow during graded cycle exercise in humans, a large number of studies have been conducted to consolidate and elaborate this finding. It is evident that an impaired blood flow regulation and O<sub>2</sub> delivery during exercise results in a lower aerobic exercise capacity, eventually leading to reductions in functional independence and overall quality of life. Thus, understanding the effects of aging on blood flow and its regulation during exercise is

important. Moreover, understanding the mechanisms behind the altered blood flow regulation with aging could aid in distinguishing if such limitations are caused by physiological age and/or long-term inactivity. In studies investigating age-related changes in blood flow during exercise where the systemic blood flow is not limited by cardiac output, most (Proctor *et al.*, 1998;Lawrenson *et al.*, 2003;Poole *et al.*, 2003;Kirby *et al.*, 2009;Donato *et al.*, 2006;Mortensen *et al.*, 2012b) but not all (Magnusson *et al.*, 1994;Proctor *et al.*, 2003;Donato *et al.*, 2006) have reported attenuated steady-state blood flow with age. In the studies reporting similar blood flow during exercise in older and young subjects, the older subjects experienced elevated mean arterial blood pressure, indicating an increased peripheral resistance during exercise (Magnusson *et al.*, 1994;Proctor *et al.*, 2003;Donato *et al.*, 2006). Overall, the majority of experimental data indicate that there is age-associated attenuation in skeletal muscle blood flow during exercise, an effect that is likely to be attributed to impaired vascular conductance (Hearon, Jr. & Dinunno, 2016).

The mechanism or, more likely, mechanisms behind the impaired regulation of vascular conductance with aging are currently unknown. Older adults experience a higher basal muscle sympathetic vasoconstriction (Sundlof & Wallin, 1978;Davy *et al.*, 1998) and it has been demonstrated that local  $\alpha$ -adrenergic blockage normalizes age differences in resting leg hemodynamics, suggesting elevated leg sympathetic tone as an underlying mechanism (Dinunno *et al.*, 2001). However, a recent study demonstrated that elevated sympathetic nervous system activity does not contribute to the age-associated impairment in exercising blood flow during graded handgrip exercise (Richards *et al.*, 2014). Another factor that could contribute to impaired exercise hyperemia is endothelial dysfunction (Taddei *et al.*, 1995), and impaired NO bioavailability as a consequence of oxidative stress has been shown to influence exercise hyperemia in the forearm (Schrage *et al.*, 2007;Crecelius *et al.*, 2010;Taddei *et al.*, 2001). This supports that NO bioavailability contributes to the age-associated impairments in exercise hyperemia; however, exercise blood flow did not improve in the leg of older subjects despite improved markers of NO bioavailability (Nyberg *et al.*, 2012). This discrepancy could be due to different vasodilatory pathways mediating exercise hyperemia in the arm and leg (Wray & Richardson, 2006).

ATP has also been suggested to play a role in impaired exercise hyperemia with age (Kirby *et al.*, 2012; Mortensen *et al.*, 2012b). The vasodilatory response to arterial infused ATP is maintained with aging in the forearm (Kirby *et al.*, 2010) but not in the leg (Mortensen *et al.*, 2012b). Furthermore, Kirby *et al.* demonstrated that venous [ATP] was lower in older than in young subjects during graded handgrip exercise, suggesting that the release of ATP from the erythrocytes and/or endothelial cells was impaired. Interestingly, the lower venous [ATP] was concomitant with a lower muscle blood flow during exercise (Kirby *et al.*, 2012). Thus, a failure to increase intravascular [ATP] could be a contributing factor in the age-related attenuation in blood flow during exercise.

Based on these observations, it may be concluded that the mechanism underlying the age-associated impairment in the regulation of skeletal muscle blood flow is not fully elucidated and possibly involves several vasoactive systems.

### Functional sympatholysis

The ability to attenuate the vasoconstrictive effect of the sympathetic activity is commonly referred to as functional sympatholysis (REMENSNYDER *et al.*, 1962). Functional sympatholysis is believed to contribute importantly to the regulation of skeletal muscle blood flow and O<sub>2</sub> delivery during exercise (Saltin & Mortensen, 2012; Hearon, Jr. & Dinunno, 2016). The mechanism underlying functional sympatholysis is likely to be compounds formed locally in the muscle that interfere with either the release or the effect of norepinephrine, thereby reducing the constrictive effect. In recent years, intra-arterial infusion of tyramine, which evokes norepinephrine release out of neuronal vesicles resulting in stimulation of adrenergic receptors (Brandao *et al.*, 1980), has been used to evoke sympathoexcitation (Hearon, Jr. & Dinunno, 2016). This method alongside simultaneous infusion of other vasoactive substances (e.g. sodium nitroprusside (NO donor), adenosine and ATP) have provided insight into the mechanisms behind functional sympatholysis.

In a series of studies, Rosenmeier and colleagues elegantly showed that neither NO nor adenosine had sympatholytic properties, and that ATP, in contrast, significantly attenuated tyramine-induced vasoconstriction in the leg (Rosenmeier *et al.*, 2003; Rosenmeier *et al.*,

2004;Rosenmeier *et al.*, 2008). The ability of ATP to attenuate sympathetic vasoconstriction has later been shown to be dependent on the concentration of ATP such that increasing [ATP] progressively limited  $\alpha$ 1-mediated vasoconstriction (Kirby *et al.*, 2008). It should be noted, that NO has been suggested to be involved in functional sympatholysis in the human forearm (Sander *et al.*, 2000;Chavoshan *et al.*, 2002) and in animal models (Thomas & Victor, 1998;Jendzjowsky & Delorey, 2013).

The ability for functional sympatholysis has been shown to be impaired in older individuals (Koch *et al.*, 2003;Dinenno *et al.*, 2005;Mortensen *et al.*, 2012b), potentially contributing to the reduced muscle perfusion observed in aging muscles (Saltin & Mortensen, 2012). However, recent studies have also provided evidence for that functional sympatholysis is related to the training status of the skeletal muscle (Nyberg & Hellsten, 2016;Mortensen *et al.*, 2012a;Mortensen *et al.*, 2012b;Jendzjowsky & Delorey, 2013). Notably, this effect of physical activity appears to be independent of age, as it has been shown that lifelong physically active men have a complete exercise-induced attenuation of vasoconstriction evoked by tyramine infusion (Mortensen *et al.*, 2012b). However, it is unclear by what mechanism exercise training affects functional sympatholysis and to what extent age affects the capacity of skeletal muscle to improve functional sympatholysis with training.

### Adaptations to exercise training in aging individuals

In young men, a large number of studies have demonstrated a number of adaptations in the cardiovascular system and skeletal muscle in response to physical activity and aerobic exercise training, including increased cardiac output, increased vascular conductance, greater perfusion capacity of the muscle, greater  $O_2$  extraction, increased oxidative capacity and increased capillary density (Hellsten & Nyberg, 2015). In contrast, the effects of exercise training on the cardiovascular system is less well described in older individuals, and most studies have been cross sectional, comparing older, highly trained athletes with more sedentary (Stratton *et al.*, 1994). Notwithstanding, longitudinal studies have demonstrated that a period of exercise training can lead to significant training adaptations (e.g.  $\dot{V}O_{2peak}$ , ejection fraction and cardiac output) that are similar in young and older men (Ehsani *et al.*, 1991;Stratton *et al.*, 1994). Cross-

sectional studies on young and older subjects have shown that regular exercise training improves vascular function as assessed by intra-arterial infusion of the endothelium-dependent vasodilator acetylcholine (Taddei *et al.*, 2000;DeSouza *et al.*, 2000;Nyberg *et al.*, 2012). Additionally, lifelong trained men have been shown to attain similar exercise hyperemic responses to young men and preserve the ability for functional sympatholysis (Mortensen *et al.*, 2012b). Notably, exercise training has been shown to lower exercise hyperemia at the same absolute workload in young individuals (Kiens *et al.*, 1993;Saltin *et al.*, 1968;Proctor *et al.*, 2001); however, unaltered (Gliemann *et al.*, 2013) or even increased (Beere *et al.*, 1999) exercise hyperemia has been observed in older subjects at the same absolute workload after a period of exercise training. Collectively, these data indicate that regular exercise training in older humans leads to significant training adaptations within the cardiovascular system; however, these adaptations may differ from adaptations in young humans.