THE ROLE OF OXYGEN-DEPENDENT SUBSTANCES IN EXERCISE

by

Christopher S. Davies

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School of Clinical and Experimental Medicine
College of Medicine and Dentistry
University of Birmingham
Birmingham
B15 2TT
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ABSTRACT

This thesis investigated the role of O_2-dependent substances in mediating the vasodilatation seen following exercise (post-exercise hyperaemia) and in fatigue development. Additionally we compared young and old subjects to investigate the effects of ageing in both of these phenomena.

Breathing supplementary 40% O_2 *during handgrip exercise* at 50% of maximum voluntary contraction had no effect of the magnitude of post-exercise hyperaemia compared to air breathing control. Furthermore, aspirin administration did not alter magnitude of post-exercise hyperaemia or the levels of prostaglandin E metabolites assayed from the forearm venous efflux. Similarly the magnitude of post-exercise hyperaemia was not affected by aminophylline administration. Collectively these suggest that prostaglandins and adenosine are not obligatory mediators of post-exercise hyperaemia.

Supplementary O_2 breathed *during recovery* had no effect on fatigue in a second bout of exercise or any of the substances proposed to mediate fatigue, in young subjects. We demonstrated that older subjects showed no changes in the magnitude of post-exercise hyperaemia, but they were more fatigue resistant. There was no O_2-dependence of either post-exercise hyperaemia or fatigue in older subjects.

In conclusion, we have found no evidence of O_2-dependent mediators in either post-exercise hyperaemia or fatigue.

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Abstracts associated with the studies presented within this thesis:

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Davies C.S., Gunchev D., Marshall J.M. (May 2009) Supplemental oxygen in recovery improves oxygen performance and attenuates the increase in Pi concentration. (Alternative Muscle Conference, Manchester).

Table of Contents

INDEX TO TABLES

INDEX TO FIGURES

COMMONLY USED ABBREVIATIONS

ACh	Acetylcholine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
COX	Cyclooxygenase
Cr	Creatine
DeoxyHb	Deoxyhaemoglobin
EDHF	Endothelium derived hyperpolarising factor
EET	Epoxyeicosatrienoic acid
FBF	Forearm blood flow
FVC	Forearm vascular conductance
Hb	Haemoglobin
HR	Heart rate
MABP	Mean arterial blood pressure
MRI	Magnetic Resonance Imaging
MVC	Maximum voluntary contraction
MVE	Maximum voluntary effort
NIRS	Near Infra-red Spectroscopy
NO	Nitric oxide
NOS	Nitric oxides synthase

OxyHb	Oxyhaemoglobin
PCr	Phosphocreatine
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
Pi	Inorganic phosphate
SNP	Sodium nitroprusside
TVE	Time to voluntary exhaustion
TTI	Tension-Time index

CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview

Muscle contraction plays an important role in all our lives, whether as part of the walking and lifting that occurs as part of our daily activity or in a more structured programme of exercise. As well as playing an important role in health, exercise dysfunction is an important factor in many diseases, such as the reduction in exercise tolerance seen in patients with chronic heart failure (CHF) (see e.g. Witte & Clark, 2007). The initiation and maintenance of cardiovascular changes associated with muscle contraction, whether sustained or rhythmic, involves a complex interplay between local vascular responses and reflex regulation of the cardiovascular and respiratory systems, with the higher parts of the central nervous system also having a role to play, especially during voluntary contractions. This makes exercise physiology a fascinating topic for the integrative physiologist.

During muscle contraction, the demand for oxygen (O_2) increases. In order to match this demand the blood flow to muscle must increase. This is partly achieved through an increase in the cardiac output, but it has been shown that vasodilatation in working muscle occurs leading to a process called exercise hyperaemia (sometimes also called active or functional hyperaemia). This phenomenon was first described by Gaskell (1877) and since then a lot of research has been performed to try to find out the cause of exercise hyperaemia. A number of mechanisms have been proposed, which are discussed in more detail below, although it seems likely that there is a degree of interdependency or redundancy within the mechanisms such that blocking one

mechanism leads to an increased contribution of others (Clifford & Hellsten, 2004; Joyner & Wilkins, 2007).

As a result of muscle contraction, fatigue occurs. For the purposes of this thesis, muscle fatigue is defined as a transient and recoverable decline in muscle force and/or power with repeated or continuous muscle contractions (Allen *et al.*, 2008). This may manifest as a decreased peak force or a decreased ability to maintain a force. Fatigue is to be at least in part caused by substances which are produced inside the muscle, several of which are similar to those implicated in mediating exercise hyperaemia (Allen *et al.*, 2008).

Previous work done in this laboratory has looked at the importance of O_2 in muscle contraction and the effects of different timings of O_2 delivery and are discussed further below (Fordy & Marshall, 2004; Win & Marshall, 2005). The conclusion of their findings were that O_2-dependent substances are produced during contraction that contribute to hyperaemia immediately post-exercise and that O_2-dependent substances produced or acting during recovery influence fatigue development.

Thus the aim of the studies described in this thesis was to further investigate the role of O_2-dependent substances in hyperaemia immediately post-exercise and fatigue. In addition, we hypothesize that O_2-dependent substances play a reduced role during exercise in elderly subjects. Therefore, the second aim of these studies was to investigate the effects of ageing on post-exercise hyperaemia and fatigue.

1.2 Muscle blood flow

When muscle contraction occurs it is accompanied by an increase in blood flow known as exercise hyperaemia, allowing close matching between O_2 delivery and consumption. The magnitude of the hyperaemia is proportional to the increase in exercise intensity (Saunders *et al.*, 2005b). When rhythmic exercise is taking place there is a hyperaemia in the periods between contractions, even though the blood flow during the contraction may be reduced due to compression by the muscle. During moderate intensity exercise this "intermittent hyperaemia" may be sufficient to meet the O_2 demands of the tissue, such that blood flow drops back to baseline levels when exercise ceases, whilst with heavier exercise the period of increased blood flow may continue after exercise has stopped (van Beekvelt *et al.*, 2001b). By contrast, during isometric contraction hyperaemia is limited by muscle compression, resulting in a rapid, large increase in muscle blood flow on release of the contraction. Since the original work of Gaskell in 1877, exercise hyperaemia has become a well-described phenomenon although there is still disagreement about what mediates it. The main theories of neural, mechanical, metabolic and shear stress are reviewed in the pages below.

1.2.1 Neural regulation of blood flow

Involvement of changes in neural activity in the regulation of blood flow is a mechanism that offers an attractive link between muscle contraction and the increase in muscle blood flow, especially in light of the rapidity of the hyperaemia (~1 sec) (Sheriff *et al.*, 1993). There have been three main theories linking neural activity to vasodilatation in exercise. The first theory considers

that withdrawal of sympathetic vasoconstriction or tone leads to an increased blood flow to the exercising muscle. This has been discounted because of evidence showing that muscle sympathetic activity actually increases to both active and resting muscle (Hansen *et al.*, 1994). The second theory suggests that sympathetic cholinergic neurones supply muscle blood vessels and that their activation contributes to the vasodilatation of exercise. The presence of these neurones has been shown in cats and dogs (Bolme & Fuxe, 1970) and they have been shown to contribute to the increased blood flow in skeletal muscle, in exercising conscious cats (Komine *et al.*, 2008). Early work seemed to suggest that these neurones were present in humans as well, with Roddie (1977) implicating them in the human vasodilator response to emotional stress and Sanders *et al.* (1989) finding evidence of cholinergic vasodilatation in the non-exercising arm of human subjects. This contrasts with histological work which shows no evidence of the presence of sympathetic cholinergic vasodilator neurones (Bolme & Fuxe, 1970), as well as more recent work using microelectrodes to measure activity in the nerves supplying the forearm muscle (Halliwill *et al.*, 1997). Halliwill *et al.* found that the dilatation during emotional stress was due to a withdrawal of sympathetic vasoconstriction and found no evidence of dilator fibres. It is now considered unlikely that there is any direct sympathetic vasodilator influence in human skeletal muscle (Joyner & Halliwill, 2000; Joyner & Dietz, 2003).

The third theory to link nerve activity with blood flow, initially proposed by Segal and Kurjiaka (1995), hypothesises that acetylcholine (ACh) released from motor neurones during skeletal muscle activation “spills over” and dilates the terminal

arterioles. Segal and colleagues obtained evidence for this phenomenon by using intravital microscopy on hamster muscle, showing that there was indeed an acetylcholine-mediated dilatation when motor neurones were stimulated in the presence of a neuromuscular–blocking drug (Welsh & Segal, 1997). However, despite the appeal of this proposal, there is no evidence to support it as an important contributor to exercise hyperaemia in whole muscle in animals or human subjects. Studies on the infusion of atropine (an acetylcholine receptor antagonist) into hindlimb muscle have found no effect on blood flow during exercise in either rats (Armstrong & Laughlin, 1986) or dogs (Buckwalter *et al.*, 1997). Further, in a study by Dyke *et al.* (1998), 5 healthy human volunteers attempted to try to contract their forearm muscle after paralysis with a neuromuscular-blocking drug. This would have the effect of causing motor neurone activation, without the metabolic factors released by contracting muscle. They found that there was only a modest increase in forearm blood flow which rules out a major role for ACh spillover in exercise hyperaemia.

In summary, although a mechanism linking neural activity with exercise hyperaemia is theoretically sound, experimental evidence suggests that it does not play a significant role in humans, although sympathetic cholinergic neurones may play a role in animals.

1.2.2 Mechanical regulation of blood flow

As indicated above, the increased blood flow that occurs in response to exercise is rapid, beginning within 1-2 seconds of the onset of exercise (Sheriff *et al.*, 1993). In a recent study, Wunsch and colleagues (2000) investigated whether it was possible for the rapid hyperaemia to be caused by vasodilator

substances by measuring the time taken to cause vasodilatation *in vitro*. They used applications of potassium chloride, adenosine, acetylcholine, and sodium nitroprusside. Their results showed that it took at least 4 seconds for any of the substances tested to cause vasodilatation, and suggested that there must be some other mechanism causing the initial rapid rise in blood flow. Mechanical mechanisms potentially meet the criteria and come under the umbrella of the so-called “Muscle Pump” theory that was popularised in a seminal review by Laughlin (1987). Put simply, this proposes that the contraction of muscle directly leads to an increased perfusion of the active muscle by increasing the arterio-venous pressure gradient and by propelling blood through the muscle (Laughlin, 1987; Tschakovsky & Sheriff, 2004). Since 1987, there has been a general consensus that the muscle pump is active in the initial stages of hyperaemia, although, as a recent Point-Counter Point discussion in the Journal of Applied Physiology shows (Sheriff *et al.*, 2005), opinion is still divided as to whether it is an important determinant of muscle blood flow in exercise. The following discussion analyses the evidence for and against an important role for the muscle pump.

There has been evidence for the muscle pump from a number of different experimental models, which were designed to isolate any mechanical effect. Sheriff and Van Bibber (1998) used a pig model in which an extracorporeal shunt was placed between the inferior vena cava and the terminal aorta so they could remove any effect of pumping of blood by the heart. Using this model they found that the isolated hindlimb skeletal muscle was capable of initiating and sustaining blood flow without input from the heart. In humans, Rådegran and

Saltin (1998) found that arterial blood flow was significantly increased during passive knee extension as compared to rest. In the same study they also showed that there was an immediate increase in blood velocity and flow following a single contraction. These results suggest that the muscle pump has the potential to contribute to the increase in muscle blood flow at the very onset of exercise.

The contribution of the muscle pump to the exercise hyperaemia of steady state exercise has been assessed by manipulating the force intensity and the frequency of contractions, such that when one increases the other decreases, thus keeping the total work rate the same. Two studies from Sheriff's laboratory on rats and dogs running on a treadmill found that doubling stride frequency (i.e. doubling contraction frequency) led to a doubling of muscle blood flow, even when the overall work rate was the same (Sheriff & Hakeman, 2001; Sheriff, 2003). A similar experiment on human cyclists showed that systemic vascular resistance decreased as pedalling frequency increased, despite overall work being the same (Gotshall *et al.*, 1996). These studies are all consistent with the muscle pump theory. By contrast, in a study performed on human volunteers using forearm exercise, lifting heavy or light weights in a slow or fast cadence respectively, so that the total work rate was the same, it was found that vascular conductance rose at the same rate in both conditions (Shoemaker *et al.*, 1998). From this they concluded that the muscle pump does not have an important effect and that there are vasodilator influences present early in the exercise period. The discrepancy between these studies may be due to the importance of gravity and a hydrostatic column of blood in the muscle pump as

described in an editorial by Laughlin and Joyner (2003). They theorise that the muscle pump may only lead to an increased blood flow when there is significant hydrostatic pressure, such as in upright exercise in humans. This is a condition that would not have been present in the forearm exercise in the study of Shoemaker *et al.* (1998), but would occur during locomotion in the studies from Sheriff's laboratory (Sheriff & Hakeman, 2001; Sheriff, 2003).

On the other hand, there have also been recent studies that support the theory of a rapid vasodilatation occurring due to metabolic substances. A study by Tschakovsky *et al.* (1996) used cuff inflation around the forearm to reproduce the mechanical effect of muscle contraction without any metabolic influence. They found no increase in blood flow when the arm was positioned above heart level, although there was an increase when the arm was below heart level, a finding that supports the idea that a hydrostatic column of blood is necessary. However, they also found that the increase in blood flow in response to muscle contraction was higher than that seen due to cuff inflation, this difference becoming noticeable after as little as 2 seconds. This indicates that there is a rapid non-mechanical vasodilator component present at the initiation of the increase in blood flow.

A recent study by Hamann *et al.* (2003) used a different approach. They gave an adenosine infusion to increase the hindlimb blood flow of dogs to a level greater than that seen performing treadmill exercise, reasoning that if there is a significant muscle pump effect, exercise would result in a further increase in blood flow. They found that exercise did not increase blood flow in this condition, a result they interpreted to show that the initial increase in blood flow

is due to vasodilation, not the muscle pump. A further study by the same group (Hamann *et al.*, 2004) used a potassium infusion to clamp the smooth muscle membrane potential so that vasodilation could not occur. When the dogs started treadmill exercise, during the potassium infusion protocol the hyperaemia was less than 10% of that seen during control exercise, indicating that vasodilatation is obligatory in exercise hyperaemia.

In summary, there is still controversy about whether the muscle pump is important in exercise hyperaemia. This may partly be due to the protocols used to investigate the phenomenon. As has already been discussed, it is believed that a significant hydrostatic pressure is necessary for the effects to be seen (Laughlin & Joyner, 2003). It has also been theorised that electrical stimulation may not be ideal to investigate the muscle pump as all the muscle fibres contract at the same time as opposed to the sequential activation seen in voluntary exercise (Laughlin, 1987). On balance it is likely that the muscle pump does play a role in exercise hyperaemia, particularly during exercise in large animals such as humans when the exercising muscle is below heart level. However, it is also likely that there is a vasodilator mechanism active in the initial stages of exercise. Potassium ions (K^+) are a possible candidate for this and evidence for their contribution is discussed below (1.2.3.5).

1.2.3 Metabolic regulation of blood flow

Given the direct relationship between intensity or frequency of contractions and the increase in blood flow, the presence of a vasodilator substance that is released as a result of muscle contraction is a logical conclusion. The idea that a vasodilator substance is released directly from the muscle fibres was first

proposed by Gaskell in the 1870s (Gaskell, 1877). More recently, studies have also provided evidence that vasodilator substances may be released from the endothelium and from red blood cells. In a recent review of the possible causes of exercise hyperaemia (Joyner & Wilkins, 2007), 5 criteria were set out for candidate vasodilator substance(s):

> The substance(s) or its precursor(s) should be present in skeletal muscle (or perhaps blood or nerves).
>
> The substance(s) should have access to the muscle resistance vessels.
>
> The concentration in the interstitial fluid (or at the endothelium) must be sufficient to cause dilation and the concentration should be proportional to the contractile activity.
>
> Exogenous administration of the substance(s) should be capable of causing prolonged dilation without sensations in humans.
>
> Pharmacologic agents or physiological manoeuvres which modify the blood flow responses to exercise should also modify the dilator responses to any putative substance given exogenously.

With these criteria in mind, one of the main problems in finding the vasodilator substance or substances is the apparent interdependency or redundancy in the system, whereby when the actions of one substance are blocked, other substances may be able to act or appear to play a greater role, as discussed below. Another factor complicating interpretation is the apparent difference in how hyperaemia is mediated during exercise and immediately post-exercise, with the different methodologies used to measure blood flow affecting which is

actually being measured. The following discussion will analyse the evidence for and against the major substances proposed to play a role in exercise hyperaemia, as well as how they might interact.

1.2.3.1 Role of oxygen

Oxygen is an obvious candidate for a metabolic regulator of muscle blood flow given the role it plays in metabolic processes in both the skeletal muscle and in the vascular smooth muscle. It is generally accepted that in the skeletal muscle bed at rest, acute systemic hypoxia causes a vasodilatation (e.g. Hutchins *et al.*, 1974; Marshall, 2000). Increasing the pO_2 of the solution bathing muscle preparations causes vasoconstriction (Duling, 1972; Pries *et al.*, 1995). Similarly it has recently been shown in human calf that vasoconstriction occurs when breathing hyperoxic gas mixes and that this effect is dose-dependent between room air (21%) and 100% O_2 (Rousseau *et al.*, 2005). The rate of O_2 delivery is a function of the O_2 content in arterial blood and the blood flow. It is therefore a reasonable theory that if O_2 delivery is inadequate for metabolic needs then this relative lack of O_2 may be sensed in either a direct or indirect way to cause an increase in blood flow to rectify it. The following discussion describes the efforts to investigate the direct and indirect effects of O_2 during exercise (see Table 1.1 for summary of human studies investigating effect of altering inspired O_2).

The efforts to assess the effects of the partial pressure of O_2 (pO_2) have for a long time been hampered by methodological difficulties in assessing pO_2 at the level of the muscle. Initial efforts used direct visualisation and O_2 electrodes placed in easily isolated muscles such as hamster cremaster muscle (Gorczynski & Duling, 1978) or rat spinotrapezius (Lash & Bohlen, 1987). Both

groups used electrical stimulation to initiate contractions in small muscle fibres. Gorczynski and Duling (1978) found that periarteriolar pO_2 remained relatively constant despite muscle contraction, although muscle tissue pO_2 declined in a progressive manner with increasing stimulation frequency. Simultaneous visualization of the arterioles supplying the muscle fibres showed that they dilated rapidly after initiation of contraction in a biphasic manner with an early peak, a return towards resting levels and then a further sustained late peak of vasodilatation. When contrasting this with the monophasic decline in muscle pO_2 and considering the fact that the latency from initiation of contraction to dilatation is shorter than the latency of tissue pO_2 decline they suggested that pO_2 may be more important in the maintenance of vasodilatation than in the initiation. Their evidence points to the O_2-dependence being due to tissue O_2 as periarteriolar pO_2 remained relatively constant. This is supported by further experiments in which bathing the muscle in O_2-rich superfusate, thus eliminating this drop in tissue pO_2, reduced the vasodilatation during contraction (Gorczynski & Duling, 1978).

Lash and Bohlen (1987) made similar findings of a small transient decline in periarteriolar pO_2 following electrical stimulation of rat spinotrapezius muscle which returned to normal as the exercise hyperaemia developed. They found a more significant decline in perivenular pO_2, with a decrease to ~50% of resting values which remained below 70% resting for the duration of the contraction. They hypothesised that instead of a direct effect of low pO_2 on the arterioles, vasodilatator substances were produced as a result of declining perivenular pO_2 and diffuse to dilate the nearby arterioles.

A similar approach was used by Bylund-Fellenius (1981) in humans. A flexible catheter with an O_2 transducer was inserted into the gastrocnemius muscle to measure tissue pO_2 at rest and at the end of a bout of foot pedal exercise. They found that in normal healthy subjects the pO_2 fell to approximately 70% of resting values following exercise at 8kg force with a further decrease at more intense levels of exercise. A similar study was performed more recently and found similar results in the tibialis anterior muscle during treadmill exercise (Jung *et al.*, 1999).

A method widely used to assess the effects on muscle blood flow of changes in Po_2 in humans is to manipulate O_2 delivery. Rowell *et. al.* (1986) showed that breathing 10% O_2 invariably augmented the increase in leg blood flow during dynamic quadriceps exercise at all workloads up to and including peak workload. This suggests that the magnitude of the hyperaemia is determined by the delivery of O_2 to the active muscle. These data have been supported in studies done breathing 11% O_2 during 2-legged knee extension exercise, where a 20-25% increase in exercise hyperaemia was seen during submaximal exercise, which was shown to be due to a vasodilatation (Koskolou *et al.*, 1997). However, hypoxia alone causes vasodilatation so these findings could simply show that exercise hyperaemia and hypoxic vasodilatation are summative.

An alternative to giving hypoxic gas is to use supplemental O_2 in order to ameliorate any decrease in pO_2, which might occur as a result of performing exercise. This was first used in exercise by Welch *et al.* (1977) in healthy active young men performing cycling exercise at either 55-70% of their maximum aerobic capacity, or just below their maximal aerobic capacity. They found that,

when compared to breathing room air, breathing 100% O_2 throughout caused an 11% reduction in exercise hyperaemia. Similar findings have been made more recently in physically active young subjects performing dynamic single leg knee extension at 70% peak workrate (Pedersen *et al.*, 1999). In this study, breathing 60% O_2 reduced the vasodilatation caused by exercise by ~12% compared to breathing room air, a value that is consistent with the findings of Welch *et al.* (1977) described above. However, another more recent study also using a dynamic leg kicking model at ~40% maximum power investigated the effects of breathing either room air, 70% O_2 (hyperoxia), or 14% O_2 (hypoxia) in healthy untrained young subjects (MacDonald *et al.*, 2000). They found no differences in leg blood flow during exercise as measured by Doppler ultrasound between any of the gas concentrations. This discrepancy between the studies could possibly be explained simply by differences in the level of hyperoxia given. However, given the findings of Fordy and Marshall (2004) described below using 40% O_2, this hypothesis is unlikely. Alternatively, it may be that trained subjects have an O_2-dependent component of exercise hyperaemia that is absent in less trained subjects.

The effect of hyperoxia on hyperaemia immediately post-exercise has been investigated further in this laboratory with respect to the timing of any O_2-dependent contribution (Fordy & Marshall, 2004). In this study, fit recreationally active young male subjects breathed 40% O_2 during a period of handgrip exercise at maximum effort until exhaustion with room air breathed before and after exercise, or simply breathed room air throughout. The major findings in this protocol were that 40% O_2, breathed during contraction only, was able to

reduce the hyperaemia seen after exercise by ~25%. This was in line with findings in a separate protocol in which 40% O_2 was breathed before, during and after static exercise at 70%MVC, whereas when 40% O_2 was breathed only during a recovery period following static handgrip exercise, there was no effect on forearm blood flow (unpublished PhD thesis: Fordy, 2007). This suggests that post-exercise hyperaemia following static handgrip exercise is partly mediated by an O_2-dependent substance, which is produced during the period of contraction itself.

This was supported by the results of a different protocol, in which the hyperaemic response to performing rhythmic handgrip exercise at 10% MVC for 5 minutes with concurrent arterial occlusion with a sphygmomanometer cuff was measured (unpublished PhD thesis: Fordy, 2007). Subjects breathed either room air or 40% O_2 for 5 minutes before starting the exercise with occlusion, in an attempt to see whether 'loading' the muscles with O_2 had any effect. There were no differences in the post-exercise hyperaemia between the 2 conditions, showing that the effect of hyperoxia on reducing post-exercise hyperaemia only occurs when administered during exercise.

Results obtained recently with high resolution proton magnetic resonance spectroscopy to detect levels of myoglobin deoxygenation as a measure of intramuscular pO_2 are also highly relevant to this issue. This methodology can be performed non-invasively and in real time and is therefore a useful tool in assessing the changes that occur during exercise and recovery. One of the initial studies by Richardson *et. al.* (1995) looked at human quadriceps muscle and found evidence of a steep O_2 gradient from blood to muscle. Six healthy

competitive cyclists were instrumented for blood flow and blood gas measurements before performing two bouts of leg extension exercise increasing from 25% to 100% peak force whilst breathing 21% O_2 (normoxia) or 12% O_2 (hypoxia). They then repeated the protocol in a 2-Tesla MRI machine to assess myoglobin deoxygenation during exercise (Richardson *et al.*, 1995). Maximum deoxy-myoglobin levels were taken to be those reached in the last two minutes of a 10-minute period of vascular occlusion and levels reached in exercise were reported as a percentage of this value. The group found that in both normoxic and hypoxic exercise deoxy-myoglobin levels rapidly increased to 50% and 60% of occlusion levels respectively within the first 20 seconds of exercise and then remained at these levels until maximum workrate was achieved. A further study by the same group again found no evidence of a progressive fall in intramuscular pO_2 with increasing workrate (Richardson *et al.*, 2001). However, another group has found a linear response with increasing intensity in the gastrocnemius muscle (Mole *et al.*, 1999). Therefore the question arises as to whether there is an O_2 gradient from the blood to the muscle and how much this gradient changes from rest.

Even in normoxia it was calculated that there was a steep O_2 gradient from the ~37mmHg in capillary blood to the ~3mmHg in muscle at maximal workrates (Richardson *et al.*, 1995). Using a higher powered magnet it has recently been possible to visualise the levels of deoxy-myoglobin even at rest (Richardson *et al.*, 2006). This has shown that in resting muscle the pO_2 is not hugely different from capillary levels (34mmHg compared to this studies capillary value of 44mmHg), indicating that during exercise there is a rapid drop in intramuscular

Po_2 to allow a larger diffusion gradient. When combined with the early findings described above from hamster cremaster muscle (Gorczynski & Duling, 1978), this gives weight to the hypothesis that any O_2 contribution to exercise hyperaemia is likely at the tissue level.

Table 1.1 Summary of studies assessing effect of altering inspired O_2 on blood flow in humans

Study	Muscle group	Exercise	Blood flow measurements	O_2 supplementation	Results
Rowell *et. al.* (1986)	Quadriceps	Dynamic - ~30% - 100% MVC.	Thermal dilution during exercise.	Normoxia vs. 10% O_2.	Exercise blood flow higher in hypoxia than normoxia at all workloads.
Koskolou *et. al.* (1997)	Quadriceps	Two-legged knee extension.	Doppler Ultrasound during exercise.	Normoxia vs. 15% O_2 vs. 11% O_2.	Exercise hyperaemia increased in hypoxia.
Welch *et. al.* (1977)	Leg	Cycling – 55-70% and just below maximum aerobic capacity	Dye dilution technique during exercise.	100% O_2 vs. normoxia.	Hyperoxia reduces exercise hyperaemia by 11%.
Pedersen *et. al.* (1999)	Quadriceps	Dynamic single leg extension – 70% peak workrate.	Thermodilution during exercise.	60% O_2 vs. normoxia.	Hyperoxia reduces exercise hyperaemia by 12%.
MacDonald *et. al.* (2000)	Quadriceps	Dynamic single leg kicking – 40% max. Untrained subjects.	Doppler Ultrasound during exercise.	70% vs. normoxia vs. 14%.	No differences in exercise hyperaemia at any level of O_2.
Fordy & Marshall (2004)	Forearm	Static – 100% MVE until exhaustion	Venous occlusion plethysmography (immediately post-exercise).	40% vs. normoxia (gas given during contraction)	Hyperoxia reduced post-exercise hyperaemia by 25%.
Win & Marshall (2005)	Forearm	Static – 60% MVC for 2 minutes.	Venous occlusion plethysmography (immediately post-exercise).	40% vs. normoxia.	Hyperoxia reduces post-exercise hyperaemia by 40%

1.2.3.2 Nitric oxide (NO)

Since the discovery of NO and the realisation of its importance in maintaining basal vascular tone in the late 1980s (Palmer *et al.*, 1987; Moncada *et al.*, 1988) there has been much interest in a possible role for NO in mediating exercise hyperaemia. NO is produced from L-arginine by the enzyme nitric oxide synthase (NOS) which is present in 2 main constitutive isoforms, endothelial and neuronal, which in humans can be found in the vascular endothelium and skeletal muscle respectively (Frandsen *et al.*, 1996; Stamler & Meissner, 2001). There have been a number of studies over the last 20 years that have attempted to elucidate the role of NO, with varied results.

The first study to look at the contribution of NO to exercise hyperaemia in humans used dynamic wrist flexion and extension at 0.2 and 0.4W and measured forearm blood flow with venous occlusion plethysmography (Wilson & Kapoor, 1993a). They found that NOS inhibition with L-NMMA reduced resting forearm blood flow from 2.5 to 1.5ml/100ml tissue/min, a finding that is consistent in all studies on the effects of NOS inhibition (Rådegran & Hellsten, 2000). However, they did not find any change in the increase in FBF during exercise, indicating that NO does not play an additional role in exercise hyperaemia.

Other studies have yielded similar findings. Shoemaker *et al.* (1997) used Doppler ultrasound to investigate the effect of prior inhibition of muscarinic acetylcholine receptors with atropine and NOS with L-NMMA on the increase in blood flow evoked by rhythmic handgrip at 10% maximum voluntary contraction (MVC). They found no difference in the steady state blood flow response

compared to control exercise without drug intervention. Similarly, Radegran and Saltin (1999) found no effect of prior L-NMMA infusion on the hyperaemia seen in response to a graded single leg knee extension protocol from ~65% peak power until peak exercise (pre-defined as the maximum force subjects could sustain for 3 minutes). However, it is worth noting that both studies noted a decrease in the total recovery blood flow response over 5 minutes (Shoemaker *et al.*, 1997) and 10 minutes (Radegran & Saltin, 1999) following exercise. Thus, these studies both appear to show that NO is important in regulating post-exercise blood flow but not during steady state exercise.

This hypothesis is supported by the findings of Frandsen *et al.* (2001) who used a different NOS inhibitor. They used L-NAME on the basis that it can enter into the interstitium, hypothesising that it would give a more complete inhibition of NOS activity as it can block neuronal NOS in the skeletal muscle as well as the endothelial NOS in the vascular endothelium. In their study 8 young men performed submaximal single leg knee extension at 30W for 30 mins (~30% peak force) and 5 performed a graded exercise protocol until exhaustion, with blood flow measured using the thermodilution method. Their findings were that leg blood flow was similar during exercise with or without NOS inhibition in both submaximal exercise and exhaustive exercise. They did find a lower level of leg vascular conductance (i.e. FBF) in the submaximal exercise following NOS inhibition, indicating reduced vasodilatation in extensor muscles. However, they attributed this to vasoconstriction in non-active tissue included in the measure of limb blood flow, a theory that is supported by the finding that this difference was not present in the intense exercise protocol. In agreement with the studies

described above, Frandsen *et al.* found a role for NO in the post-exercise period, with the LBF after submaximal exercise reduced by L-NAME compared to control.

There are however other studies which do indicate an effect of NOS inhibition on exercise hyperaemia. In a study by Gilligan *et al.* (1994) 18 subjects performed rhythmic handgrip exercise at 15, 30 and 45% of their MVC for 5 minutes each and used venous occlusion plethysmography to measure the blood flow during these relaxation periods. NOS was blocked prior to the exercise trial using an L-NMMA infusion. They found that NOS inhibition decreased the vasodilator response to exercise by ~18% compared to control and the increased exercise blood flow was reduced by 7%. It is worth noting however that venous occlusion plethysmography requires the exercise to be stopped for measurements of FBF to be made. Therefore, it is possible that they were measuring the decreased post-exercise blood flow response that the studies described above have also shown (Shoemaker *et al.*, 1997; Radegran & Saltin, 1999; Frandsen *et al.*, 2001).

A later study also using venous occlusion plethysmography during rhythmic handgrip exercise at 15% MVC for 20 minutes investigated the effect of infusing L-NMMA intra-arterially whilst steady state exercise was occurring (i.e. 5 minutes into the exercise protocol) (Dyke *et al.*, 1995). They found that NOS inhibition reduced the hyperaemia in the final 10 minutes of exercise compared to the first 5 minutes prior to the infusion and compared to a time control in which the same exercise was performed without L-NMMA infusion. This suggests that NOS inhibition during exercise may elicit a reduction in exercise

blood flow that was not visible in the studies described above where NOS inhibition occurred prior to commencing exercise.

However, two recent studies have both found a role for NO in exercise hyperaemia when using Doppler ultrasound, consistent with the findings of Dyke *et al.* (1995) above. This can give a measure of blood flow without stopping exercise, thus avoiding the limitation of venous occlusion plethysmography (Gilligan *et al.*, 1994). When using Doppler, Casey and Joyner (2009) found that L-NMMA infusion during rhythmic handgrip exercise at 10 and 20% MVC caused reductions in exercise blood flow of 12.3% and 11.3% respectively. Further, Wray *et al.* (2011) investigated the effect of blocking NOS with L-NMMA infusion prior to and during progressive handgrip exercise consisting of 3 minutes each of exercise at 4, 8, 12, 16, 20 and 24kg (mean MVC was ~50kg). Their main findings were that blood flow was significantly lower during L-NMMA infusion at all forces apart from 4kg, as well as at rest. Importantly, when the lower blood flow at rest was taken into account, the change in blood flow during exercise was only significantly lower at 20 and 24kg. This indicates that the contribution of NO to exercise hyperaemia may be intensity-dependent, and that some of the studies described above were perhaps using too low a force of exercise to demonstrate a role for NO.

Thus, taking all the studies discussed above into account, NO definitely has a role in mediating post-exercise blood flow. The evidence that NO plays a role in mediating steady state exercise blood flow is not as clear. It appears that the role of NO in mediating exercise hyperaemia is intensity dependent, only making a significant contribution at higher intensities of exercise. It would also

appear that when NO inhibition occurs either during or just prior to exercise, a reduction in blood flow may be visible, but if inhibition occurs in advance of exercise then other substances may play a greater role. This idea is supported by studies that investigate the effect of inhibition of multiple substances and suggest that there may be interdependency or redundancy between the actions of NO, prostaglandins, adenosine and ATP. These studies are discussed further below.

1.2.3.3 Prostaglandins

One of the groups of substances that have been implicated in mediating exercise hyperaemia are vasodilator prostanoids (see Table 1.2 for summary of studies investigating effect of cyclooxygenase inhibition in humans). The prostanoid family are formed from the metabolism of arachidonic acid by the enzyme cyclooxygenase (COX) into endoperoxides, which are in turn converted to the various prostanoids by specific prostanoid synthases (for review see Tang & Vanhoutte, 2009). Prostanoids can be either vasodilator such as PGE_2 or prostacyclin (PGI_2) or vasoconstrictor such as thromboxane A_2 (TXA_2). A role for prostanoids in the vasodilatation of exercise was first demonstrated by Kilbom and Wennmalm (1976). They used venous occlusion plethysmography to assess the post-exercise hyperaemia following 5 minutes of rhythmic handgrip exercise and 5 minutes of static handgrip exercise at 15% of maximum voluntary contraction (MVC) in healthy young subjects, both before and after rectal administration of the COX inhibitor indomethacin. Their main findings were that COX inhibition reduced both the peak and the total blood flow following both rhythmic and static exercise.

Efflux of prostanoids into venous blood has also been shown to increase in response to high intensity repeated static contractions in cat triceps surae (PGE_2 and PGI_2) (Symons *et al.*, 1991). Further, these findings have been replicated in human subjects in venous efflux (Wilson & Kapoor, 1993b) and by using microdialysis to measure levels of substances within the muscle interstitium (Karamouzis *et al.*, 2001a; Karamouzis *et al.*, 2001b). The first study by Wilson and Kapoor (1993b) demonstrated that the increase in forearm post-exercise blood flow elicited by rhythmic wrist flexion and extension was associated with an increase in venous efflux of both 6-keto $PGF_{1\alpha}$ (the stable breakdown product of prostacyclin) and PGE_2. Further, COX inhibition with indomethacin reduced post-exercise hyperaemia by ~20% and virtually abolished release of both 6-keto $PGF_{1\alpha}$ and PGE_2. Evidence that the increase in venous efflux of 6-keto $PGF_{1\alpha}$ and PGE_2 is due to increased release from the muscle comes from studies using microdialysis (Karamouzis *et al.*, 2001a; Karamouzis *et al.*, 2001b). When young male subjects performed either 40 minutes of plantarflexion of the foot (1.5 second contraction – 1.5 second relaxation) or 60 minutes of single leg knee extensor exercise at 20W, the interstitial levels of PGE_2 and 6-keto $PGF_{1\alpha}$ remained constant in the calf exercise and PGE_2 increased in the dynamic leg extension exercise (Karamouzis *et al.*, 2001b). Given the increase in blood flow that is present in these exercise conditions, it is likely that muscle production of both these substances actually increased during exercise. Another study by the same group used similar microdialysis techniques and demonstrated that both PGI_2 and PGE_2 increased with dynamic cycling exercise, while levels of thromboxane

B_2 (TXB_2 – a vasoconstrictor prostanoid) decreased (Karamouzis *et al.*, 2001a). Of interest given the close relationship of increasing blood flow with increasing exercise intensity, this last study also found that PGI_2 and PGE_2 production was greater at 150W cycling than it was at 100W. Collectively, these studies therefore seemed to demonstrate a conclusive role for vasodilator prostaglandins, particularly PGI_2 and PGE_2 in mediating exercise hyperaemia.

Since these studies different groups have tested the effects of COX inhibition with differing results. Duffy *et al.* (1999) used light wrist flexion and extension (similar exercise protocol to that used by Wilson and Kapoor (1993b)) and found that a different COX inhibitor, aspisol, also reduced both the peak post-exercise hyperaemia and the total volume of blood flow over 5 minutes. However, a study using ibuprofen to block COX inhibition in young men performing low load rhythmic handgrip exercise (4.4kg – approximately 10%MVC) found no effect on blood flow during exercise, as measured by Doppler ultrasound (Shoemaker *et al.*, 1996a). This was the case both in exercise performed above heart level and in exercise performed below heart level, where they had hypothesised that the increase in shear stress would increase the role of prostaglandins.

More recent studies indicate that differing results may be dependent on the muscle group studied, the intensity of the exercise and the timing of the measurements. Boushel *et al.* (2002) used Near-Infrared Spectroscopy (NIRS) to measure the microvascular blood flow in the quadriceps muscles (vastus lateralis and medialis – VL and VM) of 6 healthy young subjects whilst performing 10 minutes of dynamic knee extension at four different workloads. Combined NOS and COX inhibition (with L-NAME and indomethacin

respectively) attenuated the hyperaemia seen during control exercise at 30W, 45W and 60W exercise, but not at 15W exercise. They also used microdialysis to measure PGE_2 released into the interstitium of active muscle and found a decrease in [PGE_2] at 15 and 30W, and an increase at 45 and 60W, which was prevented when COX was blocked. These results indicate that PGE_2 plays a role in hyperaemia during more intense exercise.

Saunders at *al.* (2005a) used a novel alternative approach, using Doppler ultrasound to measure the change in exercise blood flow when transitioning from steady state rhythmic handgrip exercise at ~10% MVC to ~20% MVC. The rationale for this protocol was that they had previously demonstrated that this increase in intensity does not further reduce the minimum venous volume (Saunders & Tschakovsky, 2004) and so reasoned that this would allow them to assess any rapid contribution of vasodilator substances in the absence of any muscle pump effect. The main findings were that combined inhibition of COX and NOS had no effect on the rapid vasodilatation when exercise intensity increased (Saunders *et al.*, 2005a). However, when low intensity rhythmic handgrip exercise at 10%MVC was performed for 20 minutes, dual inhibition did reduce the blood flow and vascular conductance (Saunders *et al.*, 2005a), supporting the results from the study by Boushel *et al.* (2002).

This study served as a basis for another study by Schrage *et al*. (2004) in which 14 young subjects performed 20 minutes of rhythmic handgrip exercise at ~10% MVC with 1 second contraction-2 second relaxation. After beginning exercise, subjects received either COX inhibition with ketorolac or NOS inhibition with L-NAME to study the individual effects of prostaglandins and NO. Subjects then

received the second drug to produce a dual COX/NOS blockade. It was shown that ketorolac led to a transient reduction in FBF during exercise of ~12%, which had returned to control levels by the end of the 5 minute infusion. NOS inhibition alone caused about a 20% reduction in blood flow. When ketorolac was added to the L-NAME there was a further transient decrease which then returned to levels seen with NOS inhibition alone. NOS inhibition after COX inhibition had the same effect as NOS inhibition alone. These data combined suggest that prostaglandins normally contribute to exercise hyperaemia but when their synthesis is inhibited, another substance other than NO can restore the hyperaemic response.

It has previously been shown that hypoxia increased PGI_2 production in cultured endothelial cells (Michiels *et al.*, 1993). Further, Messina *et al.* (1992) demonstrated that removal of the endothelium in rat skeletal muscle arterioles abolished the vasodilator response to hypoxia, a response which was mirrored by administration of the COX inhibitor indomethacin. Thus they concluded that the vasodilatation in response to hypoxia was mediated via prostaglandin release from endothelial cells. On the basis of this, Win and Marshall (2005) hypothesized that ameliorating hypoxia by administering hyperoxic gas (40% O_2) would reduce the vasodilatation following static handgrip exercise by reducing release of vasodilator prostaglandins. The COX inhibitor aspirin was administered orally to block prostaglandin synthesis. The important findings of this study were that aspirin reduced post-contraction peak hyperaemia and vasodilatation by ~35% compared to control, a magnitude of reduction that was matched by both hyperoxia alone and by hyperoxia combined with aspirin. This

provides strong evidence that vasodilator prostaglandins mediate post-exercise hyperaemia and that either their production or mechanism of action is O_2-dependent. This study hypothesized that these O_2-dependent prostaglandins originated from the endothelium based on the previous *in vivo* work (Messina *et al.*, 1992; Michiels *et al.*, 1993). However, given the most recent findings by Richardson *et al.* (2006) described above that show a significant decrease in muscle interstitial pO_2, it is equally possible that the 40% O_2 acts by ameliorating hypoxia within the muscle (see Figure 1.1 for summary).

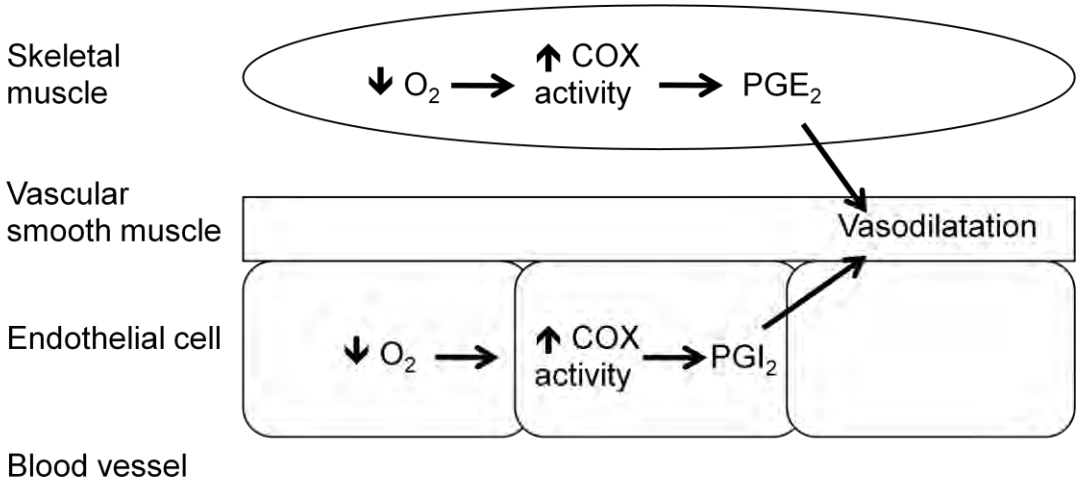

Figure 1.1. Schematic representation of the proposed mechanisms by which vasodilator prostaglandins may be released in an O_2-dependent manner. In response to hypoxia, COX activity increases in skeletal muscle to induce PGE2 release and in endothelial cells to induce PGI2. Both skeletal PGE2 and endothelial PGI2 can cause vasodilatation.

Table 1.2 Summary of studies assessing effect of COX inhibition on blood flow in humans

Study	Muscle group	Exercise	Blood flow measurements	COX inhibition	Results
Kilbom & Wennmalm (1976)	Forearm	Static and Dynamic handgrip – 15% MVC for 5 min	Venous occlusion plethysmography	Indomethacin prior to exercise.	COX inhibition reduces both peak and total blood flow post-exercise.
Wilson & Kapoor (1993b)	Forearm	Dynamic wrist flexion and extension	Venous occlusion plethysmography	Indomethacin prior to exercise.	COX inhibition reduces post-exercise hyperaemia by 20%.
Duffy *et. al.* (1999)	Forearm	Dynamic wrist flexion and extension	Doppler ultrasound	Aspisol prior to exercise.	COX inhibition reduces post-exercise hyperaemia.
Shoemaker *et. al.* (1996a)	Forearm	Dynamic handgrip – ~10% MVC.	Doppler ultrasound	Ibuprofen	Exercise hyperaemia not affected by COX inhibition.
Boushel *et. al.* (2002)	Quadriceps	Dynamic knee extension for 10 minutes – different workloads	Near-Infrared Spectroscopy.	Indomethacin (+NOS inhibition with L-NAME)	Combined NOS and COX inhibition reduced exercise hyperaemia at higher intensity (30-60W) but not at lower (15W).

Study	Muscle group	Exercise	Blood flow measurements	COX inhibition	Results
Saunders *et. al.* (2005a)	Forearm	Dynamic handgrip – 10%MVC for 20 mins and transition from 10% to 20%MVC.	Doppler ultrasound	Ketorolac (+NOS inhibition with L-NAME).	Combined NOS and COX inhibition reduces steady state exercise hyperaemia but no effect on rapid vasodilatation in transition.
Schrage *et. al.* (2004)	Forearm	Dynamic handgrip – 10%MVC for 20 minutes.	Doppler ultrasound	Ketorolac during exercise.	COX inhibition caused a transient 12% decrease in exercise hyperaemia, that returned to control levels within 5 minutes.
Win & Marshall (2005)	Forearm	Static handgrip – 60%MVC for 2 minutes.	Venous occlusion plethysmography	Aspirin.	COX inhibition decreased post-exercise hyperaemia reduced by 35%.
Mortensen *et. al.* (2007)	Quadriceps	Single leg extension – 20% MVC for 5 minutes	Doppler ultrasound	Intra-arterial indomethacin	COX inhibition alone had no effect on exercise hyperaemia.

Study	Muscle group	Exercise	Blood flow measurements	COX inhibition	Results
Heinonen *et. al.* (2011)	Quadriceps	Single leg extension – low intensity (4.5kg)	Positron emission tomography with radiolabelled water.	Indomethacin before exercise (+ NOS blockade with L-NMMA).	Combined NOS and COX inhibition reduced exercise hyperaemia by15-20%.
Mortensen *et. al.* (2009b)	Quadriceps	Knee extension – 30%MVC.	Doppler ultrasound.	Indomethacin (+NOS blockade with L-NMMA and adenosine receptor antagonism with theophylline)	Exercise hyperaemia reduced by 30% in COX and NOS dual blockade. No additional effect of theophylline.

1.2.3.4 Adenosine and ATP

The cellular source of energy is adenosine triphosphate (ATP), which is dephosphorylated to adenosine diphosphate (ADP) and can be further broken down to form adenosine monophosphate (AMP) and finally adenosine. As concentrations of these substances change significantly during muscle contraction, it is a plausible theory that they might play a role in exercise hyperaemia. Adenosine and ATP in particular have received a lot of attention (see Table 1.3 for summary of studies assessing role of adenosine in humans).

The 'adenosine hypothesis' was originally formulated by Berne (1963) when considering the coronary circulation. It stated that blood flow is regulated by interstitial adenosine generated when there is insufficient O_2 to regenerate ATP, in a negative feedback loop to increase blood flow and therefore O_2 supply. Since then evidence has emerged linking adenosine to exercise hyperaemia in skeletal muscle. Blood samples taken from dog calf muscles showed an increase in adenosine concentrations in the venous efflux following muscle stimulation (Fuchs *et al.*, 1986). More recently, microdialysis of human vastus lateralis muscle during knee extension exercise showed an increase in interstitial adenosine concentrations with exercise that increased further with increasing work rates (Hellsten *et al.*, 1998). More importantly, this study showed a very strong correlation (r=0.98) between the interstitial adenosine concentration and increased leg blood flow. Further studies have used various pharmacological agents to antagonise the effects of adenosine. Adenosine deaminase, which breaks down adenosine to inosine, reduced exercise hyperaemia in oxidative dog gracilis muscle (Kille & Klabunde, 1984) and in

oxidative cat soleus muscle (Schwartz & McKenzie, 1990), despite the fact that inosine is a vasodilator. Dipyridamole, which blocks the reuptake of adenosine thereby leading to increased extracellular concentrations of adenosine, increased exercise hyperaemia in both pigs (Laughlin *et al.*, 1989) and dogs (Kille & Klabunde, 1984). Although these studies imply that the adenosine is released directly from skeletal muscle fibres, it is equally possible that the adenosine is formed extracellularly from breakdown of ATP, ADP and AMP by ecto 5'nucleotidases and ecto-phosphatases either in the interstitium itself or within the vessel lumen before diffusing across the endothelium (for review see Marshall, 2007). See Figure 1.2 for summary of sources of adenosine.

Various experimental methods have been used to try to quantify the magnitude of the hyperaemic effect of adenosine and have generally concluded that it is responsible for 20-40% of the maintained phase of exercise hyperaemia and ~80% of post-exercise hyperaemia over 5 minutes (Marshall, 2007). For example, Ballard *et al.* (1987) perfused dog gracilis muscle at a constant high rate and took arterial and venous blood samples. They found that contraction resulted in an elevated venous adenosine concentration although arterial concentrations remained unchanged, showing that adenosine is released. They then infused adenosine and compared the vasodilatation and venous adenosine concentrations during contraction and adenosine infusion to calculate the contribution of adenosine to exercise hyperaemia. They concluded that 15% of the vasodilatation at 1 minute, 40% between 5 and 20 minutes and 80% of the vasodilatation 5 minutes after cessation of contractions was due to adenosine. These findings are supported by Poucher and colleagues (1990)

from studies in the cat, in which they observed around a 40% reduction in exercise hyperaemia following infusion of 8-phenyltheophylline (8-PT), a potent non-selective adenosine receptor antagonist, compared to a control infusion of vehicle. A second study by Poucher (1996) compared the non-selective adenosine receptor antagonist 8-PT with the A_{2A} receptor specific antagonist ZM 241395 and found that both resulted in a similar 30%, reduction in hyperaemia, showing that the A_{2A} receptor subtype is responsible for the adenosine component, at least in cats.

In humans, the only adenosine receptor antagonists available for use are theophylline or the more soluble analogue aminophylline (Rådegran & Hellsten, 2000; Marshall, 2007). Theophylline compounds have been demonstrated to blunt the vasodilatation in response to intra-arterial infusion of adenosine in the human forearm vasculature (Taddei *et al.*, 1991). It should however be noted that they are not complete inhibitors of the action of adenosine, with a recent study by Casey *et al.* (2009) demonstrating that aminophylline blunted the vasodilatation to low, medium and high doses of adenosine by approximately 60%, 50% and 30% respectively. With regards to investigating exercise hyperaemia, a study has tested the effect of theophylline infusion during single leg rhythmic knee extension exercise at 33W, equivalent to ~48% of peak power (Radegran & Calbet, 2001). They observed an approximately 20% reduction in exercise hyperaemia with adenosine receptor antagonism compared to control. They also infused adenosine at rest and found that it can mimic the rapidity and peak increase in blood flow that is seen during exercise and concluded that there is an obligatory role for adenosine in mediating

exercise hyperaemia in humans. This view is supported in the review published around the same time (Rådegran & Hellsten, 2000).

Additionally, it is worth noting that there is evidence of a bimodal distribution of responsiveness to exogenous adenosine in humans (Martin *et al.*, 2006a, b; Martin *et al.*, 2007). The initial study hypothesised that previous disparities in the literature regarding the effect of adenosine were due to differences in adenosine response between subjects (Martin *et al.*, 2006a). They tested the vasodilator response to intra-arterial adenosine infusion and to rhythmic handgrip exercise at intensities equal to ~7, 14 and 21% MVC. Responders were categorised as having a robust vasodilator response to both intra-arterial adenosine infusion and handgrip exercise, whereas non-responders had a bunted vasodilator response to intra-arterial adenosine. Additionally, NOS inhibition with L-NMMA blunted the response to adenosine infusion in responders but not non-responders, whilst exercise hyperaemia was unaffected by NOS inhibition in either group, thus confirming that NO is not obligatory to exercise (see above). A later study by the same group found that aminophylline blocked the response to adenosine infusion in responders but non-responders were unaffected (Martin *et al.*, 2006b). However, both responders and non-responders had an approximately 15% reduction in FVC during rhythmic handgrip at ~21% MVC, but not at 7 or 14% MVC. This suggests that at more intense levels of exercise, adenosine plays a modest role in mediating exercise hyperaemia. The fact that this occurs in both responders and non-responders gives weight to the hypothesis that adenosine released from muscle is responsible for mediating exercise hyperaemia. The effects of increasing

interstitial concentrations of adenosine by using dipyridamole to block the equilibrative nucleoside transporters, which transport adenosine across the cell membrane (Martin *et al.*, 2007). This failed to increase the exercise hyperaemia in either the responders or the non-responders, which combined with their other study (Martin *et al.*, 2006b) suggests that adenosine may not be obligatory. Interestingly, dipyridamole increased the response to adenosine infusion at rest in both responders and non-responders, with non-responders responding identically to the responders after administration of the dipyridamole. This suggests that the group classified as non-responders actually have a naturally increased activity of the adenosine transporter, and so are more efficient at clearing adenosine from the interstitium.

ATP is a substance for which a role in exercise hyperaemia has been suggested for years, but which has only recently been thoroughly investigated. Forrester and Lind (1969) first measured resting plasma ATP concentrations of 0.63μg/ml in blood taken from human forearm veins and reported a clear increase in plasma ATP following static handgrip exercise at 10% and 20% of MVC, although the actual amounts were difficult to assess experimentally. It has since been found that ATP is released from human erythrocytes when they are exposed in vitro to a hypoxic/hypercapnic gas mixture (Bergfeld & Forrester, 1992). Further, Dietrich *et al.* (2000) demonstrated in perfused, isolated rat cerebral arterioles that an hypoxic solution only causes vasodilatation and increase efflux of ATP when they were being perfused by red blood cells. Both the vasodilatation and the increased ATP efflux returned to baseline levels when the solution around the arterioles was returned to normoxia. Therefore, in

hypoxic conditions erythrocytes are capable of releasing ATP, which can cause vasodilatation, raising the possibility that decreased pO_2 is important in causing ATP release from red blood cells in exercise. In addition, there is evidence that ATP can be released from motor and sympathetic nerves (for review see Marshall, 2007) and from endothelial cells in response to hypoxia (Burnstock, 2006). When ATP is applied to hamster cheek pouch arterioles and found that extraluminal application causes vasoconstriction via P2X receptors, whilst intraluminal application causes vasodilatation due to stimulation of P2Y receptors (McCullough *et al.*, 1997). Thus it is likely that any direct effect of ATP on mediating exercise hyperaemia is due to ATP within the lumen, i.e. released from erythrocytes or endothelial cells.

In-vitro work using rat red blood cells found that ATP release correlated better with the percentage of relaxed haemoglobin (rHb – the haemoglobin state when either O_2 or CO is bound) than it did to the pO_2 of the blood (r^2 = 0.88 vs. 0.54) (Jagger *et al.*, 2001). This idea has been further explored in humans by Gonzalez-Alonso *et al*. (2002). They found that ATP is important in mediating exercise hyperaemia in healthy young men performing 4 minutes of 1 legged rhythmic knee extension at intensities ranging from 27% to 85% of the peak workload. They found that leg exercise raised [ATP] in both the femoral artery and vein, as well as raising the arterio-venous [ATP] difference. This was associated with an increase in thigh blood flow and vascular conductance. The increase in venous [ATP] tended to be higher in hypoxia (P=0.14), despite arterial O_2 content being similar in both the hypoxia and the normoxia+carbon monoxide conditions. As carbon monoxide causes a decreased blood O_2

content but the haemoglobin structure remains the same as if O_2 were bound, this indicates that the release of ATP from erythrocytes may be due to the conformational change which haemoglobin undergoes as it becomes deoxygenated. Additionally, infusion of ATP into the femoral artery reproduced the increase in blood flow seen during maximal exercise (Gonzalez-Alonso *et al.*, 2002). This vasodilatation has since been shown to be due to action via the $P2Y_2$ receptor (Rosenmeier *et al.*, 2008).

Recently however, there has been some evidence that the increase in ATP from erythrocytes seen in hypoxia may be an artefact of mechanical deformation. A recent study from the Gonzalez-Alonso group found that femoral venous plasma [ATP] was not altered by administration of either graded hypoxia or low intensity knee extension (Dufour *et al.*, 2010). Similarly, leg blood flow during submaximal single leg knee extension exercise was not affected by anaemia brought on acutely by withdrawing 20% of the subjects blood volume (resulting in an 11% decrease red blood cell count) suggesting that it is not simply deoxygenation which causes the hyperaemia (González-Alonso *et al.*, 2006).

Finally, Mortensen *et al.* (2011) recently used microdialysis to measure [ATP] in the femoral artery and vein, thus removing the confounding factor of ex vivo ATP release from red blood cells and reducing the time from sampling to analysis. They demonstrated that single leg knee extension breathing normoxic gas caused a progressive rise in [ATP] in the femoral artery and vein. Breathing hypoxic gas (10-12.5% O_2) caused an increase in resting venous [ATP], but did not change venous [ATP] in exercise. This group interpreted this as showing that the decrease in endothelial pO_2 in the muscle during exercise was not

significantly different when breathing normoxic or hypoxic gas. However, given the results discussed above (Dufour *et al.*, 2010), an alternative explanation may be that the mechanical deformation induced by the exercise was the same in both groups and that this was the cause for the increased [ATP] seen in exercise. They also tested the contribution of shear stress induced by the increase in blood flow by passively moving the leg at 60r.p.m. and by inflating a sphygmomanometer cuff around the thigh at 60r.p.m. (Mortensen *et al.*, 2011). Both interventions caused an increase in leg blood flow and vascular conductance, although only the thigh compressions caused [ATP] to increase, particularly in venous plasma. This supports the theory that mechanical deformation causes ATP to be released from red blood cells or endothelium.

In summary, it appears that in more intense exercise, adenosine alone can be responsible for a modest amount of the vasodilatation that occurs (15-20%) although it is not obligatory. Unfortunately, due to a lack of specific antagonists for vasodilatory P2Y receptors, it is difficult to fully test the involvement of ATP in mediating exercise hyperaemia (Clifford & Hellsten, 2004). However, the evidence provided above suggests that ATP released intraluminally by red blood cells or endothelial cells either in response to hypoxia or mechanical deformation may play a role in mediating exercise hyperaemia, specifically through actions at the $P2Y_2$ receptor. Recently however, there has been some evidence that adenosine and ATP may act interdependently with other substances such as NO and prostaglandins to mediate exercise hyperaemia. This evidence is considered below.

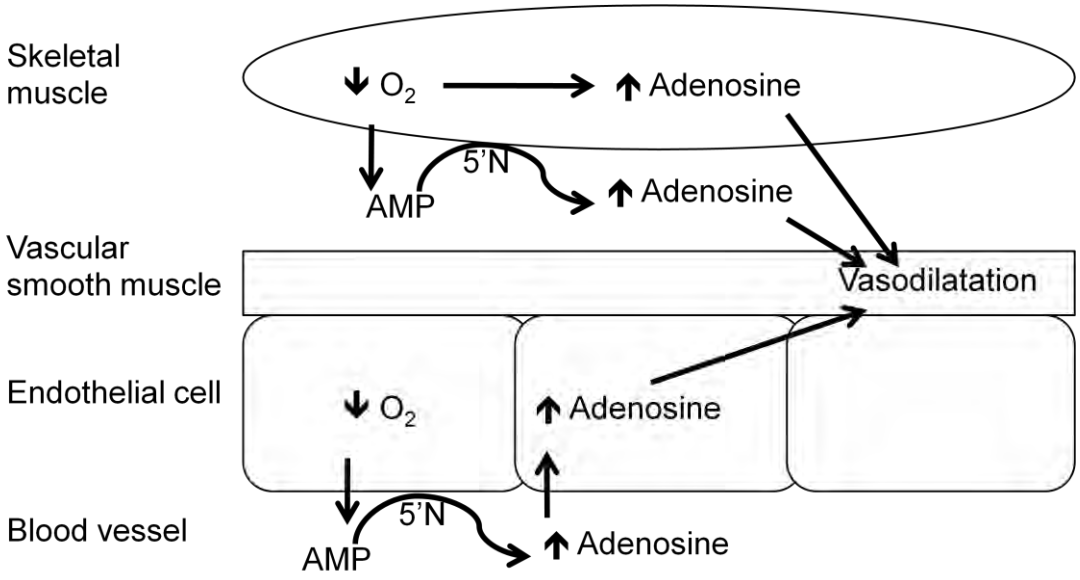

Figure 1.2. Schematic representation of the proposed mechanisms by which adenosine may be released in an O_2-dependent manner. In response to hypoxia, adenosine release may be increased in skeletal muscle or endothelial cells, or increased release of AMP may lead to increased formation of adenosine via ecto 5'nucleotidase (5'N).

Table 1.3 Summary of studies assessing role of adenosine in mediating hyperaemia in humans

Study	Muscle group	Exercise	Blood flow measurements	Drug intervention	Results
Radegran & Calbet (2001)	Quadriceps	Dynamic knee extension - ~48% peak.	Doppler ultrasound	Intravenous theophylline (adenosine receptor antagonist)	Theophylline reduces exercise hyperaemia by ~20%.
Martin *et. al.* (2006b)	Forearm	Dynamic handgrip – 21%, 14% & 7% MVC	Doppler ultrasound	Intra-arterial aminophylline (adenosine receptor antagonist)	15% reduction in exercise hyperaemia at 21% MVC, but no change at 7 or 14%.
Martin *et. al.* (2007)	Forearm	Dynamic handgrip – 21%, 14% & 7% MVC	Doppler ultrasound	Dipyridamole (increases interstitial adnosine by blocking reuptake)	No effect on hyperaemia.
Mortensen *et. al.* (2009b)	Quadriceps	Dynamic knee extension - 30% peak.	Doppler ultrasound	Intravenous theophylline (in presence of NOS and COX blockade).	Theophylline had no additive effect to the reduction in hyperaemia seen by dual NOS and COX blockade.
Hellsten *et al.* (1998)	Quadriceps	Dynamic knee extension – 10, 20, 30, 40 & 50W	Doppler ultrasound	N/A	Interstitial adenosine increase strongly correlated with increased exercise hyperaemia.

1.2.3.5 Potassium ions (K^+) and Endothelium derived hyperpolarising factor (EDHF)

K^+ has been implicated in mediating exercise hyperaemia since the 1970s with demonstration of a transient increase in venous plasma K^+ following a brief electrically evoked contraction in dog hindlimb muscle (Mohrman & Sparks, 1974). Although measuring venous plasma concentrations of K^+ led to a time delay relative to any increases in release from the muscle capillary, the authors concluded that the timecourse of this increased release was sufficient to precede the vasodilatation which occurred. Thus they concluded that increased K^+ could be causally related to the exercise hyperaemia caused by a brief tetanic contraction. The increase in K^+ seen in this study was also similar to concentrations that have been shown to cause vasodilatation (Mohrman & Sparks, 1974; Kirby & Carlson, 2008). Similarly, K^+ has been shown to be released in proportion to the magnitude of the exercise hyperaemia and work done (Hilton *et al.*, 1978).

A more robust case for a role of K^+ in mediating the rapid vasodilatation induced by exercise has recently been made using an *in situ* hamster cremaster muscle preparation (Armstrong *et al.*, 2007). In this study hamster cremaster muscle was stimulated at varying intensities (between 4 and 80Hz) for 250ms with the actions of K^+ being inhibited by one of 3 drugs – 3,4-diaminopyridine (DAP – a K_V channel blocker), ouabain (Na^+/K^+ ATPase inhibitor) or barium chloride ($BaCl_2$ - K_{IR} channel blocker). The diameter of arterioles running perpendicular to muscle fibres was visualised and any changes measured using intravital microscopy. Their major findings were that all 3 drugs blunted the rapid

vasodilatation (at 4 seconds) in response to muscle contraction by 60-65% at most stimulation frequencies (at all frequencies for DAP and ouabain and at all except 4 and 40Hz for $BaCl_2$). These data suggest a significant role for K^+ in mediating the rapid onset vasodilatation following a single short tetanic contraction.

Although there is still a lack of evidence for a role of K^+ in mediating the vasodilatation during and after more sustained exercise, it has been implicated in mediating the effects of endothelium derived hyperpolarising factor (EDHF) (Nagao & Vanhoutte, 1992).

The existence of an unidentified substance or substances which caused hyperpolarisation of the vascular smooth muscle in porcine coronary artery in the presence of NOS and COX blockade was demonstrated by Nagao and Vanhoutte (1992). Since then, the consensus is that this EDHF actually consists of a number of substances which all cause hyperpolarisation of the vascular smooth muscle (for review see Feletou & Vanhoutte, 2006). Campbell *et al.* (1996) demonstrated that in bovine coronary arteries, inhibition of the cytochrome p450 enzyme with miconazole significantly reduced endothelium dependent relaxation induced by metacholine. This suggests that epoxyeicosatrienoic acids (EETs) produced from the metabolism of arachidonic acid by cytochrome p450 are candidates for being EDHF. It was then shown in human coronary arteries that EDHF hyperpolarises the vascular smooth muscle via Ca^{2+}activated K^+ channels (Miura & Gutterman, 1998; Miura *et al.*, 1999). The contribution of EETs has since been investigated *in vivo* in the human forearm using the cytochrome p450 inhibitor miconazole (Halcox *et al.*, 2001).

In the presence of a prior combined NOS and COX blockade with L-NMMA and aspirin, these researchers compared the vasodilatation seen in response to bradykinin infusions with and without miconazole infusion. Their findings were that p450 blockade reduced the vasodilatation caused by the endothelium dependent vasodilator bradykinin, but had no effect on the endothelium independent vasodilator sodium nitroprusside. This shows that in the human forearm in the absence of NO and prostaglandins, an endothelium dependent vasodilator is present. In the same study, the researchers were able to reproduce the effects of miconazole by using an intra-arterial infusion of potassium chloride to clamp the vascular smooth muscle membrane potential and thus eliminate any effect caused by hyperpolarisation. This indicates that in the human forearm, arachidonic acid metabolites such as EETs are able to play the role of EDHF.

Of importance regarding exercise hyperaemia, Hillig *et al.* (2003) investigated the effects of inhibiting the cytochrome p450 2C9 enzyme with sulfaphenazole in healthy young men performing single leg knee extension for 10 minutes (the highest level of force all subjects could maintain for this time). They compared the effects of infusing sulfaphenazole alone with coinfusion of sulfaphenazole and L-NMMA, using a dose of L-NMMA they had previously shown had no effect on exercise hyperaemia in a similar protocol (Radegran & Saltin, 1999). Their findings were that cytochrome p450 2C9 inhibition alone had no effect on the increase in leg blood flow or vascular conductance during exercise compared to control. When combined with NOS blockade however, sulfaphenazole reduced the exercise hyperaemia by ~16%. Combined with the

results of their previous findings that NOS blockade alone had no effect on exercise hyperaemia, this suggests that NO and cytochrome p450 2C9 derived products act in an interdependent manner, such that inhibition of one can be compensated for by upregulation of the other.

1.2.3.6 Interdependency and redundancy

The discussion above has concentrated largely on the individual contributions of NO, prostaglandins, adenosine and ATP in mediating exercise hyperaemia. Individually the contributions of any one of these mediators is relatively modest. Recently however, there have been some studies which have shown evidence of interdependency between the vasodilator systems.

In one of the first studies to demonstrate interdependency, intra-arterial indomethacin was given to block COX in moderately trained young subjects performing 5 minutes of 1-legged rhythmic knee extension exercise at 20% of their maximum workload, measuring blood flow with Doppler ultrasound (Mortensen *et al.*, 2007). They found that single inhibition of COX had no effect on the increase in blood flow evoked by exercise, a finding that contrasts with the studies described above in section 1.2.3.3 in the forearm. However, when COX inhibition was combined with NOS inhibition, exercise hyperaemia was reduced by 33%, despite previous findings by the same group that the same dose of L-NMMA given as a single agent in a similar protocol did not lower blood flow (Radegran & Saltin, 1999). Similar findings were made in another recent study where positron emission tomography (PET) with radiolabelled water was used to measure the blood flow to metabolically active tissue (Heinonen *et al.*, 2011). Healthy young men performed rhythmic single leg

extension at 40rpm at a force of 4.5kg after combined NOS and COX blockade (with L-NMMA and indomethacin). They found that combined blockade reduced exercise hyperaemia to the working quadriceps femoris muscle by 15-20%. NOS inhibition alone had no effect and blood flow to the resting posterior muscle group was unaffected by either single or double blockade. Thus both studies indicate that NO and prostaglandins act interdependently to mediate exercise hyperaemia.

Further, in a relatively large study on 24 physically active young men performing knee extension at 30% of peak workload, systemic blockade of adenosine receptors using an intravenous infusion of theophylline and a local blockade of COX and NOS with indomethacin and L-NMMA infused into the femoral artery showed that dual blockade of COX and NOS and triple blockade of COX, NOS and adenosine receptors both reduced leg blood flow and vascular conductance by similar amounts (~30%) (Mortensen *et al.*, 2009b). Additionally, they found that the vasodilator response to intra-arterial infusion of adenosine was reduced by both COX and NOS inhibition separately, these reductions being additive in dual blockade. These results raise the possibility that adenosine acts to mediate exercise hyperaemia by increasing levels of vasodilator prostaglandins and nitric oxide. Interestingly, in a separate protocol designed to assess the changes in plasma adenosine concentration, neither venous nor arterial adenosine levels were altered, suggesting that interstitial adenosine mediates exercise hyperaemia rather than plasma adenosine.

A recent study by the same group in Copenhagen used microdialysis to measure changes in concentrations of adenosine, prostacyclin and NO in the

interstitium and plasma in response to both intra-arterial and interstitial infusions of adenosine (Nyberg *et al.*, 2010). They found that intra-arterial adenosine infusion not only increased leg blood flow but caused an increase in both interstitial and venous plasma levels of NO and 6-keto $PGF_{1\alpha}$. Further, when adenosine was infused into the interstitium, there was no effect on leg blood flow, but both interstitial NO and 6-keto $PGF_{1\alpha}$ increased. In other studies cultured endothelial and skeletal muscle cells produced NO in response to adenosine, but only endothelial cells produced prostacyclin. Combined, the results of these studies indicate that adenosine mediates local vasodilatation by increasing production of prostacyclin and NO from the endothelium as well as NO from muscle fibres.

Regarding interactions between ATP and other mediators, researchers have had mixed findings depending on which limb they have investigated. NOS inhibition with L-NMMA did not affect the forearm vasodilatation in response to ATP, but did reduce the vasodilatation caused by acetylcholine (Rongen *et al.*, 1994). This indicates that NO does not significantly contribute to ATP-induced vasodilatation in the human forearm. Further investigation found that individual blockade of COX and EDHF had no effect on ATP-induced vasodilatation, whereas combined blockade of COX, EDHF and NO reduced ATP-induced vasodilatation(van Ginneken *et al.*, 2004). However, combined blockade also reduced vasodilatation evoked by the endothelium independent vasodilator SNP, thus we can conclude that the reduction in vasodilatation was a non-specific effect due to the combined vasoconstrictor properties of the infused agents.

More recently however, another group has found evidence of interdependency in the leg of moderately trained young men (Mortensen *et al.*, 2009a). The increase in leg vascular conductance in response to femoral artery ATP infusion was reduced by 12% and 26% by L-NMMA and indomethacin respectively. Dual inhibition reduced LVC by ~42%, indicating that both NO and prostaglandins mediate part of the vasodilator response to ATP, although they appear to work via separate pathways. Adenosine receptor antagonism had no effect on ATP-induced vasodilatation, indicating that ATP breakdown to adenosine is not significantly responsible for mediating the vasodilatation. The authors speculate that any differences between this positive study in the leg and negative findings in forearm may either be due to vasodilatator differences between the limbs, or to the increased muscle mass in the leg resulting in a more visible response to pharmacological intervention.

In summary, the current evidence suggests that both adenosine and ATP may mediate exercise hyperaemia through production of both vasodilator prostaglandins and NO. These vasodilator prostaglandins and NO likely work in an interdependent manner. The caveat is that the role of ATP cannot be tested due to the absence of an antagonist.

1.2.4 Effects of age on hyperaemia

In ageing there is a decrease in exercise capacity in humans that is partly due to a decreased $V_{O2\text{-}max}$ and cardiac output (Ogawa *et al.*, 1992). It is also possible that decreased vasodilatation in aged humans affects skeletal muscle blood flow and thus limits exercise performance (Schrage *et al.*, 2007). The

discussion below focuses on recent evidence that vasodilatation in exercise is impaired with ageing.

Irion *et al*. (1987) first showed that exercise hyperaemia occurred to a lesser extent in old animals than in younger animals. Hind limb blood flow was assessed using injection of microspheres in 12 and 24 month old rats, corresponding to a human age of 40-50 and 70 years old respectively. Exercise hyperaemia following dynamic muscle contraction was impaired by ~30% in the older rats and this was associated with an increase in muscle fatigue. Resting blood flow did not differ between age groups, thus the source of impairment lies in the mechanisms of exercise hyperaemia.

Regarding the effects of ageing on exercise hyperaemia in humans, the results have been more equivocal. Jasperse *et al.* (1994) measured FBF in sedentary or recreationally active subjects aged 19-29 and 60-74 years old. Peak forearm vasodilator capacity, assessed by the response to 10 minutes of ischemia with isometric handgrip for the last 2 minutes, was not affected by ageing. Similarly, the peak dynamic workload and the blood flow immediately following dynamic exercise were not significantly different in either age group at any workload from 7%MVC to ~80% of MVC, thus indicating that ageing has no effect on exercise hyperaemia. This contrasts with a later study measuring leg blood flow during submaximal cycling exercise in endurance trained young and older men (Proctor *et al.*, 1998). Older men had ~25% lower blood flow than younger subjects at any of the three workloads that were assessed (~40%, 65% and 90% of V_{O2peak}), although resting values of leg blood flow were not measured, therefore it is not clear whether the deficit in exercise blood flow represented the

continuation of a deficit which was present at rest. Similarly, sedentary older subjects had a reduced leg blood flow during cycling exercise compared to sedentary young subjects, both at an absolute work rate of 99W and at the relative maximum work rate for each group of subjects (Poole *et al.*, 2003). This difference was not present at lower work rates of 15 and 30W. The authors postulated that the limited muscle perfusion in older subjects was directly responsible for the reduced maximum work rate compared to the younger subjects.

More recently Donato *et al.* (2006) investigated the effects of ageing on limb blood flow during dynamic exercise in the forearm and the leg. Exercise hyperaemia in the forearm was not altered with age when levels of hyperaemia were expressed as an absolute value. It was also true when exercise hyperaemia was expressed relative to muscle mass or to maximum work rate, in order to account for any change in these values in older subjects. In the leg however, exercise hyperaemia was reduced in old subjects compared to younger subjects at comparable workloads (although absolute workload was lower as older subjects had a lower maximum force). As resting blood flow was unaffected by age this supports the concept that exercise hyperaemia is attenuated in the leg with ageing.

Other groups have demonstrated ageing-related changes in the forearm exercise hyperaemia. Carlson *et al.* (2008) used single, one second contractions to effectively isolating any vasodilator mechanism. Exercise hyperaemia was maintained with age at 5% MVC, but at intensities greater than 10% MVC both the immediate and peak vasodilatation were reduced in the

older subjects. Importantly, there were no significant differences between young and older subjects in terms of forearm muscle mass or the maximum force they were able to attain, thus the impaired exercise hyperaemia is purely explained by an attenuated vasodilator response.

The mechanism for impairment of exercise hyperaemia in ageing may be attenuated endothelial function. In a study of 53 healthy adults (age 19-79), the magnitude of vasodilatation in response to ACh infusion was negatively correlated with the subjects age (r=-0.86), although there was no effect of age on the dilatation to sodium nitroprusside (Taddei *et al.*, 1995). This indicates that the endothelial function is impaired with ageing, despite no significant impairment of the vascular smooth muscle. More recently, it has also been shown that infusion of prostacyclin resulted in a reduced vasodilatation from baseline in older subjects compared to younger subjects, although again the response to SNP was not altered (Nicholson *et al.*, 2009). Interestingly, this group found that NOS inhibition had no effect on the older group but decreased the response to PGI_2 in the young, such that there was no longer any difference between the young and older groups, suggesting a decreased role for NO in ageing.

Similar findings for a decreased role of NO in ageing have been made looking at the blood flow response to handgrip exercise (Schrage *et al.*, 2007). 15 recreationally active subjects aged 55-81 performed dynamic handgrip exercise at 10% and 20% of MVC for 20 minutes. L-NAME and ketorolac were infused during steady state exercise in differing orders, such that the effect of single NOS and COX blockade could be assessed as well as the effect of dual

blockade. When compared to another study by the same group in which young subjects undertook the same protocol (Schrage *et al.*, 2004), the effects of NOS blockade on reducing steady state exercise FBF were reduced by about 40% in older subjects and the 12% decrease in FBF seen in the young subjects during COX inhibition was completely absent in the older subjects. This shows a reduced role for both NO and prostaglandins in mediating exercise hyperaemia in older humans. In another study, infusing the antioxidant ascorbic acid during dynamic handgrip exercise at 10% MVC increased FBF by ~25% (Crecelius *et al.*, 2010). The researchers then infused L-NMMA and ketorolac in a randomised order during steady state exercise with ascorbic acid infusion. Regardless of the order of blockade, they found that L-NMMA reduced the steady state blood flow with ascorbic acid by approximately 20%, i.e. nearly back to steady state exercise levels prior to ascorbic acid infusion. COX inhibition with ketorolac had no effect, either when infused first or second. This indicates that the reduced steady state blood flow seen in low intensity dynamic handgrip exercise in older subjects may be due to decreased NO bioavailability due to formation of reactive oxygen species.

Of relevance to the findings of a role for vasodilator prostaglandins in exercise hyperaemia in young subjects, there is some evidence for a shift from vasodilator to vasoconstrictor prostanoids. Tang and Vanhoutte (2008) measured the levels of various prostanoid synthases in 36 week old and 72 week old rats, corresponding to middle age and old age in humans. They found that expression of prostacyclin synthase was unchanged with ageing, but expression of thromboxane synthase was increased, indicating that production

of the vasoconstrictor thromboxane may be raised relative to the vasodilator prostacyclin. Importantly there is also evidence for this in aged humans with essential hypertension (Taddei *et al.*, 1993). In this group of patients, infusion of the COX inhibitor indomethacin resulted in an increase in acetylcholine mediated vasodilatation, indicating that a COX-derived vasoconstrictor such as thromboxane is produced.

In summary, there is substantial evidence of a reduction in exercise hyperaemia with ageing, which appears to be due to a complete loss of prostaglandin mediated vasodilatation and a reduction in NO mediated vasodilatation. This may be largely due to impaired endothelial function and an increase in reactive O_2 species which decreases NO bioavailability.

1.3 Mechanisms of Muscle Fatigue

Fatigue has both a central neural and a peripheral muscular component [see, e.g. (McKenna & Hargreaves, 2008)]. The central component can be defined as a progressive exercise-induced failure of voluntary activation of the muscle and refers to processes taking place proximal to the neuromuscular junction (Taylor & Gandevia, 2008), i.e. at the levels of the motor neurone, spinal cord or brain. Evidence for the presence of central fatigue can be seen in experiments in which extra force is produced as a result of motor neurone or motor cortex stimulation whilst subjects perform a maximum voluntary contraction (for reviews see e.g. Gandevia, 2001; Taylor & Gandevia, 2008). Whilst the present studies will not consider central fatigue further, it is important to note that studies involving direct electrical stimulation of muscle or motor nerve supply

avoid central fatigue mechanisms contributing to observed responses whereas experiments involving voluntary contraction may be affected by them.

Peripheral fatigue refers to any process which takes place at or distal to the neuromuscular junction (Amann & Calbet, 2008). A number of substances have been proposed to be involved in the development of peripheral fatigue. Traditionally, lactate ions produced as a result of anaerobic metabolism have been implicated in peripheral fatigue. However, experimental data show that muscle lactate levels do not correlate well with fatigue (Karlsson *et al.*, 1975; Bangsbo *et al.*, 1992a; 1992b) and thus lactate is not considered further. Briefly, the main substances that are currently implicated in the development of peripheral muscle fatigue are hydrogen (H^+) ions, inorganic phosphate (Pi) and potassium ions (K^+). Furthermore, previous work from this laboratory suggests that at least part of the mechanism of peripheral fatigue is O_2-dependent (Fordy & Marshall, 2004). This study on young, recreationally active male subjects investigated the effect of breathing 40% O_2 on the time to fatigue during 2 periods of maximal voluntary forearm contraction, separated by 7 minutes recovery. It was found that breathing 40% O_2 rather than air during the 7 minute recovery period from a period of maximum voluntary forearm contraction to exhaustion greatly improved the time to exhaustion during a second period of forearm contraction. By contrast, data in the same study showed that breathing supplemental O_2 during the period of contraction only but not during the recovery period had no effect on muscle fatigue in the second period of contraction. When combined, these results show that the O_2-dependent aspect of fatigue occurs during recovery and not during contraction. The following

discussion will analyse the evidence for and against the contributions of the substances mentioned previously and how they may be O_2-dependent.

1.3.1 Potassium ions

In humans, the concentration of K^+ within the muscle fibres ($[K^+]_i$) is approximately 165mM and has been shown to decrease to ~130mM during intense exercise (Sjogaard *et al.*, 1985). Moreover, using microdialysis techniques, concentrations increasing from ~4mM at rest to greater than 10mM have been found in the muscle interstitium (Juel *et al.*, 2000) and values of 8mmol/L have been found in arterial and venous blood of men performing exhaustive treadmill exercise (Medbo & Sejersted, 1990) even though the resting level in plasma is 4-5mmol/L. K^+ is released from muscle fibres during exercise through K_{IR} channels as a result of action potentials and via K^+_{ATP} channels, which respond to the decreased ATP by remaining open more frequently (Standen, 1992). K^+ is taken back up by the cell via Na^+/K^+-ATPase (Clausen, 2003).

A number of studies have shown a link between increased K^+ efflux from skeletal muscle cells and fatigue. Thus, Juel *et al* (2000) used graded single leg extensor exercise to measure the interstitial concentration of potassium ($[K^+]_{int}$). Their results showed that the higher the intensity of the exercise, the greater the $[K^+]_{int}$. Further, Nielsen *et al* (2004) also used single leg extensor exercise to look at the effect of training on K^+ concentrations and performance. The subjects trained one leg whilst using the other leg as an untrained control. It was found that following 6 weeks training, the time to fatigue was significantly longer in the trained leg than the untrained one and this was associated with a

greater, more rapid increase in $[K^+]_{int}$ in the control leg than in the trained leg. In other studies performed in vitro, Clausen and Nielsen (2007) incubated rat skeletal muscle in a 4mM K^+ solution. They demonstrated that pre-incubation with 10mM K^+ caused a decrease in initial force and an increased rate of force decline in muscle relative to that seen in 4mM K^+ solution. These changes became more pronounced with increasing periods of pre-incubation. Of particular interest, they showed an increased rate of force decline when they incubated muscle in 8mM K^+ solution (Clausen & Nielsen, 2007), the level that has been found in arterial blood following exhaustive exercise.

It has also been found that venous K^+ concentrations were the same at the time of exhaustion when subjects exercised to exhaustion on two separate occasions on the same day, even though the time to exhaustion was shorter in the second exercise period (Bangsbo *et al.*, 1992b). A final piece of evidence suggesting that potassium can cause fatigue comes from experiments in which subjects perform either leg exercise alone or leg exercise preceded by arm exercise. It was found that the $[K^+]_{Int}$ measured in the leg during leg exercise were greater when arm exercise was performed first consistent with a rise in plasma K^+ caused by arm exercise and this was associated with a decreased time to exhaustion (Nordsborg *et al.*, 2003). It is thought that these changes in $[K^+]$ mediate fatigue by decreasing the action potential amplitude, thus decreasing the excitability of the skeletal muscle cell membrane (Sjogaard, 1996). This results in a decrease in force development, which is measured as fatigue.

Interestingly, whilst it seems that increased $[K^+]_{Int}$ from ~8mM and above is associated with a decreased time to fatigue in exercise, it appears that from the resting level of ~4mM up to a concentration of ~8mM it may actually increase the force generated (for review see McKenna *et al.*, 2008). This raises the possibility that initially during exercise the rise in K^+ improves muscle performance. When peak tetanic force is plotted against $[K^+]_{Int}$ little decrease in force is seen as $[K^+]_{int}$ increases from ~4mM to around 8-9mM, followed by a rapid decrease in force with little further increase in $[K^+]_{int}$ (McKenna *et al.*, 2008). They describe the concentration where force rapidly decreases as the critical $[K^+]_{int}$. The $[K^+]_{Int}$ at which force rapidly decreases can be shifted to the left or right depending on the actions of Na^+/K^+-ATPase (sodium/potassium pump or NKA), which clears K^+ from the extracellular space by pumping 3 Na^+ ions out of the cell and 2 K^+ ions into the cell. Clausen and Nielsen (2007) investigated the effects of stimulating the Na^+/K^+-ATPase on fatigue in rat muscle. They found that the effect of 10mM K^+ was reduced by stimulation of the Na^+/K^+-ATPase, whether by Na^+ loading, salbutamol, adrenaline, rat CGRP or insulin. Additionally, K^+ is released from muscle cells via the K^+_{ATP} channel, which responds to low ATP by remaining open more frequently (Standen, 1992). This provides an interesting O_2-dependent hypothesis as reduced O_2 may lead to reduced ATP and hence inactivation of the Na^+/K^+-ATPase and opening of the K^+_{ATP} channel, thus increasing net efflux of K^+ (see Figure 1.3).

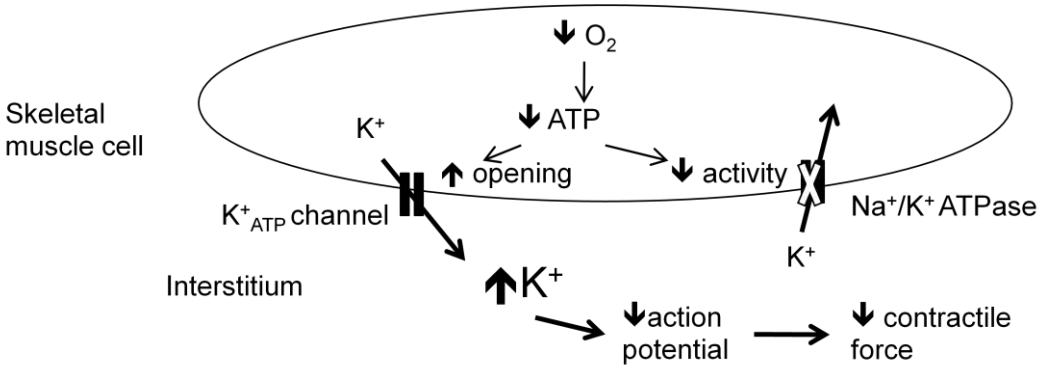

Figure 1.3. Schematic representation of the proposed mechanisms by which K^+ may mediate fatigue in an O_2-dependent manner. In response to hypoxia, decrease ATP leads to opening of K_{ATP} channels and closing of N^+/K^+ ATPase. The resulting net efflux of K^+ causes decreased contractile force by decreasing skeletal muscle action potentials.

1.3.2 Inorganic Phosphate (Pi)

Inorganic phosphate (Pi) increases in the interstitium during intense exercise when the rate of ATP (adenosine triphosphate) consumption is greater than the rate of supply from the mitochondria (Robergs *et al.*, 2004). This is due to Pi release due to ATP hydrolysis outstripping the uptake by the mitochondria.

$ATP + H_2O \rightarrow ADP + Pi + H^+ + energy$

As reviewed by Fitts (1994), the concentration of interstitial Pi ($[Pi]_i$) in skeletal muscle at rest is quite variable, with values of between 3 and 41 $mmol.kg^{-1}$ dry weight being reported and content being consistently higher in slow-twitch than fast-twitch muscles. As well as stores of Pi, phosphate is found in the cell in two important high-energy forms, adenosine triphosphate and phosphocreatine (PCr). ATP levels can be maintained during contractions by the creatine kinase reaction ($PCr + ADP + H^+ \rightarrow Cr + ATP$) and by the adenylate kinase reaction ($2\ ADP \rightarrow ATP + AMP$). As can be seen from the creatine kinase reaction, PCr

breakdown is not the cause of the increase in Pi, as is sometimes mistakenly thought (Robergs *et al.*, 2004). However, as during intense exercise ATP is constantly being synthesised from PCr and immediately broken down, the net reaction within the cell can be said to be PCr → Cr + Pi (Allen *et al.*, 2008).

Evidence to support the role of Pi in fatigue has been accumulating since the 1980s. Cooke and Pate (1985) found that adding increased concentrations of extracellular Pi led to a reduction in isometric tension of single rabbit psoas muscle fibres. This correlates well with work by Westerblad and Allen (1996), who injected phosphate into mouse muscle fibres with and found that it caused a reduction in tetanic force. Despite these findings, the impact of Pi alone has been hard to investigate as injecting it into muscle fibres causes other metabolic changes. A solution to this problem has come from Westerblad and colleagues (Dahlstedt *et al.*, 2000; 2001), who have used creatine kinase knockout (CK-/-) mice as a model to study the effects of increased Pi. In contrast with wild-type mice, the CK-/- mice have a higher myoplasmic concentration of Pi in fast twitch muscles at rest but show no increase in Pi following fatiguing contractions (Dahlstedt *et al.*, 2000). These studies showed that the whole extensor digitorum longus muscle (EDL, a predominantly fast-twitch muscle) produced ~25% lower force in the first contraction in CK-/- mice than wild-type mice suggesting that Pi has a force depressing effect. However, muscle fibres from CK-/- mice showed no decline in force production even over the course of 100 contractions, compared to fibres from wild-type mice which showed a ~15% reduction in force after 10 contractions and ~30% after 100 contractions

(Dahlstedt *et al.*, 2000). This supports the idea that increases in [Pi] mediate fatigue.

The potential mechanisms by which increased Pi may mediate fatigue have been well reviewed (Westerblad *et al.*, 2002; Allen *et al.*, 2008). Fatigue is said to have three phases (for a review see eg. Allen, 2009). Briefly, phase one is a rapid drop to 80-90% of the initial force. In phase two the force is constant and in phase three the force drops again. Pi has been mainly implicated in phase one and three. In phase one, Pi is thought to inhibit cross-bridge formation, a theory that is supported by the studies described above on CK-/- mice which showed lower initial contractile force than wild type mice. In phase three, force is thought to decline due to a reduced release of calcium (Ca^{2+}) from the sarcoplasmic reticulum and reduced sensitivity of contractile proteins to Ca^{2+}, both of which have been linked to Pi. Firstly, an initial increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) occurs which has been attributed to Pi blocking Ca^{2+} reuptake (Duke & Steele, 2000). This may result in less Ca^{2+} being available for release in later stages of contraction (Westerblad *et al.*, 2002). Secondly, it has been hypothesised that Pi enters the sarcoplasmic reticulum and causes precipitation of Ca^{2+} (Fryer *et al.*, 1995; Kabbara & Allen, 1999; Dahlstedt *et al.*, 2000). Finally, studies on skinned muscle fibres have shown that increased Pi reduces myofibrillar Ca^{2+} sensitivity (Millar & Homsher, 1990; Martyn & Gordon, 1992). This could have a greater effect when combined with reduced Ca^{2+} release due to the above mechanisms.

A potential O_2-dependent mechanism of Pi in fatigue can be hypothesised from work done by Haseler *et al* (1999; 2004) investigating the effects of

supplemental O_2 on phosphocreatine resynthesis, a mechanism for removing Pi from the muscle fibre. 100% or 21% O_2 was breathed before, during and after plantarflexion exercise. Using magnetic resonance spectroscopy, they found that the rate of phosphocreatine resynthesis following submaximal rhythmic exercise was only increased by breathing 100% O_2 in active subjects and not in sedentary subjects. This implies that O_2-delivery to muscle is more than adequate to supply muscle activity in sedentary subjects, with the authors hypothesising that an increase in mitochondrial number and efficiency allowed trained subjects to make better use of supplementary O_2. This could therefore provide a mechanism for supplementary O_2 to reduce the role of Pi in fatigue, at least in trained subjects (see Figure 1.4).

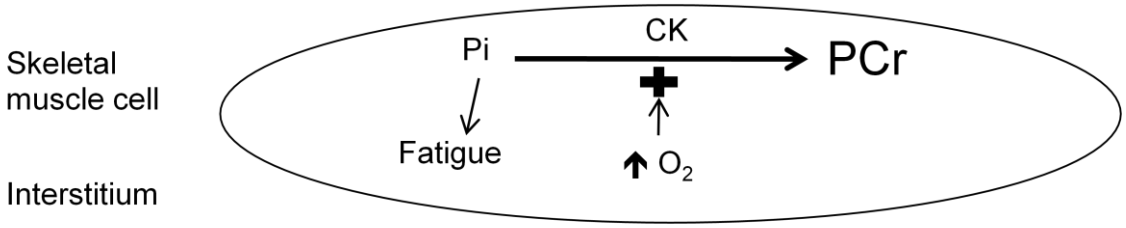

Figure 1.4. Schematic representation of the proposed mechanisms by which hyperoxia may reduce inorganic phosphate (Pi) mediated fatigue. Increased O_2 delivery to skeletal muscle increases the rate of phosphocreatine (PCr) resynthesis resulting in decreased Pi.

1.3.3 Hydrogen ions

The increase in hydrogen ions (H^+) during exercise occurs when the rate of ATP breakdown exceeds the rate of mitochondrial respiration, not as a result of lactic acid production as is often assumed (Robergs *et al.*, 2004). H^+ has traditionally been thought to be involved in fatigue based on studies that have generally been performed on single fibres at room temperature or below and shown a

decrease in shortening velocity and isometric tension with decreasing pH (Metzger & Moss, 1987; Cooke *et al.*, 1988). In more recent times this view has been questioned by a number of groups using different lines of investigation. Sahlin and Ren (1989) investigated the relationship between force and metabolic changes in the quadriceps femoris muscle following knee extension exercise in human subjects. The results showed that although force and pH both dropped, force recovered more rapidly than pH did, suggesting that hydrogen ions are not directly responsible for causing fatigue. The same findings were made in human first dorsal interosseous muscle (in the hand), where pH fell from about 7 to 6.5 and remained at 6.5 for at least 2 minutes, by which time force had recovered to ~85% of that of rested muscle (Cady *et al.*, 1989). In a similar model to Sahlin and Ren (1989), Bangsbo *et al.* (1996) used leg exercise performed on two separate occasions. On one day subjects performed arm exercise prior to leg exercise in order to increase blood and therefore leg muscle pH, and on the other they performed leg exercise without prior arm exercise. The findings of this study were that leg muscle fatigue occurred at a lower leg muscle pH following arm exercise than without, again suggesting that H^+ is not directly implicated in fatigue.

The reason for the seeming contradiction between results obtained in the original skinned muscle experiments and those on intact muscle would now seem to be due to temperature. Pate and colleagues (Pate *et al.*, 1995) studied rabbit psoas muscle fibres with the sarcolemma chemically removed ("skinned fibres") to allow the solution bathing the contractile apparatus to be precisely controlled. At 10°C they found that reducing the pH from 7 to 6.2 caused a 30%

reduction in shortening velocity and a 50% reduction in isometric tension, findings in line with the original studies. However, when the temperature was increased to 30°C, the reduction in pH only caused an 18% decrease in isometric tension and actually caused a 6% increase in shortening velocity. These findings suggest that at more physiological temperatures H^+ does not cause fatigue and similar results have been found by other groups (eg. Westerblad *et al.*, 1997).

In summary, it is now considered unlikely that hydrogen ions are directly responsible for peripheral fatigue. If they are involved at all it is likely in an indirect role such as stimulating group III-IV afferent nerves to cause a sensation of discomfort as proposed by Westerblad et al (2002).

As mentioned above, pH decreases as a result of H^+ released when ATP breakdown exceeds mitochondrial respiration (Robergs *et al.*, 2004). As a result, it is possible that pH could be a third theoretical O_2-dependent mechanism of fatigue, as increased O_2 delivery could delay the onset of H^+ formation by increasing oxidative phosphorylation, which uses up H^+ (see Figure 1.5).

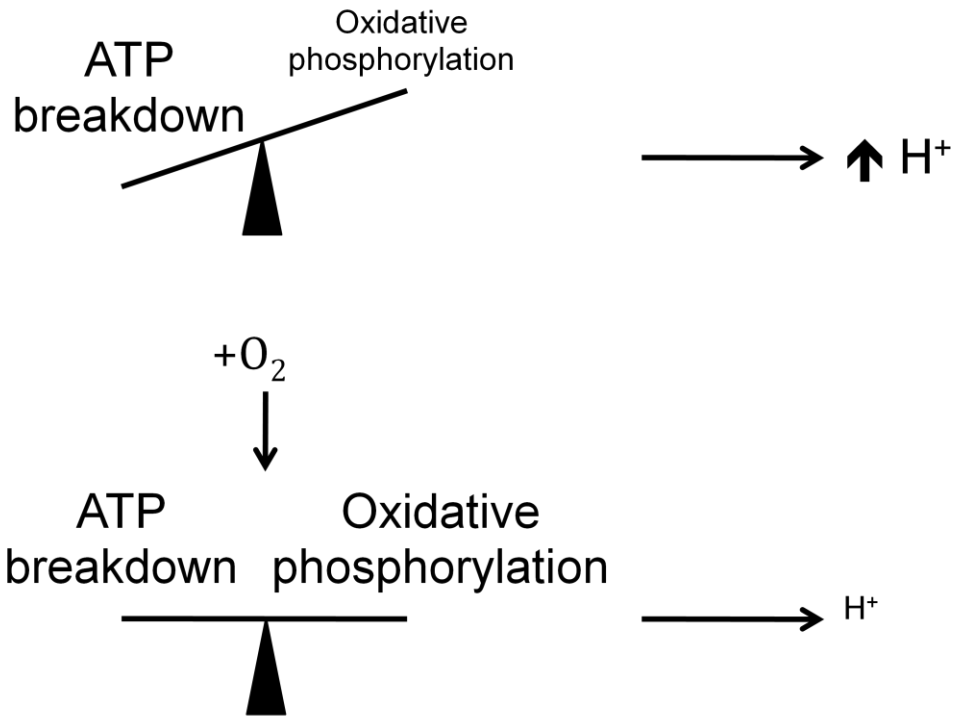

Figure 1.5. Schematic representation of the proposed mechanisms by which increasing O_2 delivery may reduce hydrogen ion (H^+) production. H^+ is produced when ATP breakdown exceeds resynthesis by oxidative phosphorylation. We propose that supplemental O_2 shifts the balance by increasing oxidative phosphorylation, thus reducing H^+ accumulation.

1.3.4 Effect of age on muscle fatigue

Despite the importance of fatigue in health and disease, the topic of whether the susceptibility of muscles to fatigue is altered with age is still an area of active research. Firstly we must consider whether any change in muscle function seen in older subjects is a physiological result of normal ageing or a functional result of reduced activity (Kirkendall & Garrett, 1998). Supporting a functional result of reduced activity, it has been demonstrated that habitual activity is inversely related to age (Aoyagi & Shephard, 1992) and it has been shown in cadavers that vastus lateralis cross-sectional area declines approximately 40% between

the ages of 20 and 80 years (Lexell *et al.*, 1988). Additionally it has recently been demonstrated that older adults have a reduction in strength which is not totally accounted for by a loss in muscle volume (Goodpaster *et al.*, 2006). Thus, it can be suggested that there is a loss of muscle quality with ageing.

Even after controlling for reduced strength by matching subjects for strength or by assessing relative strength levels, there is a considerable spread of results as to whether older subjects have altered levels of fatiguability. A majority of studies find that older subjects are more fatigue resistant than younger subjects, with this result being shown in the thumb (Chan *et al.*, 2000; Ditor & Hicks, 2000), ankle (Kent-Braun *et al.*, 2002; Lanza *et al.*, 2004) and elbow (Hunter *et al.*, 2005). Moreover, the aforementioned studies represent a range of different exercise protocols. However, other studies have found conflicting results, with for example Baudry, *et.al.* (2007) demonstrating increased fatiguability in response to repetitive maximal ankle dorsiflexion and Petrella *et.al.* (2005) finding that older adults are less capable of sustaining maximum knee extension contractions. A recent meta-analysis of the field suggests that the modality of exercise may play a role (Avin & Frey Law, 2011). They determined that older adults are more fatigue resistant than younger adults when performing relative intensity tasks when the exercise comprises of static exercise, but there is no change in fatiguability when subjects are performing dynamic contractions.

A pattern of reduced maximal strength but maintained or improved fatigue resistance in the elderly may be explained by the histological findings of work by Lexell and colleagues (Lexell *et al.*, 1986; Lexell *et al.*, 1988). These studies examined slices of vastus lateralis muscle from the quadriceps, and discovered

firstly that ageing was associated with a reduction in muscle fibre number, which may explain an overall reduction in absolute strength. This could partly explain improved fatigue resistance, as reduced muscle strength and size may lead to reduced occlusion. Secondly they found that ageing was associated with a reduction in the number of fast twitch Type II fibres leading to a relative increase in the percentage of slow twitch Type I fibres which are generally able to produce less force but are more fatigue resistant (Kent-Braun, 2009). Similar studies have not been performed looking at fibre type change in the forearm to our knowledge. However, White and Carrington (White & Carrington, 1993) investigated the changes in twitch speed in calf and biceps muscle with ageing. They found that whilst calf twitch speed decreased in elderly subjects compared to younger subjects, there was no difference in biceps twitch speed. This could be used as a surrogate measure for fibre type, thus suggesting that leg muscle switches to slow twitch Type I fibres whilst arm muscle does not.

In conclusion, overall the evidence points towards a reduction in absolute strength but an increase in fatigue resistance in older subjects, at least when performing static exercise. Whilst some of this is may be due to a reduction in muscle fibre number and a shift towards a Type 1 fibre type, there still remains research to be done investigating age-related alterations within the muscle, particularly in the arm.

1.4 Methods of measuring blood flow

The studies described above tend to measure blood flow using one of 3 techniques, these being venous occlusion plethysmography, Doppler ultrasound and thermodilution. The discussion below concentrates on the advantages and

disadvantages of these techniques, and puts forward the reasoning for using venous occlusion plethysmography in the studies discussed in later chapters.

Venous occlusion plethysmography is one of the oldest techniques used to measure limb blood flow (for reviews on methodology see e.g. Greenfield *et al.*, 1963; Wilkinson & Webb, 2001). Briefly, the technique relies on the fact that the veins in a limb supported at heart level are largely empty of blood. If venous outflow is blocked whilst arterial inflow remains unimpeded, this results in pooling of blood in these veins and a corresponding increase in limb volume, which can be measured using a strain gauge around the limb. Advantages of using venous occlusion plethysmography over other techniques are that it is cheap, widely available, non-invasive and does not require extensive training to obtain accurate, reproducible results. The major disadvantage of venous occlusion plethysmography is the fact that during the procedure the limb needs to be relaxed. This affects research on blood flow during exercise as readings can only reliably be taken during breaks in the contractions or on cessation of exercise.

Thermodilution is an alternative to venous occlusion plethysmography and involves the insertion of a catheter with a thermistor into an artery or vein serving the tissue of interest. In one widely used protocol, the thermistor is advanced through the venous catheter until the tip is located 12cm prox to the entry hole (Andersen & Saltin, 1985). Saline is then infused into the vein at a constant temperature of 0°C. Changes measured in the temperature at the thermistor are therefore due to dilution of this saline with blood, with increased blood flow resulting in a higher temperature than lower blood flows. Although

accuracy of thermodilution is reported to be comparable to plethysmography (Greenfield *et al.*, 1963), disadvantages include the increased invasiveness of the protocol, as well as the interference caused by exercise, as reported by Humphreys and Lind (1963).

A more recent technique for measuring blood flow uses a Doppler ultrasound probe to measure the velocity of the blood flow in the artery of interest whilst simultaneously or at set intervals measuring the diameter of the artery (see e.g. Shoemaker *et al.*, 1996b; Schrage *et al.*, 2004). Advantages of this technique include the ability to visualise both vessel diameter and blood velocity, the ability to observe changes in blood flow in real time and the fact that it is non-invasive. However, the technique is limited in use to vessels with a diameter of over 1-2mm and requires expensive equipment and skilled operators (Wilkinson & Webb, 2001).

Much research has been performed on the reproducibility and validity of venous occlusion plethysmography. The importance of maintaining a consistent forearm position at heart level has been underlined by recent research by Rojek *et. al.* who found that blood flow was significantly lower when the forearm was positioned ten centimeters below heart level than when positioned ten centimeters above heart level (Rojek & et al., 2007). Conversely the same study found that blood flow following exercise was actually increased when measured below compared to above heart level. There is also variation between research groups concerning whether blood flow is reported in one arm only or as a ratio of blood flow between both arms. Roberts *et. al.* performed six repeated measures of blood flow using strain gauge plethysmography in the forearm and

calf over a one month period (Roberts *et al.*, 1986). At rest they found a coefficient of variation of 10.5% and 11.5% in the forearm and calf respectively, with variation increasing slightly to 11.5% and 13% post-exercise. They concluded that this is a suitable accuracy for repeated measures within the same individual. This compares favourably to a similar study on the repeatability of Doppler ultrasound (Shoemaker *et al.*, 1996b), which found a coefficient of variation of 13.2% when measuring mean blood velocity at rest, which increased to 20.2% in the last minute of exercise.

A recent study investigated the accuracy and reproducibility of plethysmography when measuring high levels of blood flow (Wood & Stewart, 2010). Seven subjects were asked to perform five-minute periods of rhythmic handgrip exercise at various workloads and blood flow was measured using strain gauge plethysmography at rest and after exercise. The slope obtained by venous occlusion plethysmography was analysed separately over the first one, two, three and four cardiac cycles to assess whether measuring analysis over multiple cardiac cycles resulted in an underestimation of blood flow. There was no significant difference in values obtained at rest. However, after exercise at intensities over 30% of maximum voluntary contraction, forearm blood flow was significantly reduced when measured over 2-4 cardiac cycles compared to when measured for a single cycle. This indicates that when measuring high levels of blood flow, researchers should use small timeframes of measurement.

Given the independent evidence that venous occlusion plethysmography is still a reliable and accurate technique compared to alternatives, combined with the fact that it has been used successfully in this laboratory previously (Fordy &

Marshall, 2004; Win & Marshall, 2005), it was decided to continue with this methodology in the present studies. It is however recognised that the use of plethysmography measures post-exercise hyperaemia and therefore may not fully reflect the effect of interventions on exercise hyperaemia.

1.5 Aims and hypotheses of the present study

This study aims to investigate the role of O_2-dependent substances in exercise.

1. We hypothesise that in recreationally active young subjects, substances such as prostaglandins or adenosine act in an O_2-dependent manner to mediate a portion of the vasodilatation seen in exercise hyperaemia following both static and dynamic exercise.
2. We hypothesise that in older subjects, the magnitude of exercise hyperaemia will be comparable to that seen in young subjects, but the role of O_2-dependent substances will be reduced.
3. A substance or substances that acts in an O2-dependent manner during recovery mediates a component of fatigue.

The above hypotheses were tested by investigating the vasodilator and fatigue response to handgrip exercise in groups of recreationally active young and older subjects. We used supplementary 40% O_2 as compared to air to investigate O_2-dependence and aspirin and aminophylline to investigate the contribution of prostaglandins and adenosine respectively. Venous blood samples were be taken for analysis of metabolites produced in exercise and to test for changes that occur following interventions.

CHAPTER 2: GENERAL METHODS

2.1 Subjects

All experiments were carried out on healthy males aged 18 to 30 years of age for the younger groups and 50 to 70 years of age for older groups. Subjects activity levels were assessed using a self-reporting screening questionnaire (Appendix 1). The questionnaire was also used to confirm that subjects were non-smokers (classed as never smoked for young group and non-smoker for >20 years for the older group), healthy, on no medication and had no history of cardiovascular disease. Subjects were required to refrain from alcohol and strenuous exercise for 24 hours prior to the study and from caffeinated drinks such as coffee, tea and coke for 12 hours before. Females were not included in the experiment to exclude any effect of the menstrual cycle or oral contraceptive pill.

The experiments were approved by the University of Birmingham Life and Health Sciences Ethical Review Committee. Procedures were explained to the subjects before they agreed to participate and gave informed consent.

2.2 General experimental conditions and equipment

All experiments were performed in a room with a constant temperature of 21-24°C, with noise and visual distractions kept to a minimum. Subjects were seated facing away from the data acquisition equipment so as to minimise any distractions.

Subjects were seated on a clinical examination couch with the backrest at approximately 45° to the horizontal with both upper limbs resting on a pillow at around heart level. Exercise was carried out using the subject's dominant arm. Where required, venous occlusion plethysmography was also performed on this

arm to measure forearm blood flow (FBF) and a cannula was inserted in an antecubital vein to obtain venous blood samples (see below). The contralateral arm was used to measure blood pressure, heart rate and skin blood flow (see below).

2.2.1 Exercise

Each subject performed exercise by using a hand-grip dynamometer with a voltage output (Lafayette 70718, Loughborough, UK), which was linked to a computer to give accurate measurements of peak force and duration of the contraction. The 100% maximum voluntary contraction (MVC) for each subject was determined at the start of the first experimental day by asking the subject to grip the dynamometer as hard as possible for 2 seconds and 100% MVC was taken as the force at the end of this 2 second contraction to remove any effect of the initial snatch of the dynamometer. An average of 3 such readings was taken as 100% MVC for the remainder of the protocol.

For the studies in Chapters 4 and 5, a pilot study showed that our subjects were unable to maintain a static contraction at 60% MVC for 2 minutes as used previously in this laboratory (Win & Marshall, 2005), therefore 50% MVC was chosen for the exercise protocol in these studies.

2.2.2 Measurement of blood flow

Forearm blood flow was measured by using venous occlusion plethysmography, as has been used in previous experiments in this laboratory (Fordy & Marshall, 2004; Win & Marshall, 2005). To this end, a mercury-in-silastic strain gauge was placed around the forearm at the point of the largest circumference to measure changes in the forearm circumference. A paediatric

sphygmomanometer cuff was placed around the wrist and inflated to >150mmHg to occlude blood flow to the hand just prior to measurement of FBF. This excludes a large component of the cutaneous circulation thereby focussing measurements on the skeletal muscle of the forearm. A second, adult sphygmomanometer cuff, which was placed around the upper arm, was inflated to 50mmHg to occlude venous return from the forearm but still allow arterial inflow in order to obtain a recording of FBF. The wrist cuff was inflated manually using a hand-pump (Mityvac, St. Louis, USA) approximately 10 seconds prior to measurements being taken and the upper arm cuff was inflated using an automatic cuff inflator and air source (Hokanson E20 and AG101 respectively, Washington, USA).

At the beginning of each experimental session, with the strain gauge in place, the plethysmography was calibrated 3 times by producing a deflection on the trace equal to a change of 1% of forearm volume.

Blood flow was calculated offline by calculating the average slope for the initial linear portion of the plethysmography trace following inflation of the upper arm cuff. This value was then divided by the average of the 3 calibration deflections and multiplied by 60 to give a value of blood flow in units of ml.100ml tissue^{-1}.min^{-1}. Forearm vascular conductance (FVC) was also calculated as a measure of vasodilatation by dividing FBF by mean arterial blood pressure, which was averaged over 10 seconds at same time as plethysmography trace was analysed for FBF. The recording of arterial blood pressure is described below.

2.2.3 Delivery of 40% oxygen or room air

Subjects were given either 40% O_2 or room air through a secure facemask (Intersurgical, Wokingham, UK), with all protocols designed so that the subject was unaware which gas he was breathing. The 40% O_2 mixture was obtained by using two commercially available O_2 concentrators (Oxygen Leisure Products Ltd, London, UK). Briefly, they each concentrate room air and produce 5L.min^{-1} of 95% O_2. This was directed through rubber tubing to a Venturi valve attached to the facemask. The valve is designed so that when 10 L.min^{-1} oxygen is pumped through it draws ~25 L.min^{-1} of room air into it giving a total flow of 35 L.min^{-1} at a final O_2 concentration of ~40%. As a control intervention, room air was pumped through a similar mask and Venturi valve system by a Reciprocator Pump (Stanhope Seta Ltd, Denmark). The gas delivery was set up before the subject arrived so he was unaware which mixture he was breathing. When no gas intervention was being administered, the mask remained in place with the subject breathing ambient room air (i.e. there was no flow through the mask).

2.2.4 Measurement of heart rate and blood pressure

Arterial blood pressure (ABP) was monitored continuously using a Finapres cuff connected to a Finapres monitor (Ohmeda 2300, Englewood, USA), which was placed on the middle finger of the non-exercising hand and connected to a PC via a Maclab (Maclab, AD instruments, Hastings, UK). From the ABP trace, heart rate (HR) was calculated on-line by ChartPro 5 software (AD instruments, Hastings, UK) using the time interval between consecutive systolic peaks. All data was collected on a PC via Maclab hardware. 10 seconds traces were taken at the same point as each FBF measurement for calculation of FVC as

described above. HR and MABP were also measured in 30 second blocks before, during and after exercise.

2.2.5 Blood sampling

A 20 gauge IV cannula (BD Venflon, Becton Dickinson Infusion Therapy, Helsingborg, Sweden) was inserted into a vein in the antecubital fossa of the exercising arm. The cannula was placed proximal enough that it would not interfere with the placement of the mercury-in-silastic strain gauge used for venous occlusion plethysmography. A 3-way extension valve (BD Connecta, Becton Dickinson Infusion Therapy, Helsingborg, Sweden) was attached to the cannula. The procedure used for blood sampling was that 2.5ml blood was quickly removed and disposed of to ensure the blood actually sampled was representative of the venous blood at the intended moment in time. 5ml blood was then drawn into a 10ml syringe (using a larger syringe reduces the withdrawal pressure required and therefore reduces the risk of haemolysis). This sample was used to anaerobically fill a 170μl heparinised capillary tube which was capped on either end; a metal flea was placed in the tube to allow mixing and stop clotting. This tube was placed on ice for blood gas analysis after the experiment had finished (see below). The remainder of the 5ml blood was transferred to a vacutainer (yellow-capped Vacuette, Kremsmuenster, Austria) and placed on ice. The cannula was then flushed with 7ml 0.9% sodium chloride solution (Maco Pharma, Twickenham, UK) to prevent clotting. At the end of the experiment, the 5ml vacutainer of blood was centrifuged for 15 minutes at ~3500rpm to separate the blood components and the serum was extracted and snap frozen in liquid nitrogen before being stored at -80°C for later analysis.

2.3 Analysis of venous blood samples

Blood samples were stored in heparinised capillary tubes on ice as described above until the subject finished the protocol. Following this samples were analysed using an automated blood gas analyser (GEM Premier 4000, Instrumentation Laboratory Ltd.) to obtain measurements for pO_2, pCO_2, K^+ and lactate concentrations and pH. A pilot study was performed to investigate the effect of storing blood samples on ice. A venous blood sample was taken from a cannula and transferred to five separate capillary tubes. One tube was analysed immediately, whilst the remaining four were placed on ice, with one sample analysed every 30 minutes until 2 hours post-sampling. This was repeated with 5 separate venous blood samples. This pilot study showed no change in any of the variables measured in blood gas analysis over the course of 2 hours, compared to when analysed immediately after sampling.

2.5 Statistical analysis

All data are expressed as the mean±S.E.M. of the subjects. Statistical analysis was carried out using SPSS 17.0 for Mac.

CHAPTER 3: FOREARM SKELETAL MUSCLE BLOOD FLOW IS NOT COMPLETELY OCCLUDED BY STATIC HANDGRIP EXERCISE AT 100% MAXIMUM VOLUNTARY EFFORT

3.1 Introduction

It has long been hypothesised that the mechanical forces developed within the muscle during contraction may act to constrict the blood vessels, thus reducing the O_2 delivery. However, efforts to investigate this effect have been hampered by experimental difficulties and by apparent differences in the muscle group examined. One of the most frequently cited references is that of Humphreys and Lind (1963), who investigated forearm blood flow during sustained handgrip exercise. Four subjects performed exercise until fatigue at intensities from 30-70% MVC, in 10% increments. Gross limb blood flow was measured using venous occlusion plethysmography, a technique unsuited to use during exercise, as discussed in Chapter 1. Their results showed a clear graded increase in blood flow with increasing exercise intensity until 60% MVC, but found that artefacts from muscle tremors precluded accurate measure of blood flow at 70% MVC. By extrapolating the levels of hyperaemia at lower intensities, the authors concluded that forearm occlusion would occur at some point greater than 70% MVC. Similarly Barnes (1980) also used venous occlusion plethysmography to measure blood flow 15 seconds into handgrip exercise at intensities from 10-80% MVC. He reported that exercise blood flow increased above resting values until 20% MVC, then began to decrease, with intensities over ~30% MVC resulting in sub-resting values. This supports other findings that contractions of greater than 60% MVC completely occlude intramuscular circulation (Royce, 1958; Clark, 1962).

More recently, Kagaya and Homma (1997) used pulse-echo Doppler ultrasound to measure brachial artery blood flow during handgrip exercise in 7 young

women. They observed no change in brachial artery blood flow at any load from 10-70% MVC (20% increments), although they did find that post-exercise blood flow increased at intensities above 10% MVC and further increased at 70% compared to 50% MVC. They interpreted this to show an increasing imbalance between O_2 supply and demand within the forearm musculature, possibly due to partial occlusion. In contrast to the forearm, it has been reported that blood flow in calf muscle is occluded at much lower relative intensities, with Richardson *et al.* (1981) reporting that occlusion occurs as low as 30% MVC . However, these results were obtained with the calf below heart level, thus casting the validity of results obtained using plethysmography into doubt (see Section 1.4).

In recent years newer methodologies have been utilised in an attempt to control for some of the difficulties inherent with using plethysmography during exercise. Wigmore *et al.* (2004) used functional MRI (fMRI) to investigate the relationship between intramuscular pressure and perfusion during ankle dorsiflexion. This technique allows visualisation of perfusion in regions of the muscle rather than global observations. They observed a plateau in hyperaemia at around 60% MVC and therefore suggested that this is the force at which complete occlusion occurs.

Previous work from our laboratory seems to contrast with the idea of occlusion occurring at high intensities of exercise (Fordy & Marshall, 2004; discussed fully in Section 1.2.3.1). This study demonstrated that breathing supplementary O_2, only during a bout of handgrip exercise at 100% maximum voluntary effort (MVE), attenuated post-contraction hyperaemia. Thus we can infer that there was at least adequate perfusion of the muscle. Moreover, given that the O_2

reduced the level of exercise hyperaemia, we can conclude that the O_2 is specifically reaching areas of active muscle. This is not due to any effect of supplementary O_2 reducing levels of fatigue as peak force and rate of force decline were unaffected. Further, it has been shown that even when force declines due to fatigue, there is no effect on levels of hyperaemia measured immediately post-contraction (Wigmore *et al.*, 2006).

Recently a number of groups have investigated the usefulness of Near Infra-Red Spectroscopy (NIRS) in monitoring blood flow to active skeletal muscle (Boushel & Piantadosi, 2000; van Beekvelt *et al.*, 2001a; van Beekvelt *et al.*, 2002). NIRS is non-invasive, continuous and can operate in real time (Boushel & Piantadosi, 2000) and has been used to measure changes in skeletal muscle tissue oxygenation (Abozguia *et al.*, 2008). It works by measuring the absorption of near infrared light by the iron content of haemoglobin and myoglobin. Work by van Beekvelt and colleagues (van Beekvelt *et al.*, 2001a; van Beekvelt *et al.*, 2002) has demonstrated that NIRS works well even during handgrip exercise.

The rationale for the study in this chapter was to determine whether skeletal muscle circulation is occluded during maximum handgrip exercise. We felt that NIRS would be well suited to investigating this question. This is important in relation to the studies in this thesis, as if it was shown that skeletal blood flow is totally occluded during exercise this would invalidate the methodology of administering supplemental O_2 during the period of contraction only. However, if the muscle is not occluded at 100% MVE as used in previous studies from this

laboratory (Fordy & Marshall, 2004), it is a reasonable assumption that it is not occluded at 50% MVC.

3.1.1 Hypotheses

1. Muscle blood flow still occurs in active muscle, even during the initial stages of handgrip exercise at 100% MVE.

We tested this hypothesis by using NIRS to measure the oxygenation of the flexor digitorum superficialis muscle, which is one of the major muscle groups active during handgrip exercise (Humphreys & Lind, 1963; van Beekvelt *et al.*, 2002). We compared the level of oxygenation during sustained contraction at 100% MVE and during complete forearm circulatory occlusion at rest with a sphygmomanometer inflated to 200mmHg. We anticipated that if muscle circulation were occluded during contraction, the change in haemoglobin O_2 saturation in muscle would be greater than that seen in arterial occlusion, due to the increased metabolic demand of the active muscle compared to resting muscle.

3.2 Methods

10 healthy young subjects attended the laboratory on two separate occasions. At the beginning of the first visit, the subject was familiarised with the equipment and given an opportunity to ask any questions. Subjects were seated comfortably on an examination couch at an angle of ~45° with their forearms supported in front of them on a pillow. Forearm circumference was measured around the thickest part of their forearm and MVC was measured as described in Chapter 2. An OxiplexTS NearInfrared tissue oximeter (ISS Inc., Champaign,

IL, USA) was used for measurement of skeletal muscle oxygenated haemoglobin (Oxy-Hb), deoxygenated haemoglobin (deoxy-Hb) and total haemoglobin (HbT). The source-detector distance was 30-44mm to limit the contribution of skin and non-muscular subcutaneous tissue to measurements taken. The probe was fitted securely over the flexor digitorum superficialis muscle of the dominant forearm.

A facemask was fitted to the subject, although initially no gas was switched on. Following a 15 minute rest period subjects were required to perform a single static handgrip contraction at maximum voluntary effort (i.e. gripping as hard as possible) for 2 minutes or until exhaustion if sooner. During the period of contraction only, subjects breathed either 40% supplemental O_2 or room air such that they were unaware of the experimental gas mixture. Study days were randomised in a single blinded manner. The 2 minute cut-off was chosen as this had been found to be greater than the time to voluntary exhaustion in the majority of young subjects (data discussed in Chapter 6). Subjects then recovered for 7 minutes to allow perfusion to return to baseline before a sphygmomanometer cuff was inflated around the upper arm at 200mmHg for 3 minutes at rest. This timescale was chosen in line with the protocol used by other members of the laboratory. NIRS recordings were made throughout.

3.2.1 Analysis

Data was analysed using SPSS for mac (version 17.0). Baseline for exercise was taken as the average of the resting measurements and baseline for occlusion was taken as the peak reached during recovery. Changes from baseline were measured to the greatest change during exercise and occlusion.

Changes from baseline, differences between changes in exercise and occlusion and differences between air and O_2 breathing in exercise were analysed using repeated measures ANOVA. Two comparisons were made for time (exercise and occlusion against baseline) and one comparison for exercise vs. occlusion. Correlations were analysed using a two-tailed bivariate correlation with Pearson's correlation coefficient.

3.3. Results

3.3.1 Subject characteristics

Subjects were aged 28.6±1.6 (mean±S.E.M). They had an average forearm circumference of 26.1±0.9cm and were able to produce a mean maximum force of 28.6±3.3kg.

3.3.2 NIRS changes in exercise

On commencing handgrip exercise, haemoglobin O_2 saturation and oxyhaemoglobin concentrations rapidly decreased, reaching a nadir by 30 seconds. Levels then remained around this level or slightly higher for the remainder of exercise. Deoxyhaemoglobin concentrations followed an inverse of this pattern, reaching a peak by 30 seconds and then falling slightly for the remainder of exercise. Force produced during exercise remained at peak force for 10-15 seconds before gradually declining over the remainder of the exercise period.

Table 3.1 shows the mean ± SEM values for variables measured by NIRS in rest, exercise, recovery and occlusion. Figure 3.1 shows the maximum change in deoxyhaemoglobin and oxyhaemoglobin concentration during handgrip

exercise at 100%MVE until exhaustion and during complete occlusion for 3 minutes. There was a large variation in the maximum change of deoxyhaemoglobin from baseline between subjects, with the mean±S.D. being 25.4±20.7 for exercise and 34.7±21.7 for occlusion. Due to this variation, the increase from baseline and the difference between exercise and occlusion was not significant ($p=0.325$). However, levels of oxyhaemoglobin decreased significantly ($p<0.001$) from baseline in both exercise and occlusion. There was no significant difference in oxyhaemoglobin during forearm circulatory occlusion compared to during exercise ($p=0.088$).

Changes in haemoglobin O_2 saturation are shown in figure 3.2. O_2 saturation fell significantly during both static handgrip exercise and during forearm circulatory occlusion ($p<0.001$). The decrease during occlusion was greater than that seen in handgrip exercise ($p=0.043$), despite the O_2 requirements in exercise being greater than the requirement during occlusion at rest.

Breathing supplemental 40% O_2 during contraction compared to room air had no effect on changes in any NIRS measurements. Peak exercise change in haemoglobin O_2 saturation was 25.7±4.7% in air (mean±S.E.M.) vs. 20.1±3.5% when O_2 was breathed ($p=0.16$). Similarly, peak changes in oxyhaemoglobin concentration were 17.7±2.7% in air vs. 18.0±3.8 in O_2 ($p=0.94$) and peak changes in deoxyhaemoglobin concentration were 25.4±6.6 in air vs. 22.0±4.8 in O_2 ($p=0.68$).

3.3.3 Correlations between forearm circumference, strength and change in haemoglobin O_2 saturation

Figure 3.3 shows the relationship between forearm circumference and peak force in handgrip exercise. There was a highly positive relationship, such that subjects with an greater forearm circumference produced a greater peak force (Pearson correlation coefficient = 0.919; $p<0.001$).

Figure 3.4 shows the relationship between peak force in handgrip exercise and the maximum change in haemoglobin O_2 saturation seen during handgrip exercise at 100% MVE. There is a weakly positive correlation (Pearson correlation coefficient = 0.291) which does not reach statistical significance. Similarly, the relationship between forearm circumference and the maximum change in haemoglobin O_2 saturation seen during handgrip exercise at 100% MVE (not shown) was positive but not significant (Pearson correlation coefficient = 0.328).

3.4. Discussion

The main findings of this study were that the change in haemoglobin O_2 saturation did not decrease as much during static handgrip at 100% MVE until exhaustion as it did during total forearm circulatory occlusion. This was despite the fact that we can expect the O_2 requirement during exercise to be greater than that during occlusion at rest. In agreement with our hypothesis, this indicates that blood flow to active muscle must continue even during exercise at maximal voluntary effort. Breathing supplemental O_2 during exercise had no effect on changes in any of the variables measured by NIRS. In addition, our

data show a highly positive correlation between forearm circumference and muscle strength. Although the study was not designed to investigate this effect and therefore was underpowered, there was a positive correlation between both forearm circumference and peak force and the maximum change seen in haemoglobin oxygen saturation. This suggests that there may be greater levels of circulatory occlusion in subjects who are able to produce greater force.

3.4.1 Circulatory occlusion during exercise

Previous research investigating whether skeletal muscle circulation is occluded has had differing results depending on the technique used and the muscle bed investigated. It appears that in calf muscle, occlusion occurs at relatively low intensities of exercise (Richardson, 1981). Early studies investigating forearm muscle during handgrip used venous occlusion plethysmography. Royce (1958) and Clark (1962) calculated the level of complete occlusion as occurring at 60% MVC, whereas Humphreys and Lind (1963) extrapolated data measured up to 60% MVC and calculated that occlusion would occur at around 70% MVC. Finally Barnes (1980) calculated that in contractions greater than 30% MVC the blood flow had decreased to levels below resting values. Venous occlusion plethysmography has the advantage that it measures blood flow primarily to skeletal muscle in the forearm. However, due to the fact that the variable measured is the change in circumference of the limb under investigation, it is not ideally suited to measurements taken during periods of contraction.

More recently the technique of pulse echo Doppler ultrasound has been utilised (Kagaya & Homma, 1997). This technique calculates the diameter of the artery supplying a limb (in this case the brachial artery) and the velocity of the blood

flowing through it to give a measure of limb blood flow. Kagaya and Homma (1997) found no change in blood flow through the brachial artery at exercise intensities of between 10% and 70% MVC. However, this is possibly to be expected as there would need to be significant force production to occlude the brachial artery. Therefore it seems likely that any occlusion that does occur will occur locally at the level of the active muscle rather than the entire limb.

Local muscle occlusion has been measured to some extent using the new technique of functional MRI with Wigmore (Wigmore *et al.*, 2004) using it to investigate blood flow in the ankle dorsiflexors. They observed an increasing hyperaemia at intensities up to 60% MVC before it plateaued at around 50% of the reactive hyperaemia seen after 5 minutes of occlusion. They interpreted this as being the level at which complete occlusion occurs, although given that they still observing these maximal levels of blood flow at 90% MVC, it is equally probable that the hyperaemia seen at 60% MVC represented the maximum level of hyperaemia in response to ankle dorsiflexion.

To our knowledge, there have not been any studies that have used reliable techniques to investigate local levels of occlusion in forearm muscle during handgrip exercise. Previous work from our laboratory has provided some indirect evidence that there is not complete occlusion of forearm vasculature to the active muscles (Fordy & Marshall, 2004). In that study, 40% O_2 breathed during the period of contraction only was able to have an effect on the magnitude of post-exercise hyperaemia, therefore blood flow must have been reaching the active muscle. As noted above (section 3.1), this was not due to any effect of O_2 on force production or fatigue levels.

The present study repeated the handgrip exercise protocol used by Fordy (2004) using NIRS to investigate blood flow. This is a technique which has been used to measure forearm blood flow and found to be reproducible (van Beekvelt *et al.*, 2002). Advantages of using this technique were that it allowed real time visualisation of the active flexor digitorum superficialis muscle. By using a source-detector distance of 30-44mm we limited the contribution made by skin and non-muscular subcutaneous tissue to the measurements made. We demonstrated that the magnitude of the decrease in haemoglobin O_2 saturation that occurred during exercise was not as great as that seen during forearm occlusion. This indicates that blood flow to the active muscle was not compromised during handgrip exercise at 100% MVE. Indeed, the results presented in figure 3.2 do not show the entire picture, as we can expect the rate of O_2 uptake to be greater during the exercise protocol than the occlusion protocol.

3.4.2 Impact of force production on levels of occlusion

It would appear logical that if there is any occlusion taking place within active muscle, greater force production by the muscle would produce greater levels of occlusion. In the studies described above, there is some evidence that this is the case. Barnes (1980) examined 20 active young men performing static handgrip exercise. He then split them into the ten strongest and ten weakest and found evidence that occlusion was occurring at lower levels of MVC in the 10 stronger subjects, thus levels of occlusion were dependent on absolute force rather than relative force. This supported earlier findings that stronger subjects occluded at 45% MVC compared to 60% in weaker subjects (Heyward, 1975).

However, Wigmore and colleagues (2006) more recently used levels of fatigue as a surrogate for blood flow, determining that occlusion of blood flow would produce greater levels of fatigue. They observed no correlation between levels of fatigue and either muscle volume or initial peak strength. Although this study was not designed to investigate the relationship between muscle size and strength and the levels of occlusion, the results shown in figure 3.4 and discussed above do give us some insight. Although the results did not reach statistical significance, the data show a small positive correlation between muscle strength and the decrease in haemoglobin O_2 saturation and a medium positive correlation between forearm circumference and the decrease in haemoglobin O_2 saturation. As the current study used the magnitude of the fall in haemoglobin O_2 saturation as a measure of occlusion, this indicates that there is likely to be an effect of muscle size and strength on the relative level of occlusion seen, although as discussed above we have no evidence of a total occlusion occurring.

3.4.3 Effect of supplemental O_2 on NIRS variables

This study found no evidence of any difference in changes in haemoglobin O_2 saturation, oxyhaemoglobin or deoxyhaemoglobin concentrations during exercise when 40% supplemental O_2 was breathed during contraction compared to air breathing. This concurs with findings by Ekblom *et al*. (1975) showing that there is only a small change in haemoglobin O_2 saturation from 98-99% to 100% when 50% O_2 is breathed. In their study they found that the majority of the changes which occur are a result of P_aO_2 rising from ~98 to 240mmHg, which would not be picked up by NIRS which concentrates

specifically on haemoglobin and myoglobin O_2 saturation. Of interest in the context of the studies described in the following chapters is the finding that this presumed increase in P_aO_2 caused by breathing 40% O_2 did not appear to have any effect on levels of tissue oxygenation. The possible implications of these results are discussed in detail in Section 4.4.2.

3.4.4 Critical evaluation of experimental protocol

Although the NIRS methodology was chosen due to the ability to measure changes in haemoglobin and myoglobin in real time, some limitations are caused due to the small sample area. Firstly, measurements made with NIRS correspond to local changes within an area of the active muscle, not to changes within the whole muscle. Therefore, we cannot rule out the possibility that the measurements taken in this study miss the most active areas of muscle and that there may be small local areas of occlusion. Secondly, it is possible that the changes shown during exercise are underestimated due to the area of measurement encompassing areas of muscle not actively involved in contraction as well as contracting muscle. Finally, the data in the present study was was based on measurements of the flexor digitorum superficialis muscle. This was based on data showing that this muscle is active during handgrip exercise (Humphreys & Lind, 1963). However, we again cannot rule out the possibility that other muscles involved in handgrip are more effected by occlusion.

A further limitation of the current protocol concerns the appropriateness of comparing the exercise and occlusion interventions. This protocol was chosen in an attempt to answer whether forearm occlusion occurred during an exercise

protocol used previously in this laboratory (Fordy 2004). The first potential limitation concerns the use of 100% MVE handgrip exercise. Although the force is the maximum the subject is able to achieve throughout the entire contraction, the absolute force achieved begins to drop from approximately 10 seconds into the protocol. The possibility remains that the level of occlusion seen during exercise is underestimated due to the reduction in absolute force during the protocol. The second limitation concerns differences in time between exercise and occlusion, as the timescale for peak changes in NIRS variables to occur was different between the two interventions. This potentially confounds interpretation of data as it may be that a small level of occlusion occurs during the initial stages of exercise, but is missed by comparing NIRS changes with those seen after 3 minutes occlusion. For future work, both potential limitations could be alleviated by picking an exercise protocol that involves subjects maintaining a set force for a given time, then matching the length of occlusion to this. A different approach to answering the question of whether occlusion occurs during exercise could be to compare NIRS changes during exercise at the same force, with and without forearm occlusion.

3.4.5 Summary

In conclusion, the present study has demonstrated that significant blood flow occurs to active muscle even during the initial stages of maximal static handgrip exercise, when peak force is achieved. This validates our method of administering supplementary O_2 during the period of contraction only (as used in Chapters 4 and 5), as we can reasonably assume that there will also be significant blood flow when performing exercise at 50% MVC.

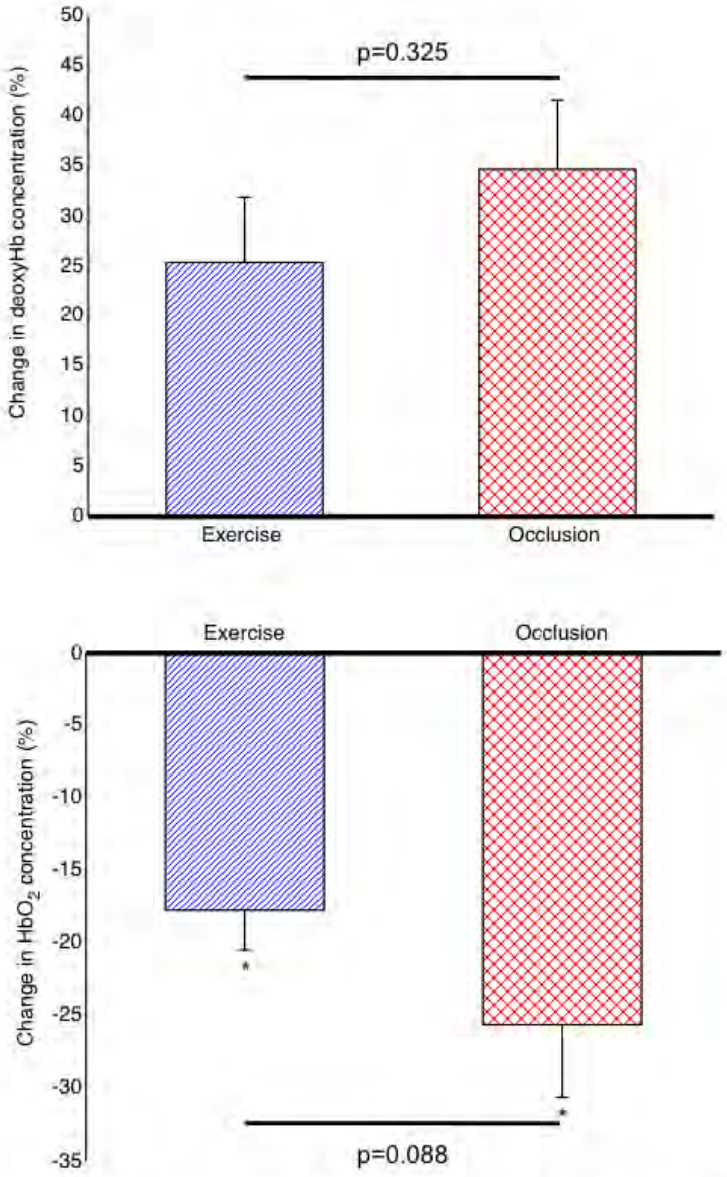

Figure 3.1. Maximum changes in concentration of deoxyhaemoglobin (a) and oxyhaemoglobin (b) from baseline during 100%MVE static handgrip exercise and complete forearm circulatory occlusion. Deoxyhaemoglobin levels were on average higher than baseline although due to the large variation this change is not significant. HbO_2 decreased significantly from baseline in both exercise and occlusion; the decrease in occlusion tended towards being greater than the decrease in exercise. n=10 subjects, *=p<0.001 compared to baseline. Horizontal bar compares change in exercise to change in occlusion.

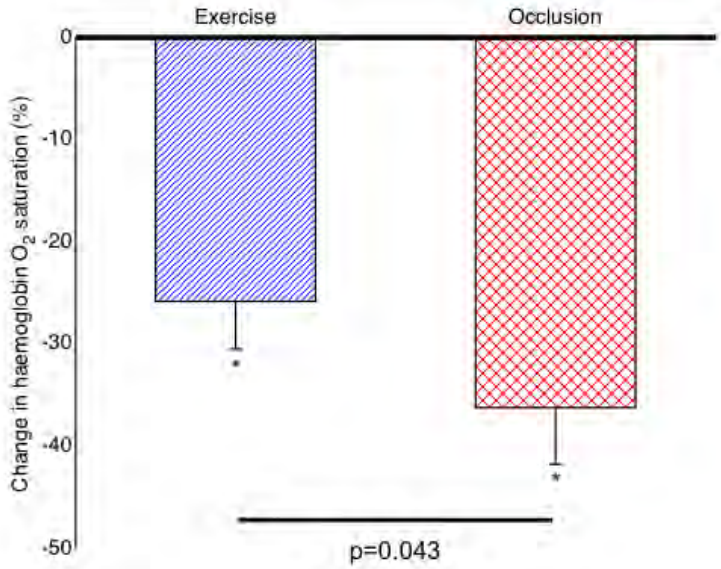

Figure 3.2. Maximum changes in haemoglobin O_2 saturation from baseline during 100%MVE static handgrip exercise and complete forearm circulatory occlusion. Haemoglobin O_2 saturation was significantly decreased from baseline in both exercise and occlusion; the decrease in occlusion was greater than that seen in exercise. n=10 subjects, *=p<0.001 compared to baseline. Horizontal bar compares change in exercise to change in occlusion.

	Rest	Exercise	Recovery	Occlusion
Hb O_2 saturation	72.8±12.2	47.1±12.9*	78.4±9	38.3±17.3*
OxyHb concentration	53.3±11.3	37±12.3*	63.5±16.8	33±15.6*
DeoxyHb concentration	22.1±13.1	23.8±11.1	26.4±11.3	21.8±11.8

Table 3.1. Mean ± SEM values for NIRS variables Haemoglobin O_2 saturation and OxyHb concentration were significantly decreased from rest in both exercise and occlusion; DeoxyHb concentrations were not significantly changed. n=10 subjects, *=p<0.05 compared to rest, measured by ANOVA.

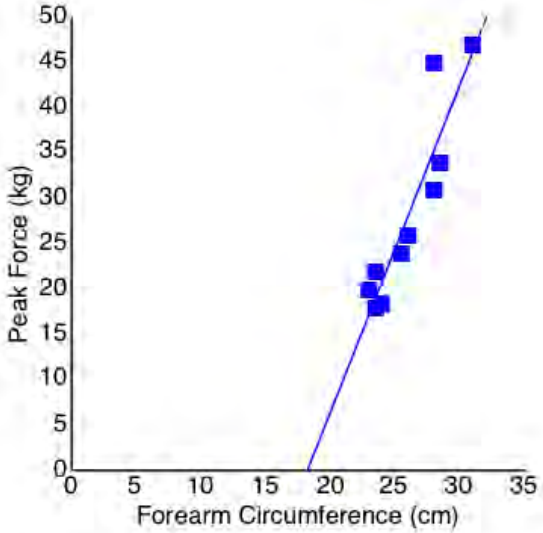

Figure 3.3a Correlation between forearm circumference and peak force using a handgrip dynamometer. There was a highly positive correlation between forearm circumference and grip strength. Pearson correlation coefficient = 0.919; p<0.001. n=10 subjects.

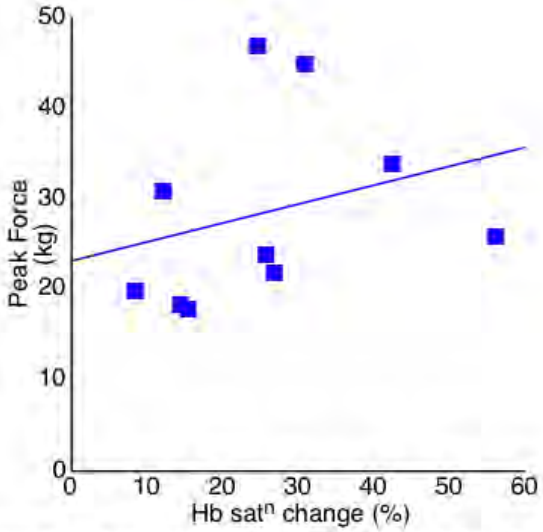

Figure 3.3b Correlation between peak force using a handgrip dynamometer and maximum change haemoglobin O_2 saturation during exercise. There was a positive correlation between forearm circumference and grip strength, although this was not statistically significant. Pearson correlation coefficient = 0.291; p=0.414. n=10 subjects.

CHAPTER 4: THE O_2-DEPENDENCE OF PROSTAGLANDINS IN MEDIATING EXERCISE HYPERAEMIA

4.1 Introduction

As described in Chapter 1, vasodilator prostaglandins produced by the COX enzyme have been found to play a role in hyperaemia during and after exercise by some groups (e.g. Kilbom & Wennmalm, 1976; Duffy *et al.*, 1999; Win & Marshall, 2005), but not all (Shoemaker *et al.*, 1996a). For a summary see Table 1.2. Other groups have found evidence of vasodilator COX products playing an interdependent role with other substances such as NO (Mortensen *et al.*, 2007), adenosine (Mortensen *et al.*, 2009b; Nyberg *et al.*, 2010) and ATP (Mortensen *et al.*, 2009a).

Previous findings from this laboratory showed that in fit recreationally active young male subjects, COX inhibition with aspirin reduced the vasodilatation caused by 2 minutes of static handgrip exercise at 60% MVC by 40-50% (Win & Marshall, 2005). This reduction in post-exercise hyperaemia could be replicated by administering 40% supplementary O_2 before, during and after the exercise and there was no additive effect when COX inhibition and hyperoxia were combined. This implies that a vasodilator COX product produced or acting in an O_2-dependent manner mediates a portion of post-exercise hyperaemia (see Figure 1.1). Since venous blood samples were not collected in this study these possibilities could not be differentiated. In previous studies, PGI_2 and PGE_2 have been assayed in venous efflux and interstitium and have been implicated in exercise and post-exercise hyperaemia (Symons *et al.*, 1991; Wilson & Kapoor, 1993b; Karamouzis *et al.*, 2001a; Karamouzis *et al.*, 2001b). Recent data also suggests that the site of production of PGI_2 and PGE_2 may be

different, with PGI_2 being produced by the endothelium and PGE_2 produced by the skeletal muscle (Testa *et al.*, 2007; Nyberg *et al.*, 2010).

In their discussion, Win and Marshall (2005) proposed that the stimulus for prostaglandin production is a fall in pO_2 at the level of the endothelium. This seemed the most likely explanation, for in healthy young subjects haemoglobin saturation would be ~98-99% and so supplementary O_2 would only increase the arterial partial pressure of O_2 (PaO2) from ~100mmHg to ~240mmHg without having a significant effect on O_2 carriage. It was judged that a relatively small increase in arterial O_2 content would be unlikely to have much effect on the pO_2 at the level of the skeletal muscle fibre. However, recent data (see Chapter 1.2.3.1) showed that the pO_2 within muscle fibres is ~34mmHg at rest (Richardson *et al.*, 2006). In light of earlier findings that muscle fibre pO_2 falls to ~3mmHg when performing increasing dynamic leg exercise and capillary pO_2 is ~37mmHg at maximal exercise (Richardson *et al.*, 1995; 2001), it is now clear that a large O_2 gradient develops between the capillary and the skeletal muscle fibre in exercise. Thus we hypothesise that increasing the pO_2 in the capillary by administering hyperoxic gas would increase this gradient and could lead to greater diffusion of O_2 into the muscle. Based on the findings that skeletal muscle produces PGE_2 (Testa *et al.*, 2007; Nyberg *et al.*, 2010), we can hypothesise that this will be the prostanoid affected. Indirect evidence of whether supplementary O_2 is effective at the level of the muscle fibre could be obtained with blood gas analysis of venous blood samples. If supplementary O_2 does ameliorate hypoxia, it is a reasonable hypothesis that this would result in a

reduction in lactate production and a reduction in the acidosis which occurs when ATP breakdown exceeds resynthesis (Robergs *et al.*, 2004).

Further work from this laboratory has investigated the timing of supplementary O_2 which causes this attenuation of post-exercise hyperaemia (Fordy & Marshall, 2004). The major findings of this research (see Chapter 1.2.3.1) were that O_2 only has this effect when administered during the period of contraction, and that administration during recovery or preloading the muscle with O_2 prior to exercise had no effect on the levels of post-exercise hyperaemia. Therefore we hypothesise that an O_2-dependent mediator of post-exercise hyperaemia is produced during the period of exercise.

Regarding the effect of ageing on exercise and post-exercise hyperaemia, some groups have shown a reduced vasodilatation during or after exercise (Proctor *et al.*, 1998; Carlson *et al.*, 2008), whilst others have found no alteration compared to younger subjects (Jasperse *et al.*, 1994) (See Chapter 1.2.4). In the context of prostaglandin mediated exercise hyperaemia, Schrage *et al.* (2004; 2007) showed that COX inhibition in young subjects reduced exercise hyperaemia evoked by dynamic forearm exercise at 10% MVC by ~12%, whereas it has no effect in older subjects, thus ageing completely abolishes prostaglandin mediated exercise hyperaemia. In addition, there is evidence that production of vasodilator prostaglandins is switched to production of vasoconstrictor prostanoids in older rats (Tang & Vanhoutte, 2008), as well as humans with hypertension (Taddei *et al.*, 1993). However, neither of these studies directly measured production of vasodilator and vasoconstrictor

prostanoids and this switch in prostanoid production has not been investigated in young and older healthy human subjects.

4.1.1 Hypotheses

1. In recreationally active young men, prostaglandins contribute to post-exercise hyperaemia in an O_2-dependent manner following both static and dynamic handgrip exercise.

2. This O_2-dependence is mediated by hypoxia within the skeletal muscle.

3. The contribution of dilator prostaglandins to mediating post-exercise hyperaemia is reduced in older subjects.

4. The magnitude of hyperaemia is not altered by ageing.

We tested these hypotheses by measuring blood flow before and after handgrip exercise in healthy young and older subjects. Over four separate visits subjects were randomly assigned to COX inhibition with aspirin or placebo and breathed either 40% O_2 or room air through a facemask during the period of exercise only. Venous blood samples were obtained for measurement of venous PGE metabolites and venous blood gases.

We anticipated that both aspirin and breathing supplementary O_2 would reduce the magnitude of post-exercise hyperaemia and the venous efflux of PGE metabolites in young subjects but not in older subjects. We anticipated that ameliorating hypoxia within skeletal muscle with supplementary O_2 would result in a reduction in the venous concentrations of lactate and hydrogen ions.

4.2 Methods

12 young (21.5+/-0.4 years) and 12 older (60.5+/-5.7 years) subjects attended the laboratory on 4 separate days, at least a week apart. Subjects were set up and MVC was measured on the first visit as described in chapter 2. Either 40% O_2 or room air was breathed through a mask during the period of contraction only, for the rest of the protocol, subjects breathed room air without a mask. Once the subject was seated comfortably, 600mg aspirin in 250ml orange squash or 250ml orange squash alone as a placebo was administered. Subjects then rested for 30 minutes prior to the first period of exercise, as this has been shown to cause maximal inhibition of the COX enzyme (Heavey *et al.*, 1985). The order of all interventions was randomised in a single-blinded manner, such that upon completion of the study all subjects had received air/placebo (i.e. control), air/aspirin, O_2/placebo and O_2/aspirin.

On the first visit to the laboratory, the subject was familiarised with the equipment and given an opportunity to ask any questions. The activity questionnaire was administered at this point. 30 minutes after administration of the aspirin or placebo a venous blood sample was taken and three recordings of baseline FBF were made by venous occlusion plethysmography. The facemask was then fitted to the subject although the gas was not switched on. The subject then began the period of static exercise at 50% MVC for two minutes, with the experimental gas mixture pumped through the facemask during the exercise period only. Immediately after the subject released the dynamometer another recording of FBF was taken. By inflating the wrist cuff in the last 10 seconds prior to cessation of exercise, it was possible to consistently

take these measurements at ~1 second after relaxation of the contraction. This therefore allowed as close an estimation of true exercise hyperaemia as possible using venous occlusion plethysmography. A venous blood sample was taken immediately following the measurement of FBF (approximately 10-15 seconds after completion of the exercise). Further venous blood samples and FBF recordings were taken at 3 and 6 minutes after the end of the exercise. 30 minutes following the first period of exercise this protocol was repeated with the subject performing two minutes of rhythmic exercise at 50% MVC, at a rate of 1 second contraction/1 second relaxation.

4.2.1 PGE metabolite assay

Serum was extracted from venous blood samples and stored as explained in the general methods (section 2.2.5). Samples taken at rest and immediately following static exercise from all subjects were assayed using a Prostaglandin E metabolite EIA Kit (Cayman Chemicals, Ann Arbor, MI). Due to the rapid metabolism of PGE2, this assay converts the metabolites of PGE into a single stable derivative, which can then be quantified.

The assay was performed as per the manufacturer's instructions. Briefly, samples were derivatized overnight with carbonate buffer, before being phosphate buffer and EIA buffer were added (all buffers were supplied with the kit). Samples were not diluted as typical values for PGE2 given by the manufacturer were 3-12pg/ml (based on findings by Granstrom et. al. (1980)) and these values would occur in the most sensitive range of the assay. 50μl of sample were combined with tracer and antibody and incubated for 18 hours; absorbance was then read using a colorimeter. These values were then used to

calculate the concentration based on the absorbance of known concentrations of assay standard.

4.2.2 Analysis

All data was analysed using SPSS for mac (version 17.0). Data are presented as mean ± S.E.M.

Differences between subject characteristics were analysed using an independent sample T-test, with the exception of activity levels and hours per week of activity due which were analysed using a Mann Whitney U test.

FBF and FVC were analysed using 3-way repeated measures ANOVA with within-subject factors for gas breathed, drug intervention and time. Differences in FBF and FVC between young and old subjects were analysed using mixed between/within ANOVA using an additional between-subjects factor of age. Main factor effects are presented using Huyne-Feldt correction to control for any differences in variance between groups. In cases where significance was detected, Bonferroni adjustment was performed in order to locate where differences occurred within or between groups. 3 comparisons were made for time (3 post-exercise timepoints vs. rest), 1 comparison was made for gas (O_2 vs. air) and 1 for drug (aspirin vs. placebo).

Heart rate and mean arterial blood pressure were analysed using repeated measures ANOVA with special contrasts defined to perform 3 pairwise comparisons of the 3 groups of time points (baseline, exercise and recovery).

pO_2, pCO_2, pH and lactate were analysed using Generalised Estimating Equations to correct for data missing due to failure of cannulation (missing

values for entire experimental visit) or malfunction of the blood gas analyser (missing single values within visit). A table showing proportions of missing data points is included in Appendix 2.

Tables showing data as Mean±S.E.M. are included in Appendix 3 for reference.

4.3 Results

4.3.1 Subject characteristics

Subject characteristics are shown in Table 4.1. Young subjects were aged 21.5±0.4 (mean +/-SEM) years old compared to 60.5±5.7 years for the older subjects. Young subjects had a mean forearm circumference of 25.7±0.7cm, a mean peak force of 32.6±2.1kg. This was similar to the older group, with a mean forearm circumference of 26.1±0.5cm and peak force of 32.8±1.8kg. Young subjects rated themselves as less active than older subjects on a subjective scale, although this difference was not significant ($p=0.075$). They also reported less hours of activity per week than the older subjects although this was not statistically significant ($p=0.17$). Both young and old subjects reported predominantly leg-based activity.

4.3.2 Heart rate and MABP

The heart rate and MABP responses for both young and old subjects in response to 2 minutes handgrip exercise are shown in Figure 4.1 (response to static exercise) and 4.2 (response to dynamic exercise). In young subjects, both heart rate and MABP were significantly greater during static exercise than during baseline or recovery ($p<0.001$). There was not a significant increase in heart rate from baseline during dynamic exercise ($p=0.114$) but there was a

significant decrease from exercise levels during recovery (p=0.03). MABP was raised in exercise compared to both baseline and recovery (p<0.001). There was no significant effect of breathing supplementary O_2, aspirin or the two in combination on either heart rate or MABP.

In older subjects, both heart rate and MABP were higher during exercise than baseline or recovery (static: all p<0.01; dynamic: all p<0.001). There was an interaction of gas and drug on MABP during the static exercise protocol (p=0.004), such that supplementary O_2 and aspirin given in combination resulted in a lower MABP over the whole time course than the air/placebo control. There was otherwise no effect of either supplementary O_2 or aspirin or combination on heart rate or MABP in older subjects.

4.3.3 FBF and FVC

Figure 4.3a and b show the FBF and FVC respectively for both young and older subjects before and after static exercise. There was no significant difference between resting blood flow with age (p=0.178). Static handgrip exercise at 50%MVC for 2 minutes resulted in a significant rise in FBF measured immediately post-exercise in young subjects (Fig 4.3a; p<0.001). This rise was comparable in the older subjects (Fig 4.3a; p<0.001), with no significant differences in the peak values measured in the young or older subjects. Figure 4.3a shows that in both groups the FBF fell from these peak values at 3 minutes and further at 6 minutes, although still remained increased from baseline (p<0.001 for young; p=0.027 for older). In young subjects there were no significant main effects of breathing supplementary O_2 (p=0.63), taking aspirin (p=0.804) or the two combined (p=0.616). Similarly, there was no individual

effect of 40% O_2 (p=0.278) or aspirin (p=0.897) in older subjects, although there was a significant interaction of gas and drug (p=0.047) for FBF following static handgrip exercise, which is attributable to the data for all the interventions being lower than the air/placebo control.

Figure 4.3b demonstrates that the trend for changes in FVC was similar to that for FBF, indicating that the changes in FBF were due to changes in vascular tone. Similarly there were no significant main effects of 40% O_2, aspirin or the two in combination in young (respectively p=0.843; p=0.899; p=0.725) or older subjects (respectively p=0.236; p=0.565; p=0.386).

Dynamic handgrip exercise (Figure 4.4 a and b) elicited a similar pattern to static exercise; both FBF and FVC were increased immediately after exercise and fell by 3 and 6 minutes, whilst remaining higher than baseline. Similarly, there were no significant main effects of supplementary O_2, aspirin or both combined in either young or older subjects.

4.3.4 Venous blood gas analysis

4.3.4.1 pO_2

Figure 4.5a&b show the values for venous pO_2 before and after static and dynamic exercise. In young subjects both static and dynamic exercise resulted in a peak venous pO_2 at 3 minutes post-exercise, such that pO_2 was significantly elevated from both baseline and from immediately post-exercise (Fig. 4.5a; $p<0.001$); pO_2 then began to return to baseline by 6 minutes post-exercise. There were no significant main effects of breathing 40% O_2 (static

p=0.968; dynamic p=0.647), aspirin (static p=0.549; dynamic p=0.566) or the two in combination (static p=0.265; dynamic p=0.926).

As can be seen from Fig. 4.5b the pattern was similar in older subjects, with pO_2 significantly peaking at 3 minutes post-exercise ($p<0.001$); the rise from baseline was also significant immediately post-exercise ($p<0.001$). As with young subjects, there were no significant main effects of supplementary O_2, aspirin or both in combination.

4.3.4.2 pCO_2

Figure 4.5c&d show the values for venous pCO_2 before and after static and dynamic handgrip exercise. In both young (Fig. 4.5c) and older subjects (Fig. 4.5d), both modes of exercise resulted in a peak venous pCO_2 immediately post-exercise and returned to baseline by 3 minutes post-exercise ($p<0.001$ compared to baseline and 3 minutes post-exercise).

There were no significant main effects of supplementary O_2, aspirin or both combined in either mode of exercise in both young and older subjects.

4.3.4.3 Lactate

Figure 4.6a&b show venous lactate concentrations before and after static handgrip exercise. In young subjects (Fig. 4.6a), venous lactate concentrations were raised from baseline at all time points following both static and dynamic exercise ($p<0.001$). There were no significant main effects of breathing 40% O_2 (static p=0.195; dynamic p=0.688), aspirin (static p=0.996; dynamic p=0.253) or the two in combination (static p=0.485; dynamic p=0.064).

The time-course of changes in venous lactate concentrations was the same in older subjects for both static and dynamic exercise (Fig. 4.6b; also $p<0.001$). As with young subjects, there were no significant main effects of supplementary O_2, aspirin or both in combination.

4.3.4.4 pH

Both young (Fig. 4.6c) and older subjects (Fig. 4.6d) demonstrated similar changes in venous pH in response to static and dynamic exercise, such that pH was significantly decreased from baseline immediately post exercise ($p<0.001$), with pH returning towards baseline at 3 minutes and further recovered by 6 minutes.

There were no significant main effects of supplementary O_2, aspirin or both combined in either mode of exercise in both young and older subjects.

4.3.5 Venous serum PGE metabolite assay

Figure 4.7 shows the concentrations of the PGE_2 metabolites assayed in serum taken before and after static handgrip exercise. In young subjects (Fig. 4.7a), levels of PGE metabolites measured from venous serum remained unchanged after exercise compared to resting levels. The same pattern occurred in the older subjects (Fig. 4.7b). As would be expected from COX inhibition, Figure 4.7 demonstrates a reduction in the resting concentration of PGE metabolites on the 2 experimental occasions involving aspirin administration 30 minutes prior to blood sampling, with a non-significant decrease in the young subjects ($p=0.092$) and a significant reduction in the older group ($p=0.027$). Breathing supplementary O_2 in recovery had no effect (young $p=0.988$; older $p=0.763$).

Analysis by repeated measures ANOVA shows no significant effect of age on PGE metabolite levels ($p=0.134$) or the effect of aspirin ($p=0.623$).

4.4 Discussion

The studies presented in this chapter were the first to investigate the O_2-dependence of vasodilator prostaglandins in mediating post-exercise hyperaemia in both static and dynamic exercise in the forearm. The data also add to the literature concerning the effects of ageing on the mediation and magnitude of post-exercise hyperaemia.

The main findings of this study were:

1. In contrast with our first hypothesis, neither COX inhibition nor hyperoxia had any effect on FBF or FVC following static exercise at 50% MVC for 2 minutes in young subjects.
2. Similarly in young subjects, neither aspirin nor 40% O_2 had an effect on FBF or FVC following dynamic exercise at 50% MVC for 2 minutes at a rate of 0.5Hz; this was also in opposition to our first hypothesis.
3. Breathing supplemental O_2 had no effect on the pH or lactate measured in venous blood, suggesting that O_2 delivery to muscle was not increased by this protocol.
4. In agreement with our third hypothesis, COX inhibition and 40% O_2 had no effect on FBF or FVC following exercise in older subjects.
5. In agreement with hypothesis four, there was no difference in FBF or FVC evoked following static or dynamic exercise between young and older groups.

6. Venous efflux of PGE_2 was not increased by exercise in either young or older subjects.

4.4.1 Control findings in young subjects

As expected, both static and dynamic exercise resulted in an increase in FBF immediately post-exercise, attributable to a decrease in vessel tone, which decreased steadily over the next 6 minutes (Fig. 4.3 and 4.4). This contrasts with venous pO_2 levels (Fig. 4.5), which rose immediately post-exercise and peaked at 3 minutes. Given that levels of exercise and post-exercise hyperaemia are widely considered to be tightly coupled to metabolic demand (Clifford & Hellsten, 2004), the finding that the level of FBF is more than adequate to meet the O_2 demands of the muscle suggests that, in the post-exercise period, pO_2 is not a factor in mediating FBF. Secondly, the disconnect in the time course of changes in FBF and pO_2 suggests that alterations in pO_2 are not obligatory for changes in FBF. This refutes our second hypothesis, although it supports the present findings that there is no O_2-dependent mediator of FBF, discussed further below. In contrast to venous pO_2, the peak changes in venous pCO_2, pH and lactate concentration all occur immediately post exercise, indicating that anaerobic metabolism is occurring within the skeletal muscle.

Contrary to our third hypothesis, results of the present PGE metabolite assay indicates that exercise did not cause release of PGE from forearm muscle into the venous efflux. This contrasts with findings by Boushel *et al.* (2002) that PGE_2 has been shown to play a role in the hyperaemia of intense dynamic quadriceps exercise. In that study however, PGE_2 had no role in mediating the hyperaemia in lighter exercise, with the change in concentration of PGE_2

occurring between 30 and 45W of exercise. Therefore we can hypothesise that the 50%MVC used in the present study is below the threshold at which PGE_2 is released and mediates post-exercise hyperaemia. A further possibility is that an increase in PGE_2 occurs within the interstitium, but release into the venous efflux is not sufficient to detect.

4.4.2 Effect of hyperoxia on FBF and FVC in young subjects

In young subjects, breathing 40% O_2 during the period of contraction only had no effect on FBF or FVC after either static or dynamic handgrip exercise at 50% MVC for 2 minutes. This is consistent with results using dynamic leg exercise at 40% MVC (MacDonald *et al.*, 2000), where subjects were also classified as physically untrained by the researchers. In that study, leg blood flow measured by doppler ultrasound was unchanged by either hyperoxia at 70% O_2 or hypoxia at 14% O_2 compared to the response in normoxia.

However, the findings of the present study and that of MacDonald *et al.* (2000) contrast with those of other groups (summary of all studies assessing effect of altering O_2 can be found in Table 1.1). Briefly, researchers assessing the effect of hyperoxia on the hyperaemia in leg exercise at 55-70%MVC (Welch *et al.*, 1977) and 70%MVC (Pedersen *et al.*, 1999) found a modest decrease in hyperaemia of 11-12% whilst using higher levels of O_2 than the present study. In the forearm, decreases in post-exercise hyperaemia of 25-40% have been shown when administering 40%O_2 to subjects performing static handgrip exercise at 100%MVE until exhaustion (Fordy, 2007) and 60%MVC for 2 minutes (Win & Marshall, 2005).

When analysing the exercise intensities used in the literature, it seems likely that part of the discrepancy between findings is due to levels of exercise performed. In the studies (including our data presented herein) finding no effect of supplemental O_2 on blood flow, exercise intensity was 50%MVC or lower. Conversely, all of the studies that reported an effect of hyperoxia used exercise intensities greater than 60%MVC. On this basis we can hypothesise that in intense exercise, an O_2 deficit occurs which leads to the production of O_2-dependent mediators of hyperaemia. Supplemental O_2 would alleviate this deficit and thus reduce production of an O_2-dependent vasodilator. In less intense exercise this O_2 deficit does not occur, therefore supplemental O_2 has no effect. Based on the previous literature and the current data, we can hypothesise that the threshold at which this occurs is greater than 50% MVC.

A further explanation for the opposing observations seen in the literature may be differences in the training status of the subjects used in the various studies. The present study and that of MacDonald *et al.* (2000) both used sedentary, untrained subjects. The above studies by Welch and Pedersen both stated that they used active subjects. Similarly, comparisons between the present study and that of Fordy (2007) suggest a difference in training status between subjects. For example, in the present study, the mean force exerted at MVC was ~33kg and forearm circumference was ~26cm in subjects with a mean bodyweight of 70kg. In comparison, the subjects used in a Fordy's study (Fordy, 2007) had a mean forearm circumference of 28.4cm, mean bodyweight of 74kg and force exerted at MVC was 50kg. Anthropometric measurements performed on male cadavers suggests that forearm circumference is highly correlated with

total muscle mass (Martin *et al.*, 1990). It therefore seems likely that the study population used by Fordy was more physically active than the population selected in this study. On the face of it the study by Win and Marshall (2005) seemed to follow an identical protocol to that described herein. Although the forearm circumference and weight were similar in the two studies, Win and Marshall (2005) made no report of the peak force obtained for the subjects or stated sports participation as an exclusion criterion. Furthermore, our pilot study showed that subjects were unable to hold 60%MVC for 2 minutes (Section 2.2.1), hence why we selected the 50%MVC in the current protocol. Thus it suggests that Win and Marshall (2005) investigated more active subjects than in the present study.

Evidence for an activity related difference in O_2 dependence can be seen in studies performed by Haseler *et al.* (1999; 2004) investigating the effects of supplementary O_2. Using magnetic resonance spectroscopy, they found that the rate of phosphocreatine resynthesis following submaximal rhythmic exercise was only increased by breathing 100% O_2 in active subjects and not in sedentary subjects. This implies that O_2-delivery to muscle is more than adequate to supply muscle activity in sedentary subjects, with the authors hypothesising that an increase in mitochondrial number and efficiency allowed trained subjects to make better use of supplementary O_2.

On the basis of the present data and the previous literature, we can hypothesise that in active subjects with more efficient muscles, supplementary O_2 can be utilised to reduce production of vasodilator metabolites which are produced during normoxic exercise; in less active or sedentary subjects, the reduced

efficiency of the muscle means that the supplementary O_2 can not be utilised and so the increase in vasodilatation is not affected. This could also tie in with the suggestion made above, that force of contraction could explain some of the differences in findings between the present and previous studies, if there is a threshold of intensity above which forearm muscles become O_2-dependent.

A further consideration with the present study is the success of supplemental 40% O_2 in relieving muscle hypoxia. Whilst haemoglobin O_2 carriage is unlikely to be affected significantly by supplemental O_2, going from 98-99% to 100%, the main effect of hyperoxia in the present methodology is likely to be the increase in P_aO_2 from ~98 to 240mmHg (Ekblom *et al.*, 1975; discussion of methodology in Win & Marshall, 2005). On the basis of this, previous work from this laboratory hypothesised that any effect of supplemental O_2 was at the level of the endothelium (Win & Marshall, 2005). Following recent data showing an O_2 gradient from blood to skeletal muscle (Richardson *et al.*, 1995; 2001; 2006) the present study hypothesised supplemental O_2 acted by ameliorating hypoxia within the skeletal muscle (see Figure 1.1 for possible sources of prostaglandins). Whilst there were no direct measurements of PO_2 within the skeletal muscle in this study, peak venous lactate or H^+ levels seen post-exercise could be used as a surrogate marker of hypoxia. It is of note that administration of supplementary O_2 had no significant main effect on either of these values, suggesting that O_2 delivery to the skeletal muscle was not sufficient to ameliorate any hypoxia within the muscle. Further evidence for this can be found in results shown in Chapter 3. In that study the change in tissue haemoglobin O_2 saturation during exercise at 100%MVE was not significantly

altered by breathing 40% O_2 during contraction compared to room air, as measured using Near Infra-red Spectroscopy. This would appear to demonstrate that despite findings of an increased O_2 gradient from blood to muscle tissue (Richardson *et al.*, 1995; 2001; 2006), increasing the P_aO_2 in the present manner does not significantly affect tissue oxygenation. Thus, it would seem that our second hypothesis was incorrect and that effects of hyperoxia seen in previous studies may have been due to increased O_2 delivery to the endothelium and not to the skeletal muscle. The methodology in the current study cannot rule out the presence of an O_2-dependent mediator produced from within the muscle fibres as supplemental O_2 did not reach the level of the muscle.

4.4.3 Effect of COX inhibition on FBF and FVC

In contrast with our first hypothesis, COX inhibition with oral aspirin had no effect on either FBF or FVC immediately following either static or dynamic exercise. These findings agree with previous investigators who have found no effect of COX inhibition. For example, Shoemaker *et al.* (1996) found no effect of oral ibuprofen on the vasodilatation caused by dynamic handgrip at 10%MVC and Mortensen *et al.* (2007) observed no effect of intra-arterial infusion of indomethacin during light dynamic knee extension, although there was an effect when combined with NOS inhibition.

However, other studies have found differing results (summarised in Table 1.2) Vasodilator prostaglandins such as PGI_2 and PGE_2 have both been implicated in mediating exercise hyperaemia due to the observed increase of both in the venous efflux (Karamouzis *et al.*, 2001a; 2001b). Kilbom and Wennmalm (1976)

were the first to use indomethacin to reduce post-exercise hyperaemia following 5 minutes static exercise at 15% MVC. Later, Wilson and Kapoor (1993) observed an attenuation of the post-exercise hyperaemia in response to dynamic wrist exercise to the magnitude of ~20%, also using ibuprofen. Similar findings have been made using a similar exercise protocol by another group using aspisol (Duffy *et al.*, 1999) and an attenuation of post-exercise hyperaemia of approximately 35% has been observed using oral aspirin (Win & Marshall, 2005).

As discussed in the General Introduction (Section 1.2.3.3), recent research has suggested that these discrepancies in the literature may be caused by differences in the intensity of the exercise used and the timing of interventions. Evidence for a role of vasodilator prostaglandins in exercise and post-exercise hyperaemia of intense exercise but not in less intense exercise has been discussed above in section 4.4.1. In terms of timing of drug administration, Schrage *et al.* (2004) showed that intra-arterial administration of the COX inhibitor ketorolac during exercise resulted in a transient 12% attenuation of exercise hyperaemia which was recovered by 2 minutes post-infusion. When designing the protocol for the present study, the 30 minute lag time between oral aspirin administration and full COX inhibition was not considered an issue in light of the previous findings from our group (Win & Marshall, 2005). However, given that vasodilator mechanisms to exercise appear to be different in the present group than previous groups studied in this laboratory, it may be the case that any contribution of vasodilator prostaglandins has been compensated for by another mechanism here.

In light of the current findings and other studies in the literature, it would seem that vasodilator prostaglandins do not play an obligatory role in mediating the hyperaemia immediately post-exercise, although on the basis of previous literature they may play a role in exercise of an increased intensity.

4.4.4 PGE metabolite assays

Although PGE_2 was the target prostaglandin in the present study, 90% is broken down in the first pass through the lungs (Granstrom *et al.*, 1980; Bothwell *et al.*, 1982). As a result we assayed levels of the stable PGE breakdown products. Our control resting levels of PGE metabolites were comparable to the values reported by Granstrom *et. al.* (1980), thus validating our technique for collecting and assaying venous plasma samples.

30 minutes following oral administration of aspirin, the present study found a non-significant fall in levels PGE metabolites compared to control exercise. However, the ~32% decrease in resting PGE metabolites seen in young subjects is not as great a decrease as that seen in previous studies by other groups. Wilson and Kapoor (1993) demonstrated a near abolishment in venous efflux of PGE_2 following intra-arterial indomethacin. Similarly, oral indomethacin has been shown to cause a decrease in interstitial PGE_2 from 4.3 to 0.4ng.ml^{-1} in the vastus medialis (Boushel *et al.*, 2002). It is thus unclear whether aspirin has had the desired effect of reducing prostaglandin production in normoxia. However, the protocol of aspirin administration that we used has previously been shown to lead to complete COX inhibition 30 minutes post-administration (Heavey *et al.*, 1985). Further, the same protocol has also been shown to have an effect on blood flow (Win & Marshall, 2005). Given the previously shown

efficacy of the current aspirin administration, we propose that it is likely that the smaller decrease in PGE metabolites is due to the longer half-life of the more stable breakdown products, rather than failure of COX inhibition. In light of this we can question the appropriateness of assaying a stable PGE metabolite in this protocol as it would seem likely that dynamic changes in PGE release from forearm skeletal muscle may be missed due to the high background presence of stable breakdown products from tonic PGE_2 production.

Contrary to our hypothesis, we found no change in the levels of PGE metabolites in plasma following static exercise compared to baseline in either young or older men. This contrasts with work Wilson and Kapoor (1993) in which they demonstrated a fivefold increase in forearm release of PGE_2 following dynamic wrist flexion at 0.2 and 0.4W. Similarly, more recent work from other groups has shown an intensity-related increase in PGE_2 in muscle interstitium following exercise. Karamouzis and colleagues (2001a) used radioimmunoassay to measure levels of PGE_2 in 30 minutes worth of microdialysis fluid from the interstitium of the vastus lateralis. They found that there was an increase in PGE_2 in fluid collected over 30 minutes of cycling exercise at 100W compared to fluid collected at rest. Additionally, they showed that the levels of PGE_2 measured increased further when exercise was performed at 150W. Another study from the same group also observed that increases in PGE_2 are dependent on the intensity of exercise (Karamouzis *et al.*, 2001b). That study observed no increase in PGE in microdialysis fluid collected from the gastrocnemius (calf) during intermittent static calf exercise designed to mimic walking, and reported as being a light exercise protocol.

However, the same study reported an increase in PGE_2 in the interstitium of the vastus lateralis during single knee leg extension exercise, used as a more severe exercise protocol.

A similar graded release of PGE_2 with increasing intensity has also been reported by Boushel *et. al.* (Boushel *et al.*, 2002). They again measured PGE_2 from the muscle interstitium (vastus lateralis and medialis) using radioimmunoassay. Following knee extensor exercise, they observed no change in PGE_2 from baseline following moderate exercise at 15 and 30W, but there was a significant increase following exercise at higher intensities (45 and 60W). Unfortunately they did not report the maximum values subjects were able to achieve. Thus, we can hypothesise that the 50% MVC exercise used in the present study was not of sufficient intensity to produce a measurable increase in PGE metabolites in the forearm venous efflux. Alternatively, as discussed above, any changes in PGE release from forearm muscle may be hidden by the background levels of stable PGE metabolites.

Finally, the present study found no effect of supplementary O_2 on PGE metabolite levels. Considering the lack of effect of supplementary O_2 on FBF and the lack of change in PGE metabolites post-exercise, we can conclude that PGE_2 does not play an O_2 dependent-role in mediating vasodilatation in exercise in the present protocol.

A further question that can be raised is the possibility that COX inhibition affected the production of COX products other than PGE_2, such as the vasodilator PGI_2 or vasoconstrictor thromboxane (TXA_2). Although neither of these substances were directly assayed, it seems unlikely that they were

significantly affected, as there were no changes in either resting or post-exercise FBF following aspirin compared to placebo. We also consider it unlikely that aspirin could have had simultaneous effects on the cytochrome P450 pathway, a source of EDHF. Work investigating the contribution of EDHF in the human forearm used the cytochrome P450 inhibitor miconazole to demonstrate the presence of EDHF dependent vasodilatation (Halcox et al., 2001). Importantly for this study, this was performed in the presence of COX inhibition with aspirin, demonstrating that aspirin does not inhibit the cytochrome P450 pathway.

4.4.5 Effect of ageing

We hypothesised that prostaglandins would play a reduced role in mediating post-exercise hyperaemia in older subjects compared to young subjects. Our data support this hypothesis in that we also found no role for them in older subjects. Also of interest is the observation that the magnitude of hyperaemia is not significantly different from that observed in the younger subjects.

Previous literature concerning the effect of ageing on the gross changes in exercise hyperaemia gives mixed results and is discussed in the general introduction (section 1.2.4). In human leg muscle there is a general consensus that exercise hyperaemia is reduced with ageing (Proctor *et al.*, 1998; Poole *et al.*, 2003; Donato *et al.*, 2006). Current data concerning the effect of ageing on forearm exercise hyperaemia is more equivocal, with Jasperse *et. al.* (1994) concluding that the maximum vasodilator capacity was not altered with age. In the same study, different intensities of exercise were also compared and no differences in blood flow were seen at any workload from 7-80%MVC. Similarly

the study by Donato *et al.* (2006) that observed attenuated blood flow in the leg found no evidence was found of a similar age-related decline in exercise hyperaemia in the forearm. In contrast, Carlson *et al* (2008) observed an attenuated peak blood flow in older subjects at intensities greater than 10%MVC.

The present study concurs with the evidence that there is no age-related change in post-exercise hyperaemia in the forearm, after either static or dynamic handgrip. The difference between the findings from the present study and those of Carlson *et al.* (2008) are most likely down to the significant differences in the methodology. They used single 1 second contractions in order to isolate local vasodilator factors. As well as a reduced peak vasodilatation, they also observed a reduced immediate vasodilatation at intensities greater than 10%MVC. Therefore we can hypothesise that the when forearm exercise is prolonged as in the present study and others (Jasperse *et al.*, 1994; Donato *et al.*, 2006), any immediate age-related impairment has been overcome.

The current finding that vasodilator prostaglandins do not play a role in older subjects supports previous results from other groups (Schrage *et al.*, 2007; Crecelius *et al.*, 2010). In the study by Schrage *et al.* (2007), older subjects performed dynamic handgrip exercise at 10 and 20%MVC. They found that COX inhibition with intra-arterial infusion of ketorolac had no effect on exercise hyperaemia, either given singly or in combination with the NOS inhibitor L-NAME. This contrasted with their previous data in young subjects demonstrating a transient ~12% decrease in exercise hyperaemia when ketorolac was infused. Thus they concluded that any vasodilator effect of

prostaglandins in exercise was completely abolished with ageing. This is consistent with the present findings in older subjects.

4.4.6 Critical evaluation of experimental protocol

With the benefit of hindsight, there are a number of aspects of this study that could be improved upon in future work. These aspects are considered below along with possible solutions.

The first limitation when comparing the results of the present study with previous work concerns the activity level of subjects. As discussed in 4.4.1 both the previous studies from this laboratory, along with the present study, have attempted to recruit “recreationally active” subjects, a factor also common to studies of other groups. The problem with this classification is clear from the discussion above, in that the strength and activity levels of subjects are clearly different in different studies. This issue was also highlighted in a recent presentation by Mortensen from the Copenhagen Muscle Research Centre, in which it was acknowledged that recreationally active subjects recruited in Copenhagen largely commute by bicycle and therefore tend to be fitter than subjects recruited as “recreationally active” in countries such as the UK and US (Muscle blood flow regulation in small and large muscle mass exercise; a role of age and training status. Session 412, Experimental Biology, Washington 2011). One method of accurately assessing a subject’s activity would be to use a physical activity questionnaire such as the Minnesota Leisure Time Physical Activity Questionnaire (MLTPA). This requires subjects to answer detailed questions about their activity in the last week, both recreational activity and daily activities of living. Utilisation of the MLTPA questionnaire was considered when

designing the current protocol, but was rejected in favour of the simpler questionnaire in Appendix 1 due to the time taken to answer it and the inapplicability of some of the questions to a UK population. An alternative to using a subjective questionnaire would be to use a device such as a pedometer or an accelerometer to record activity in the previous week to obtain an objective measure of the amount of activity a subject was performing. However, this would be especially useful if assessing leg blood flow and likely of more limited value in the study of the forearm. Disadvantages would be the time taken to get accurate results and issues with subject compliance.

A second issue regarding activity level concerns possible discrepancies between the activity level of subjects at the time they participate in the study and their previous training history. Whilst this was not considered as an issue when designing the present study, in view of the disparity between the present results and those of Win and Marshall (2005), future studies should probably assess lifetime training.

An issue encountered in the present study was difficulty recruiting suitable healthy older subjects. Initially, recruitment was intended to be limited to sedentary older subjects. However, subjects who were healthy enough to be included (i.e. had no cardiovascular disease or other health problems) tended to be those who were more physically active. Therefore it soon became apparent that the activity levels needed to be extended to include more active subjects if sufficient numbers were to be recruited, although this still resulted in a ratio of over 7 respondents per eligible subject. This can be seen from the subject characteristics (Table 4.1). When comparing findings between young and older

subjects, it must be noted that the older subjects are more active as a group than the younger subjects. This is also a point to consider in the future design of studies.

Concerning the protocol of the study, both trial experiments and questioning of subjects on completion of the study indicate that the blinding of gas and drug administration was satisfactory. In the present protocol, as in previous work from this laboratory, the administration of O_2 can only be described as supplementary due to the use of a Venturi system at a rate of 10 litres per minute. Whilst this clearly does not explain the lack of effect of breathing O_2 seeing as the same apparatus has had an effect in previous studies, utilising a reservoir bag would enhance the quality of the study by standardising the O_2 concentration given.

A limitation of the present study is the use of samples of venous blood only for measurement of muscle metabolites. One method of improving upon this would be to use simultaneous arterial blood sampling. This would allow both measurement of forearm O_2 consumption and give a more accurate measurement of metabolite production by subtracting the concentration in the arterial influx from that in the venous efflux. A different approach, widely used by the Copenhagen Muscle Research Centre, is to use microdialysis to measure the concentration of metabolites within the muscle interstitium. Disadvantages of both methods would be the increased invasiveness of the protocol.

Although using venous occlusion plethysmography to measure forearm blood flow has the advantage of being non-invasive and relatively simple to

administer, there were some limitations which became apparent in the present protocol. Firstly, the mercury-in-silastic strain gauge is extremely sensitive to any change in forearm circumference. This was an issue in the present study as subjects were often in some discomfort as a result of maintaining the handgrip and had to be reminded to remain still. It was also important to take into account the increase in forearm volume that occurred during exercise when calibrating the strain gauge, as if the resting voltage was set too high the reading could go off the scale during forearm blood flow measurement. A limitation of using venous occlusion plethysmography on 4 separate experimental days is that care must be taken to place the strain gauge around the same part of the forearm. An attempt to control for this in the present study was made by placing the strain gauge around the forearm at the point of the greatest circumference. Care also needed to be taken in placing of the cannula for venous blood sampling to avoid any interference with equipment touching the strain gauge.

4.4.7 Summary

In conclusion, the present study was novel in the use of both static and dynamic exercise to investigate the role of O_2-dependent prostaglandins in mediating the hyperaemia seen immediately post-exercise. We found no evidence for O_2-dependent mediators of post-exercise hyperaemia and no evidence that prostaglandins mediate post-exercise hyperaemia following handgrip exercise at 50%MVC. Our findings in older subjects support our hypothesis that levels of post-exercise hyperaemia would be the same in healthy older subjects as in younger subjects. Our data do not support a role for PGE_2 in mediating

vasodilatation after exercise, as concentrations assayed from venous plasma are unchanged after exercise compared to rest.

	Young (Mean±S.E.M.)	Older (Mean±S.E.M.)
Age	21.5±0.4	60.5±5.7*
Height (m)	1.79±0.02	1.77±0.02
Weight (kg)	70.09±5.7	80.4±3.0
BMI	21.9±1.5	25.8±1.0*
Forearm Circ. (cm)	25.7±0.7	26.1±0.5
Peak force (kg)	32.6±2.1	32.8±1.8
Heart rate (B.P.M)	73.7±3.2	64.9±2.1
MABP (mmHg)	90.6±4.2	96.5±2.3
Self reported activity	2.18±0.26	2.67±0.19 (p=0.075)
Hours activity/week	2.09±0.61	4.29±1.21

Table 4.1 Subject characteristics. * = p<0.05 vs. young group.

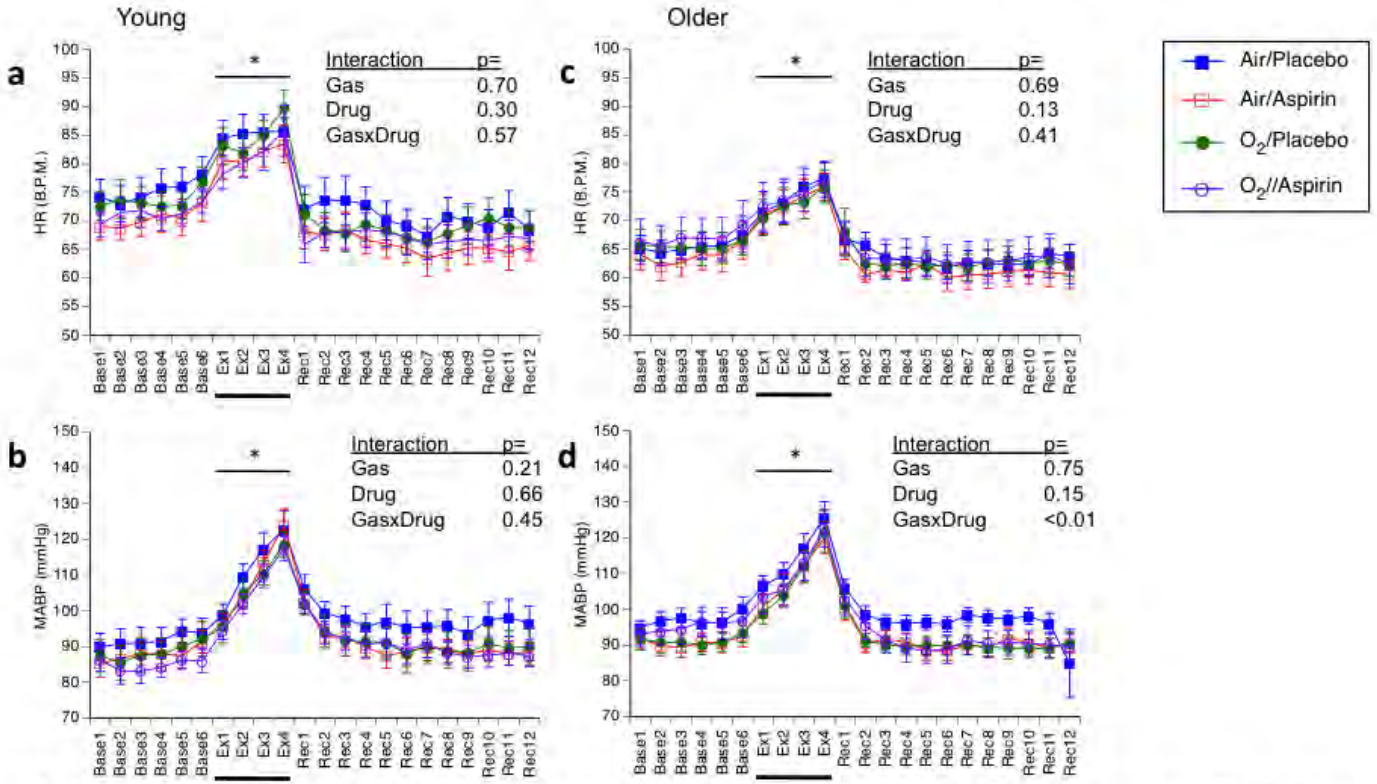

Figure 4.1. Heart rate and blood pressure before, during and after 2 minutes of static handgrip exercise. Graphs show mean ± S.E.M. over 30 second blocks from 3 minutes pre-exercise to 6 minutes post-exercise. Black bars indicate the period of exercise. Heart rate in young (**a**) and older (**c**) subjects rose above baseline within 30 seconds of exercise and remained there for the duration of exercise. MABP rose steadily over the period of exercise in both young (**b**) and older (**d**) subjects. Both HR and MABP returned to baseline within 30 seconds of ceasing exercise. * signifies $p<0.01$ compared to baseline, analysed with repeated measures ANOVA with special contrasts to group time points as baseline, exercise or recovery for statistical analysis.

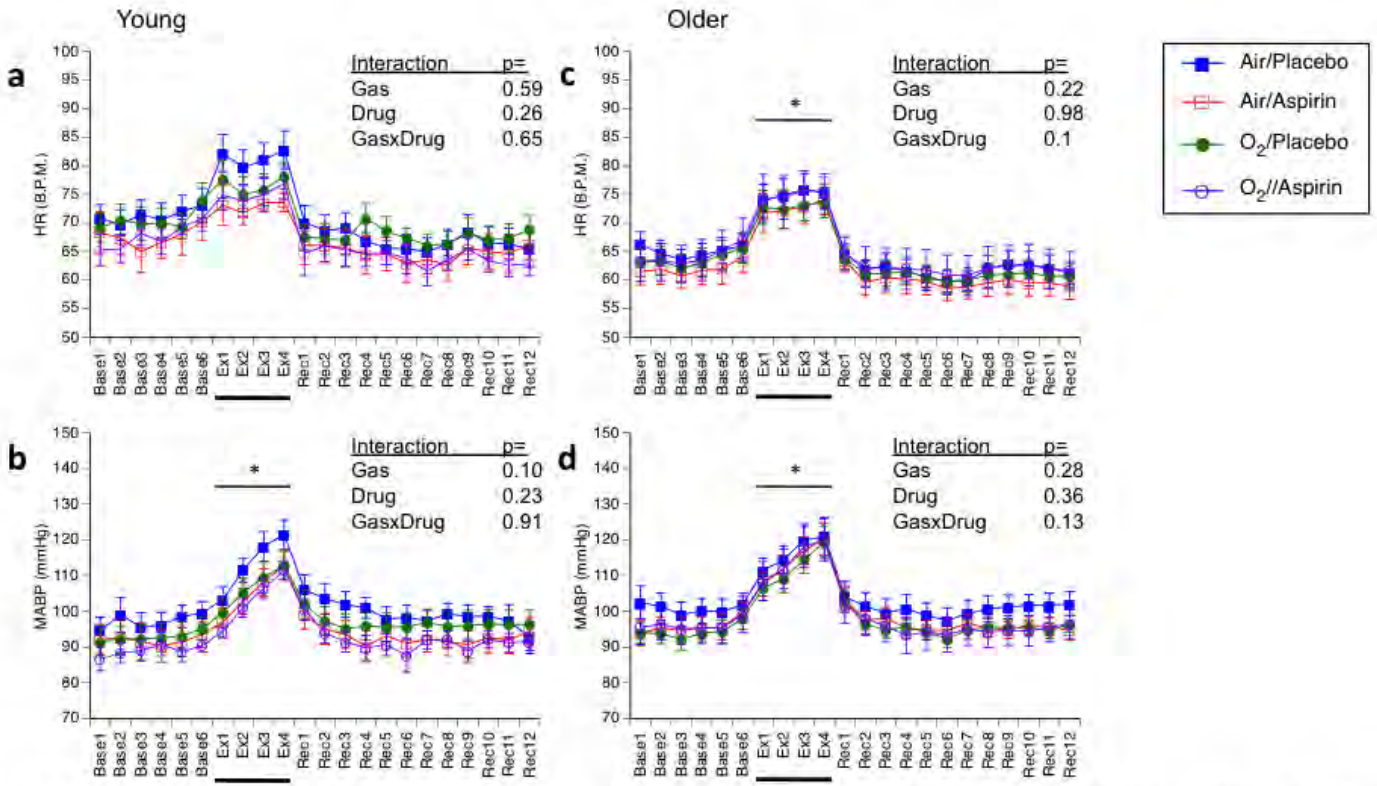

Figure 4.2. Heart rate and blood pressure before, during and after 2 minutes of dynamic handgrip exercise. Graphs show mean ± S.E.M. over 30 second blocks from 3 minutes pre-exercise to 6 minutes post-exercise. Black bars indicate the period of exercise. Heart rate in young (**a**) and older (**c**) subjects rose above baseline within 30 seconds of exercise and remained there for the duration of exercise. MABP rose steadily over the period of exercise in both young (**b**) and older (**d**) subjects. Both HR and MABP returned to baseline within 30 seconds of ceasing exercise. * signifies $p<0.01$ compared to baseline, analysed with repeated measures ANOVA with special contrasts to group time points as baseline, exercise or recovery for statistical analysis.

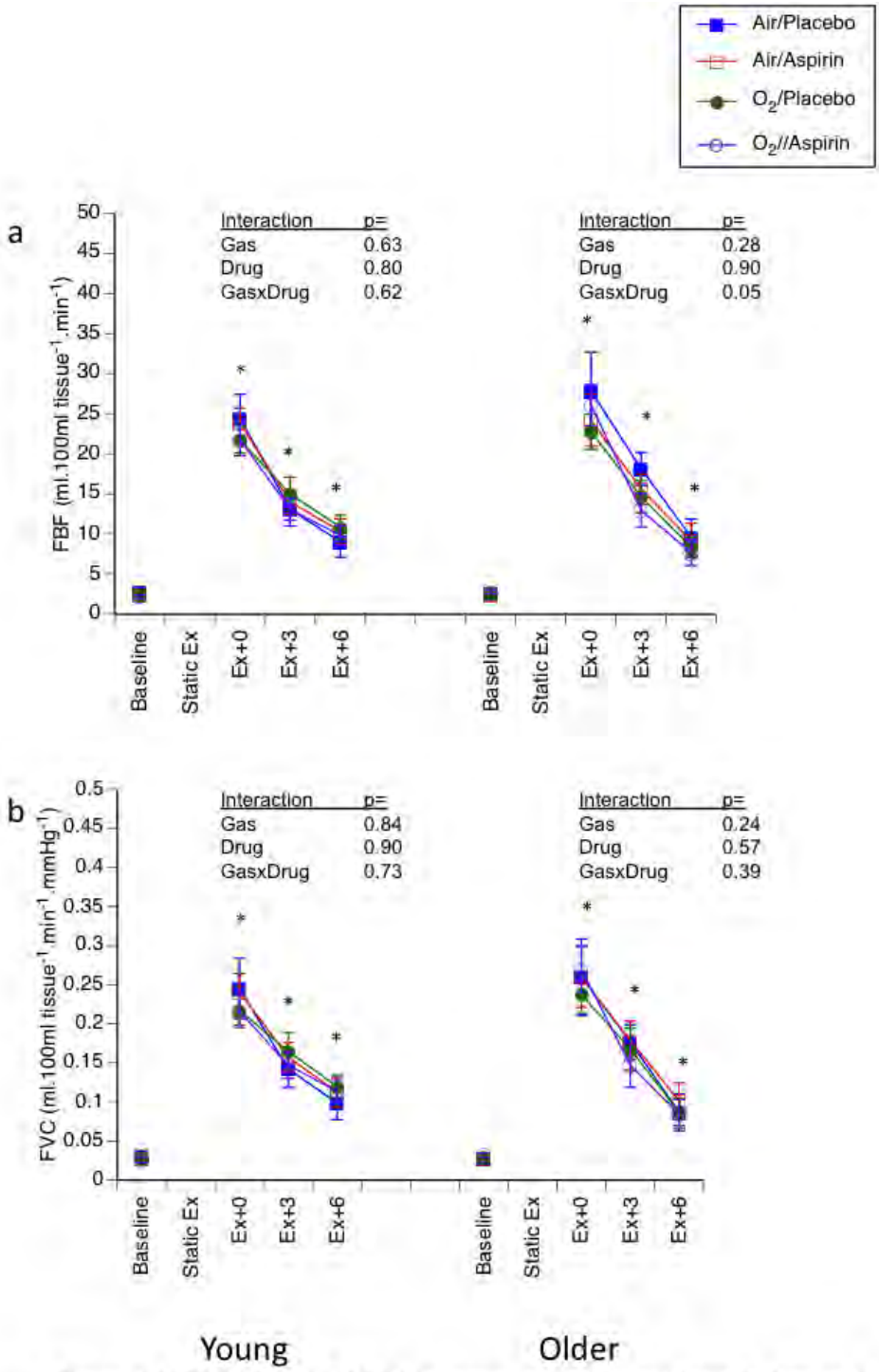

Figure 4.3. FBF (a) and FVC (b) in young and older subjects before and after 2 minutes of static handgrip exercise at 50% MVC. Both FBF and FVC increase significantly after exercise and then decline over the next 6 minutes, but remain elevated from baseline. n = 11 young, 11 older for FBF. n = 10 young, 7 older for FVC. * = $p<0.001$ compared to baseline.

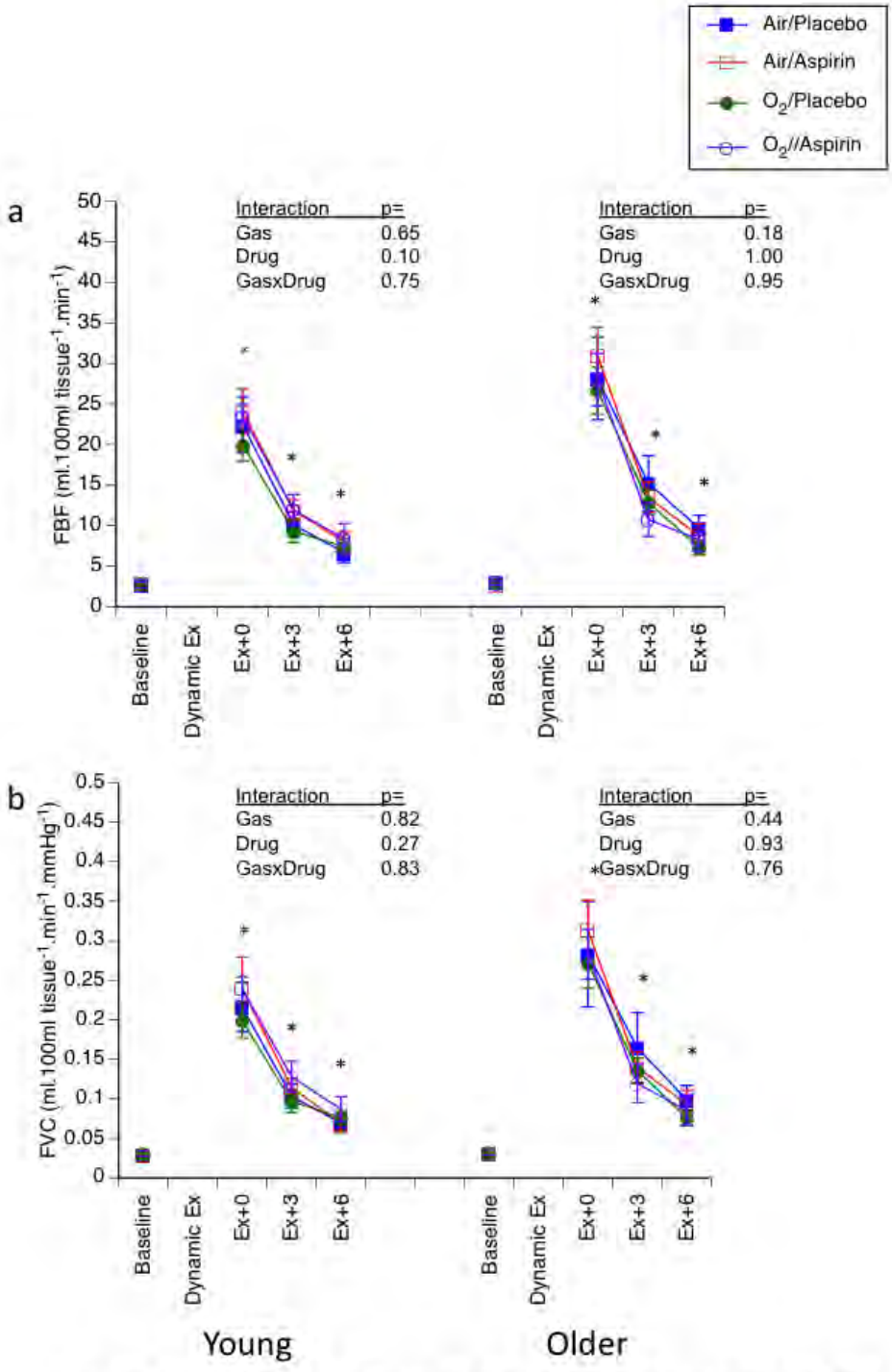

Figure 4.4. FBF and FVC in young and older subjects before and after 2 minutes of dynamic handgrip exercise at 50% MVC. Both FBF and FVC increase significantly after exercise and then decline over the next 6 minutes, but remain elevated from baseline. n = 11 young, 12 older for FBF. n = 9 young, 11 older for FVC. * = p<0.001 compared to baseline.

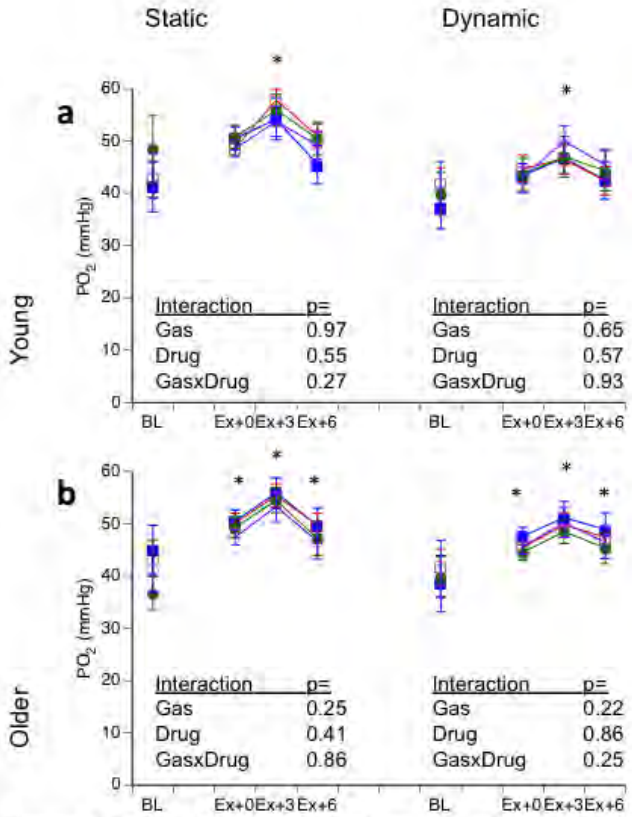

Figure 4.5a&b Venous pO_2 before and after static and dynamic exercise in young (a) and older (b) subjects. Venous pO_2 was above baseline at all points post-exercise and peaked at 3 minutes post-exercise. Data are mean ±SEM for 12 young and 12 older subjects, analysed using GEE. * = p<0.001 compared to baseline.

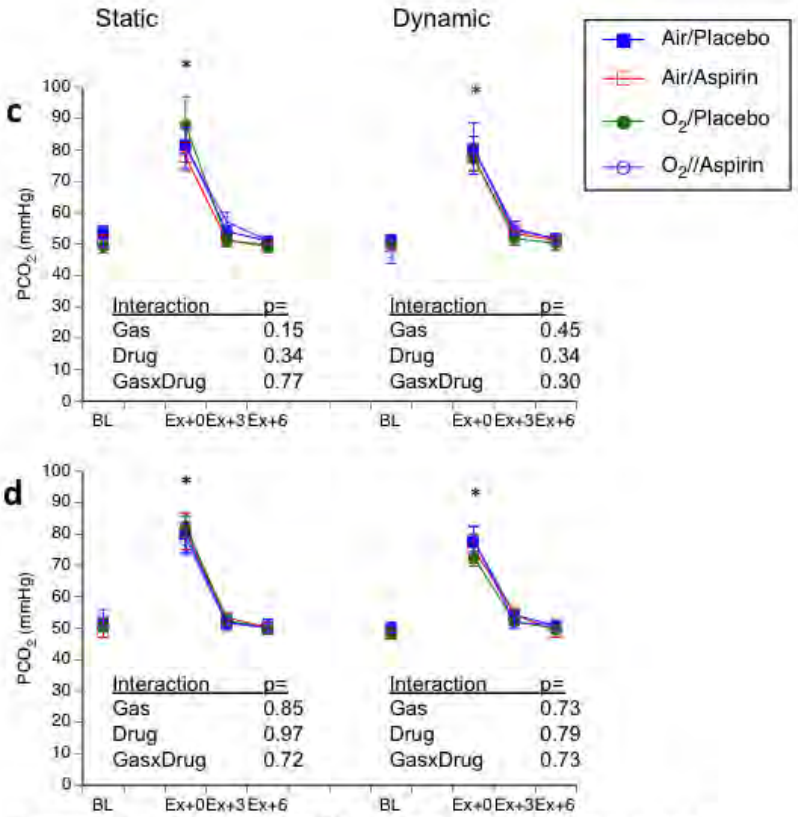

Figure 4.5c&d Venous pCO_2 before and after static and dynamic exercise in young (c) and older (d) subjects. Venous pCO_2 was above baseline immediately post-exercise and had recovered by 3 minutes post-exercise. Data are mean ±SEM for 12 young and 12 older subjects, analysed using GEE. * = p<0.001 compared to baseline.

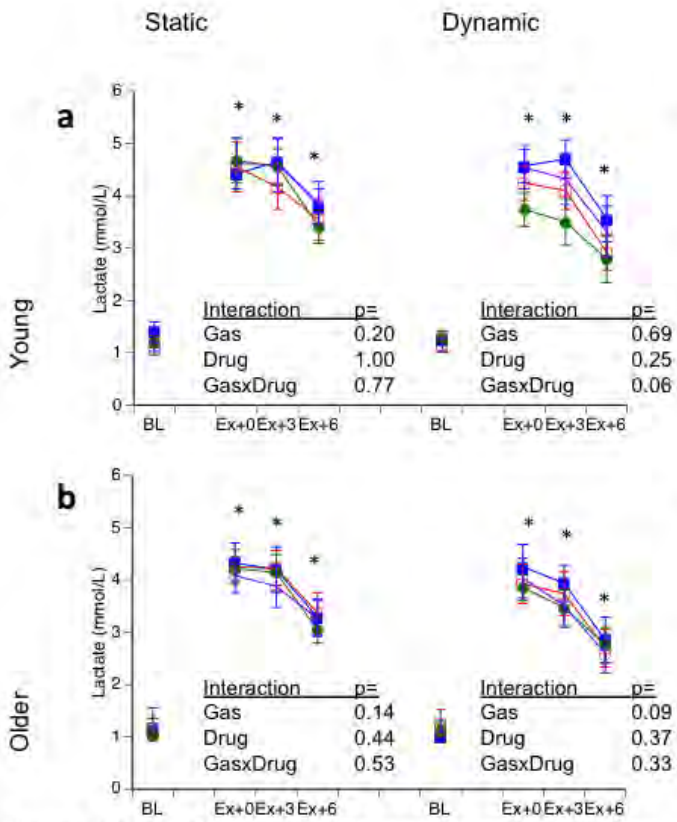

Figure 4.6a&b Venous lactate concentrations before and after static and dynamic exercise in young (a) and older (b) subjects. Lactate was above baseline levels at all points post exercise. Data are mean±SEM for 12 young and 12 older subjects, analysed using GEE. * = p<0.001 compared to baseline.

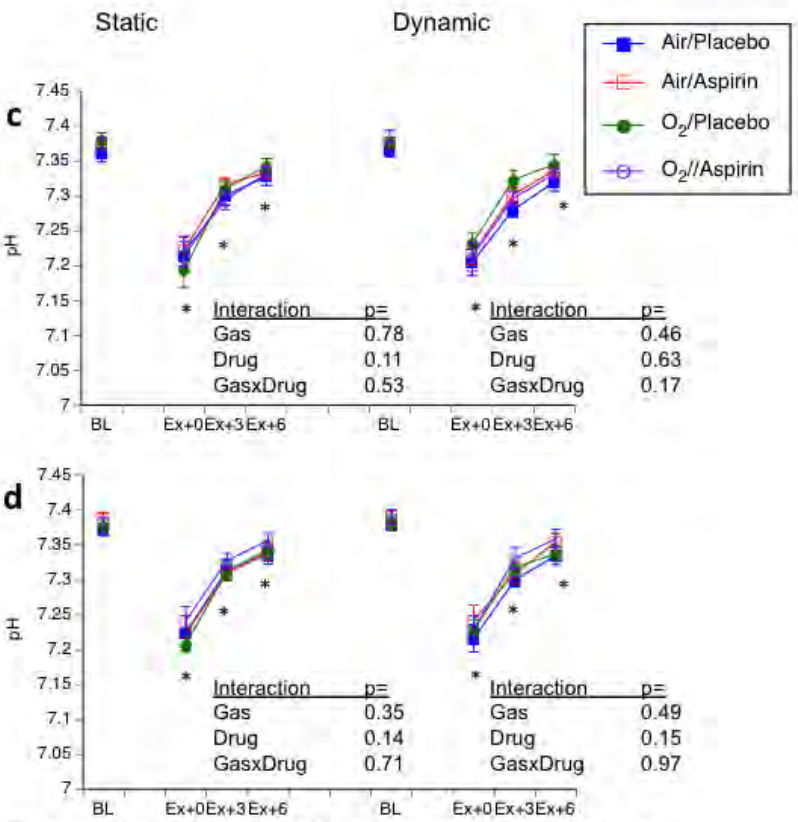

Figure 4.6c&d Venous pH before and after static and dynamic exercise in young (a) and older (b) subjects. Venous pH was below baseline at all points post-exercise and peaked immediately post-exercise. Data are mean ±SEM for 12 young and 12 older subjects, analysed using GEE. * = p<0.001 compared to baseline.

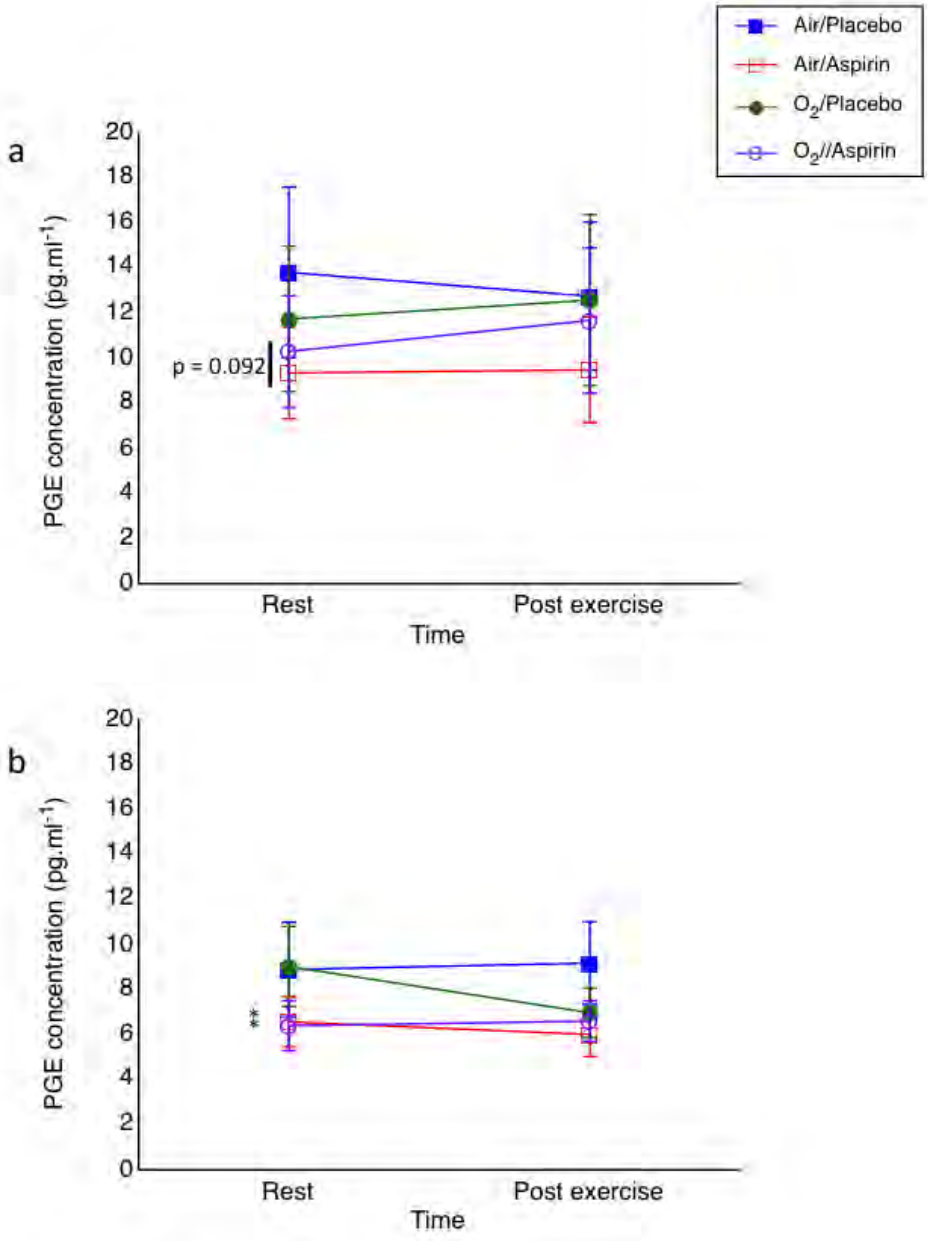

Figure 4.7. Venous PGE metabolite concentrations measured from venous serum samples taken before and after static exercise in young (a) and older (b) subjects. n = 9 young, 8 older. * = p<0.05 when compared to air/placebo control.

CHAPTER 5: THE O_2-DEPENDENCE OF ADENOSINE IN MEDIATING EXERCISE HYPERAEMIA

5.1 Introduction

In chapter 1.2.3.4 the evidence behind a role for adenosine in exercise and post-exercise hyperaemia was discussed. A role for adenosine in linking O_2 delivery and O_2 consumption was first proposed by Berne (1963) in relation to the coronary circulation. Since then, adenosine has been found to be increased in the interstitium of the exercising quadriceps muscle in humans to an extent which strongly correlated with the exercise hyperaemia (Hellsten *et al.*, 1998). Experiments performed in animals have generally concluded that 20-40% of the maintained phase of exercise hyperaemia and ~80% of the hyperaemia for 5 minutes post-exercise is attributable to adenosine (Marshall, 2007). Findings of a role for adenosine in mediating exercise hyperaemia have been corroborated in dynamic single leg extension exercise in humans by using theophylline, the adenosine receptor antagonist available for use in humans (Radegran & Calbet, 2001). Additionally it has also been shown in human forearm muscle that the vasodilatation during dynamic handgrip exercise is partly mediated by adenosine at 21% MVC, but not at lower intensities (Martin *et al.*, 2006b). For a summary of human studies see Table 1.3.

Recently it has been hypothesised that the separate mechanisms responsible for mediating exercise hyperaemia are interdependent (discussed in 1.2.3.5). With respect to adenosine mediated vasodilatation in response to exercise, researchers in Copenhagen have recently provided evidence that adenosine may primarily act via increasing levels of vasodilators such as prostaglandins and NO (Mortensen *et al.*, 2009b; Nyberg *et al.*, 2010). Specifically, they propose that adenosine in the interstitium is more important in exercise

hyperaemia than the adenosine in plasma and that in exercise interstitial adenosine acts on endothelium to increase prostacyclin production by the endothelium.

To date, the O_2-dependence of adenosine in exercise hyperaemia has not been investigated. Berne's original 'adenosine hypothesis' (1963) proposed that interstitial adenosine increased due to a lack of ATP regeneration when there was an insufficient O_2 supply. In light of the data described above in which interstitial adenosine is implicated in the vasodilatation of exercise, we can hypothesise that adenosine release into the interstitium is greater when O_2 supply and demand are mismatched (see Figure 1.2). Further, in light of evidence for O_2-dependence of vasodilator prostaglandins by Win and Marshall (2005), a second mechanism through which the role of adenosine could be O_2-dependent may be via decreased adenosine mediated prostacyclin release.

5.1.1 Hypotheses

1. In recreationally active young men, adenosine contributes to post-exercise hyperaemia in an O_2-dependent manner following both static and dynamic handgrip exercise.
2. This O_2-dependence is mediated by hypoxia within the skeletal muscle.
3. The contribution of adenosine to mediating post-exercise hyperaemia is reduced in older men.
4. The magnitude of hyperaemia is not altered by ageing.

We tested these hypotheses by measuring blood flow before and after handgrip exercise in healthy young and older subjects. Over four separate visits subjects

were randomly assigned to adenosine receptor antagonism with aminophylline or no antagonism and breathed either 40% O_2 or room air through a facemask during the period of exercise only. Venous blood samples were taken to measure venous blood gases.

We anticipated that both aminophylline and breathing supplementary O_2 would reduce the magnitude of post-exercise hyperaemia in young subjects but not in older subjects. We anticipated that ameliorating hypoxia within skeletal muscle with supplementary O_2 would result in a reduction in the venous concentrations of lactate and hydrogen ions.

5.2 Methods

Initially it was planned to recruit 12 young and 12 older subjects as for the study assessing the O_2-dependence of prostaglandin mediated exercise hyperaemia. A similar protocol to that described in chapter 4.2 was used, with the oral aspirin replaced with intravenous infusion of aminophylline as described below. Both studies were carried out concurrently with interested volunteers initially being recruited for aminophylline infusion and then being offered the less invasive aspirin administration if they felt uneasy about an I.V. drug infusion. When the Queen Elizabeth hospital moved site in the summer/autumn of 2010, it was felt that the Department of Cardiovascular Medicine where studies took place was no longer adequately covered by the medical support, thus infusion of aminophylline was no longer ethically safe. At this point 7 young subjects (21.0±0.7 years; mean± S.E.M.) and 4 older (63.3±0.5) subjects had completed all 4 study visits, and 3 young subjects had completed 2 or 3 visits of which one was the air/no infusion control visit. Due to the surprising finding that subjects

taking part in the prostaglandin study did not exhibit any O_2-dependence, it was decided to analyse the data from these subjects; on the basis of these results, described below, it was decided that it would be unethical to continue with aminophylline infusions.

As with the protocol described in chapter 4.2, subjects attended the laboratory on 4 separate days, at least a week apart. On the first visit to the laboratory, the subject was familiarised with the equipment and given an opportunity to ask any questions. Subjects were set up and MVC was measured on the first visit as described in chapter 2. Either 40% O_2 or room air was breathed through a mask during the period of contraction only, for the rest of the protocol, subjects breathed room air without a mask. On 2 visits, subjects had a 22gauge blue cannula inserted into a vein in the ante-cubital fossa of the non-exercising arm. This was then used to infuse aminophylline at a dose of 5mg/kg body weight (diluted in 40 ml saline) slowly over 20 minutes. This dose was chosen as it is the maximum safe loading dose used clinically (British National Formulary 49, March 2005). On completion of infusion, subjects rested for 10 minutes. On non-infusion days, subjects rested for 30 minutes. The order of all interventions was randomised in a single-blinded manner, such that upon completion of the study all subjects had received air alone (i.e. control), air/aminophylline, O_2 alone and O_2/aminophylline.

Prior to commencing exercise, a venous blood sample was taken and three recordings of baseline FBF were made by venous occlusion plethysmography. The facemask was then fitted to the subject although the gas was not switched on at this point. The subject began the period of static exercise at 50% MVC for

two minutes whilst breathing the experimental gas mixture through the facemask. Exercise was timed to begin 10 minutes after cessation of the aminophylline infusion to keep the 30 minute total rest on the couch consistent with the study described in Chapter 4. Immediately after the subject released the dynamometer another recording of FBF was taken. A venous blood sample was taken immediately following the measurement of FBF (approximately 10-15 seconds after completion of the exercise). Further venous blood samples and FBF recordings were taken at 3 and 6 minutes after the end of the exercise. 30 minutes following the first period of exercise this protocol was repeated with the subject performing two minutes of rhythmic exercise at 50% MVC, at a rate of 1 second contraction/1 second relaxation. In addition to the above blood samples, on 2 visits blood samples were taken immediately prior to static and dynamic exercise (i.e. approximately 10 minutes and 40 minutes post infusion) for measurement of plasma aminophylline levels. These assays were performed by the Biochemistry department at the QE hospital.

5.2.1 Analysis

Analysis was performed as described in section 4.2.1. Briefly, all data was analysed using SPSS for mac (version 17.0). Data are presented as mean ± S.E.M. FBF and FVC were analysed using 3-way repeated measures ANOVA with within-subject factors for gas breathed, drug intervention and time. Main factor effects are presented using Huyne-Feldt correction to control for any differences in variance between groups. In cases where significance was detected, Bonferroni adjustment was performed in order to locate where differences occurred. 3 comparisons were made for time (3 post-exercise

timepoints vs. rest), 1 comparison was made for gas (O_2 vs. air) and 1 for drug (aminophylline vs. no aminophylline).

Heart rate and mean arterial blood pressure were analysed using repeated measures ANOVA with special contrasts defined to perform 3 pairwise comparisons of the 3 groups of time points (baseline, exercise and recovery).

pO_2, pCO_2, pH and lactate were analysed using Generalised Estimating Equations to correct for data missing due to failure of cannulation (missing values for entire experimental visit) or malfunction of blood gas analyser (missing single values within visit). A table showing proportions of missing data points is included in Appendix 2.

Theophylline levels were analysed using a paired t-test.

Due to the low subject numbers in the older group, no statistics have been performed on the differences in ageing.

Tables showing data as Mean±S.E.M. are included in Appendix 4 for reference.

5.3 Results

5.3.1 Subject characteristics

Subject characteristics are shown in Table 5.1. Young subjects were aged 21.0±0.7 years old (mean ± S.E.M.) compared to 63.3±0.5 years for the older subjects. Young subjects rated themselves as less active on a subjective questionnaire (Appendix 1) and reported less hours activity per week than the older subjects. Both young and old subjects reported predominantly leg based activity.

5.3.2 Heart rate and MABP

The heart rate and MABP responses for both young and older subjects in response to 2 minutes handgrip exercise are shown in Fig. 5.1 and Fig. 5.2 (response to static and dynamic exercise respectively). In young subjects, there was a significant main effect of time on heart rate in static exercise ($p<0.001$), although there were no significant differences in pairwise comparisons of baseline, exercise and recovery. There was a significant increase in MABP from baseline during static exercise ($p=0.006$) and a significant decrease from exercise levels during recovery ($p=0.008$). There were no significant main effects of supplementary O_2 (heart rate $p=0.093$; MABP $p=0.794$), aminophylline (heart rate $p=0.811$; MABP $p=0.057$) or the two in combination (heart rate $p=0.849$; MABP $p=0.650$).

In dynamic exercise, both heart rate and MABP were significantly raised from both baseline and recovery (all main effect of time $p<0.001$; all pairwise comparisons $p<0.05$). As with static exercise there were no significant main effects of supplementary O_2, aminophylline or the two in combination.

Statistics were not performed on older subjects due to the early curtailment of the study but mean heart rate and MABP are shown for those 4 subjects in Figures 5.1c&d (static exercise) and 5.2c&d (dynamic exercise). It can be seen that older subjects show a similar pattern to younger subjects.

5.3.3 FBF and FVC

Figure 5.3a and b show the FBF and FVC respectively for both young and older subjects before and after static exercise. Static handgrip exercise at 50% MVC

for 2 minutes resulted in a significant rise in FBF measured immediately post-exercise in young subjects (Fig 5.3a; $p<0.001$). Figure 5.3a shows that FBF then falls from these peak values at 3 minutes and further at 6 minutes post-exercise, whilst remaining elevated from the resting baseline ($p=0.041$). In young subjects there were no significant main effects of breathing supplementary O_2 ($p=0.88$), taking aminophylline ($p=0.84$) or the two combined ($p=0.054$).

FVC behaved in a similar manner (Fig. 5.3b), although the differences from resting baseline at 3 and 6 minutes post-exercise were not significant. There were no significant main effects of supplementary O_2 ($p=0.57$), aminophylline ($p=0.27$) or the two in combination ($p=0.21$). The results for dynamic exercise for both FBF and FVC (Fig. 5.4a and b) were comparable to the static exercise protocol. Again there were no significant main effects of supplementary O_2, aminophylline or the two in combination.

Although the lack of older subjects means the study is underpowered, Figure 5.3 and 5.4 both clearly show that the pattern for older subjects is similar to the younger subjects.

5.3.4 Venous blood gas analysis

5.3.4.1 pO_2

Figure 5.5a&b show the values for venous pO_2 before and after static and dynamic exercise. In young subjects, resting pO_2 prior to static exercise (i.e. ~ 10 minutes post-infusion) was lower by ~12mmHg after aminophylline infusion (Fig. 5.5a; $p<0.001$). However, when resting pO_2 was measured prior to

dynamic exercise (approximately 40 minutes post infusion), there was no longer any significant difference between drug conditions (p=0.689).

Both static and dynamic exercise resulted in a peak venous pO_2 at 3 minutes post-exercise, such that pO_2 was significantly elevated from both baseline and from immediately post-exercise (Fig. 5.5a; $p<0.001$); pO_2 returned to baseline by 6 minutes post-exercise. In the static exercise protocol, there was no significant main effect of breathing supplementary O_2 (p=0.55), although aminophylline resulted in a significantly lower post exercise pO_2 ($p<0.001$). There was no significant main effect of supplementary O_2 and aminophylline in combination (p=0.07). In the dynamic exercise protocol there were no significant main effects of either supplementary O_2, aminophylline or both in combination (respectively p=0.99; p=0.85; p=0.76).

Although results must be treated with caution due to the low number of subjects (n=4), analysis using GEE indicates that venous pO_2 was significantly increased from all other time points at 3 minutes following both static and dynamic exercise ($p<0.01$). There were no significant differences between baseline and immediately post exercise. There were no significant main effects of supplementary O_2, aminophylline or both in combination in either the static or dynamic exercise protocol.

5.3.4.2 pCO_2

Figure 5.5c&d show the venous pCO_2 for young and older subjects respectively. In young subjects both modes of exercise resulted in a peak venous pCO_2 immediately post-exercise (i.e. p_vCO_2 reached a peak before p_vO_2). Following both static and dynamic exercise, pCO_2 returned to baseline by 3 minutes post-

exercise ($p<0.05$). There were no significant main effects of breathing supplementary O_2 (static $p=0.89$; dynamic $p=0.23$), aminophylline (static $p=0.49$; dynamic $p=0.62$) or both in combination (static $p=0.36$; dynamic $p=0.33$) in either protocol.

As can be seen in Figure 5.5d, pCO_2 in older subjects follows the same pattern as the younger subjects, being significantly raised immediately post exercise compared to all other time points ($p<0.001$), with the values at 3 and 6 minutes being fully recovered to baseline levels. Analysis by GEE showed that the increase in pCO_2 immediately post-dynamic exercise was significantly increased during aminophylline infusion ($p<0.001$), although this is likely an artefact due to the low numbers of subjects.

5.3.4.3 Lactate

Venous lactate concentrations are shown in Figure 5.6a&b. In young subjects (Fig. 5.6a) both static and dynamic exercise resulted in an increase in venous lactate concentrations immediately and 3 minutes post-exercise compared to the resting baseline ($p<0.001$), which began to recover by 6 minutes post-exercise but remained raised from baseline. In the static exercise protocol, there were no significant main effects of either supplementary O_2 ($p=0.21$) or aminophylline ($p=0.86$). There was a significant effect of both O_2 and aminophylline in combination ($p=0.03$) due to post-exercise lactate concentrations being increased in the O_2+aminophylline protocol. There were no significant main effects of any interventions in the dynamic exercise protocol.

Lactate concentrations changed in a similar manner in older subjects (Fig. 5.6b) after both static and dynamic exercise (all post exercise values $p<0.002$ from

baseline). There were no significant main effects of supplementary O_2, aminophylline or both in combination.

5.3.4.4 pH

Figure 5.6c&d shows the venous pH before and after static and dynamic handgrip exercise for young and older subjects respectively. In young subjects, pH decreased significantly immediately post-exercise before gradually recovering towards, but not reaching, resting baseline values (all time points $p<0.001$ c.f. all other time points). In the static exercise protocol, there were no significant main effects of supplementary O_2 ($p=0.95$), aminophylline ($p=0.16$) or both in combination ($p=0.23$). During the dynamic exercise protocol however, aminophylline had the effect of reducing the decrease in pH from resting baseline at all time points post-exercise ($p=0.02$). There were no significant main effects of supplementary O_2 or O_2 and aminophylline in combination.

The time course of changes seen in the older group (Fig. 5.6d) was the same as that seen in the younger group. Aminophylline had the overall effect of increasing pH compared to no infusion (static $p<0.001$; dynamic $p=0.027$).

5.3.5 Theophylline levels

Figure 5.7 shows venous theophylline levels measured before static and dynamic exercise (~10 and 40 minutes following infusion of $5mg.kg^{-1}$ aminophylline respectively). Mean levels were $11.4mg.L^{-1}$ prior to static exercise and and there was no significant change compared to levels measured prior to dynamic exercise ($p=0.074$).

5.4 Discussion

The studies presented within this chapter are novel in that they are the first to investigate the O_2-dependence of adenosine as a mediator of the hyperaemia seen immediately post-exercise and how this role changes with ageing. It is also novel in the investigation of both static and dynamic exercise. Despite being stopped early, this study has presented some interesting findings:

1. In contrast with our first hypothesis, breathing supplementary O_2 during both static and dynamic handgrip exercise had no effect on post-exercise hyperaemia.
2. Also in contrast to our first hypothesis, adenosine receptor blockade with an intravenous infusion of aminophylline had no effect on post-exercise hyperaemia following static or dynamic handgrip exercise.
3. Adenosine receptor blockade results in an acute reduction in resting venous pO_2, which appeared to be compensated for 40 minutes following blockade, despite theophylline levels not being significantly lower.
4. This study found little evidence of either aminophylline or supplementary O_2 altering metabolic activity during exercise, as evidenced by the lack of differences between blood gas measurements.
5. Despite being significantly underpowered, analysis of older subjects indicates no effects of ageing on post-exercise hyperaemia.

5.4.1 Effect of hyperoxia on FBF and FVC in young subjects

At the onset of the studies presented within this thesis, our overriding hypothesis was that in healthy recreationally active young subjects a

component of the vasodilatation seen following exercise was O_2-dependent. However, the results presented herein suggest that using the current methodology this is not the case.

Potential reasons for finding a lack of O_2-dependence were dealt with in section 4.4.1. In the present study the hyperoxia/no infusion experimental visit is analogous to the hyperoxia/placebo visit in Chapter 4 and as subjects were recruited in an identical manner the discussions in Section 4.4.1 are valid here. Briefly, the likely reasons for discrepancies within the literature are a difference in the activity levels of subjects or differences in intensities between different studies. The fact that the study presented in this chapter supports the findings of the data in Chapter 4 adds weight to the hypothesis that O_2 only plays a role in mediating post-exercise hyperaemia following intense exercise or in trained individuals.

As with the findings in Chapter 4, the present results show no effect of supplementary O_2 on peak venous lactate or pH measurements after exercise, which we were using as a surrogate of anaerobic metabolism. Therefore we cannot rule out the presence of an O_2-dependent substance being produced within the muscle fibres.

5.4.2 Effect of aminophylline on FBF and FVC in young subjects

In contrast with our first hypothesis, this study observed no effect of adenosine receptor antagonism with aminophylline on the hyperaemia seen after either static or dynamic handgrip exercise. This supports the findings of Martin *et al*. (2006b; 2007) in the forearm, which demonstrate that adenosine may not be an obligatory mediator of exercise hyperaemia. Firstly they showed that the

hyperaemic response to dynamic handgrip exercise at ~21% MVC was reduced by 15% when aminophylline was infused intra-arterially, although adenosine receptor blockade had no effect at lower intensities (Martin *et al.*, 2006b). Secondly, they demonstrated that increasing interstitial concentrations of adenosine, by using dipyridamole to block equilibrative nucleoside transporters, had no effect on the magnitude of the exercise hyperaemia (Martin *et al.*, 2007). This was even the case in non-responders in whom they demonstrated that equilibrative nucleoside transporter activity was enhanced leading to more rapid adenosine clearance. In contrast however, Radegran and Calbet (2001) observed a reduction in exercise hyperaemia of ~20% in subjects performing dynamic knee extension at ~50%MVC with adenosine receptor antagonism with an intravenous infusion of theophylline (studies summarised in Table 1.3). A limitation of the comparison between the current data and the studies mentioned above is the difference in methodology of measuring blood flow. Both Radegran and Calbet (2001) and Martin *et al.* (2006b) used Doppler ultrasound, thus measuring hyperaemia during exercise. In contrast, we used venous occlusion plethysmography, measures post-exercise hyperaemia, although the timescale of readings was designed to minimise this difference.

In analysing the results of the present study, we must consider the efficacy of aminophylline as an adenosine receptor antagonist. Radegran and Calbet (2001) used an intravenous infusion of theophylline at a dose of 6.9mg.kg^{-1} body weight. In the present study we used aminophylline, a more soluble analogue of theophylline, at a dose of 5mg.kg^{-1} body weight given over 20 minutes. This drug and dose was chosen on the basis of clinical guidelines and

availability of adenosine receptor antagonists in the UK. In the UK, theophylline is only available in oral preparations (BNF 49), while aminophylline is available for intravenous infusion (the addition of ethylenediamine to theophylline confers greater solubility in water). Intravenous infusion of aminophylline was chosen for the present study due to difficulty in obtaining a stable plasma level of theophylline when using oral administration (personal communication: Prof. Michael Frennaux). Regarding dosing of aminophylline, care must be taken due to the narrow margin between therapeutic and toxic doses. Due to clinical and ethical considerations, dosing in the present study was limited to the maximum loading dose recommended in clinical settings. When comparing the plasma theophylline levels obtained in this study (11.4 $mg.L^{-1}$) with values of 13.3$mg.L^{-1}$ (73.8μM) obtained by Radegran and Calbet (2001), it is apparent that the present study may have under dosed the aminophylline. Levels were not reported in the studies by Martin *et al.* (2006b; 2007), although it is worth noting that they used intra-arterial infusions of aminophylline, which may give a greater local adenosine receptor antagonism in the absence of systemic side-effects.

In addition, it has been demonstrated that aminophylline (and therefore theophylline) is not a complete inhibitor of adenosine receptors (Leuenberger *et al.*, 1999; Casey *et al.*, 2009). Leuenberger *et. al.* (1999) showed a maximum of 40% reduction in the vasodilatation caused by adenosine infusion. Similarly, Casey and colleagues (2009) found that aminophylline blunted the vasodilatation caused by infusion of adenosine by 60% at low doses of adenosine, with this decreasing to 30% inhibition with higher doses. Thus it appears likely that some of the discrepancies seen within the literature are due

to the absence of an effective adenosine receptor antagonist. Methods for ascertaining the level of adenosine receptor blockade are discussed in section 5.4.5.

It is also worth noting that the study by Radegran and Calbet (2001), whilst using a similar percentage of peak force as the present study, was performed using leg exercise. When compared to our findings and those of Martin *et al.* (2006b; 2007) that adenosine is not an obligatory mediator of hyperaemia, we can hypothesise that the increase in leg muscle mass compared to the forearm muscle mass results in a greater adenosine release for a similar workload. Therefore adenosine may be an important mediator in leg exercise hyperaemia, but not in forearm exercise hyperaemia.

However, an alternative interpretation could be placed on the present results when taken in the context of the findings of Chapter 4. Recent studies from the Copenhagen Muscle Research Centre have produced data that suggests that the actions of infused adenosine are dependent on prostaglandins and NO (Mortensen *et al.*, 2009b; Nyberg *et al.*, 2010). In Chapter 4 we showed that in the present group of subjects using these exercise protocols, vasodilator prostaglandins do not play a role in mediating post-exercise hyperaemia. Therefore we can hypothesise that the lack of prostaglandin-mediated vasodilatation following exercise in these subjects is consistent with the reduced contribution of adenosine, such that there was a reduction in adenosine-mediated prostaglandin release.

5.4.3 Effect of ageing

Whilst it is recognised that the premature cessation of the present study means any interpretation on the effects of ageing must be treated with caution, the data from 4 older subjects can be discussed in the context of the results above and in Chapter 4.

We hypothesised that older subjects have a decreased reliance on adenosine in mediating post-exercise hyperaemia. The present results from older subjects did appear to show that there is no adenosine-mediated vasodilatation, although this was also be the case with the younger subjects. In addition, the data presented in Chapter 4 showed no change in post-exercise hyperaemia between young and old subjects. Therefore it seems likely that had the group of older subjects in the present study been larger, we would still see no effect of aminophylline or hyperoxia and there would have again been no significant difference between young and older subjects.

5.4.4 Effect of adenosine receptor blockade on resting venous pO_2

The present study had the unexpected finding that infusion of aminophylline at a dose of 5mg. kg^{-1} resulted in an acute reduction in resting venous pO_2 measured ~10 minutes after cessation of the infusion. Interestingly, resting venous pO_2 recovered back to control levels when measured prior to dynamic exercise (~40 minutes post-infusion). Although this has not been reported in the human forearm to our knowledge, research in other species and organs supports this finding.

In 1950 Fottz *et al.* reported an increase in cardiac O_2 consumption in dogs following an infusion of aminophylline at a dose of 4mg.kg^{-1}. In the same study they observed that coronary venous pO_2 dropped and remained low for varying lengths of time up to 16 minutes. Similarly, 6mg.kg^{-1} of aminophylline has been shown to cause a decrease in jugular venous pO_2 of ~6mmHg in men with chronic obstructive pulmonary disease, despite causing no change in p_aO_2 (Nishimura *et al.*, 1992). They theorised that this may be due to either a decrease in cerebral blood flow, which they had not measured, or due to an increase in metabolic rate resulting in an increased O_2 uptake. In light of our findings in the present study that aminophylline caused no change in resting FBF, it would seem most likely that the alterations in resting venous pO_2 are due to an increase in metabolism.

This is supported by findings of other researchers that methylxanthines (which include caffeine and theophylline/aminophylline) increase metabolic rate (Dulloo *et al.*, 1989; Ide *et al.*, 1995). Dulloo *et al.* (1989) found that ingesting 100mg of caffeine increased the metabolic rate of lean human volunteers by ~4%. It was also shown that the increase occurred within 20 to 40 minutes and then began to recover to baseline levels. Given that the caffeine in that study was administered orally compared to intravenous infusion in the present study, this may explain the apparent recovery by 40 minutes that we observed. Further evidence supporting an increase in metabolism due to aminophylline infusion can be found in a study by Ide *et al.* (1995). They used magnetic resonance spectroscopy to assess levels of inorganic phosphate (Pi) and phosphocreatine (PCr) in piglet diaphragm muscle. They reported that aminophylline slowed the

increase in Pi/PCr ratio, whilst arterial pO_2 remained unchanged. They therefore concluded that aminophylline increased levels of aerobic metabolism.

In conclusion, our results show a novel finding of a transient decrease in venous pO_2. In light of research in other fields, it seems most likely that this is due to an increase in aerobic metabolism.

5.4.5 Critical evaluation of the protocol

Due to the large overlap between the methodologies used in the present study and those used in Chapter 4, limitations raised in section 4.4.4 are also applicable here.

Additional considerations of the present protocol concern the use of aminophylline as an adenosine receptor antagonist. As discussed above, it has been shown that aminophylline does not fully inhibit adenosine receptors (Casey *et al.*, 2009) and this may explain the apparent lack of adenosine mediated exercise hyperaemia in the present study. The efficacy of adenosine receptor blockade could be tested by directly infusing adenosine into the brachial artery and compare the vasodilatation before and after aminophylline infusion as used by Casey *et al.* (2009). Although this allows the ability to quantify levels of adenosine receptor blockade, a major disadvantage is the increased invasiveness of the protocol. Further, insertion of a brachial artery needle would interfere with placement of venous occlusion plethysmography equipment, and if removed before beginning the exercise protocol there would be an increased risk of bleeding. An alternative protocol, with much the same disadvantages, would use infusion of aminophylline directly into the brachial artery as used by Martin *et al*. (2006b). This may allow increased doses of

aminophylline to be targeted to the forearm vasculature whilst reducing the systemic effects of high dose intravenous aminophylline.

5.4.6 Summary

In conclusion, this study was novel in that it was the first to investigate the O_2-dependent role of adenosine as a mediator of the hyperaemia seen immediately post-exercise. The present study found no evidence for a role of adenosine in mediating post-exercise hyperaemia following either static or dynamic handgrip exercise. There was also no evidence that this hyperaemia was mediated by any substance in an O_2-dependent manner. We did observe a transient decrease in pO_2 following aminophylline infusion, which is most likely due to an increase in aerobic metabolism.

	Young (Mean±S.E.M.)	Older (Mean±S.E.M.)
Age	21.0±0.7	63.3±0.5
Height (m)	1.79±0.02	1.74±0.04
Weight (kg)	70.9±3.5	82±5.3
BMI	22.0±0.9	26.8±1.0
Forearm Circ. (cm)	24.5±0.7	26.5±0.7
Peak force (kg)	28.5±1.5	30.3±3.5
Heart rate (B.P.M)	72.4±3.6	69.5±1.8
MABP (mmHg)	84.3±4.9	93.0±8.9
Self reported activity	2±0.2	2.3±0.3
Hours activity/week	2.4±0.8	3.9±0.5

Table 5.1 Subject characteristics

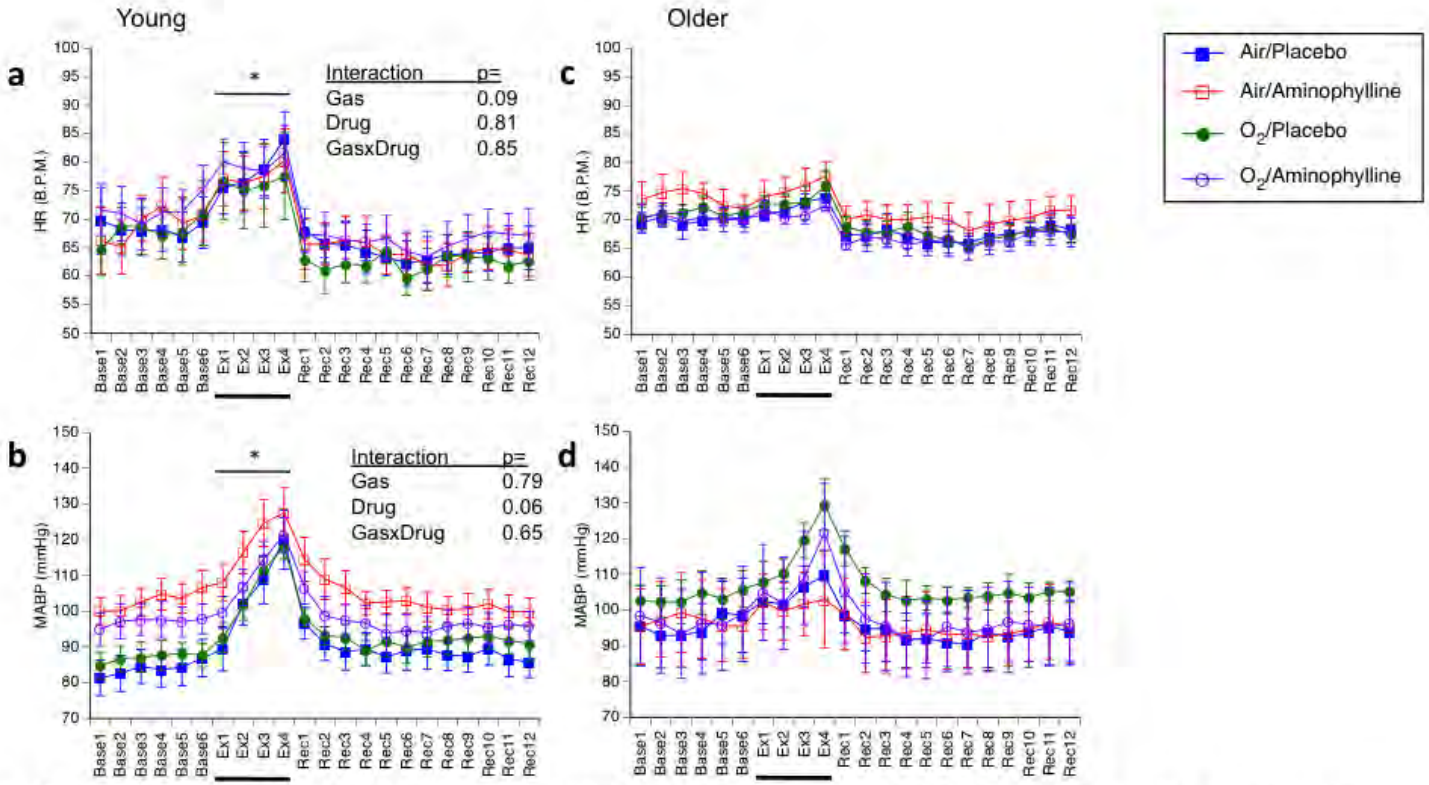

Figure 5.1. Heart rate and blood pressure before, during and after 2 minutes of static handgrip exercise. Graphs show mean ± S.E.M. over 30 second blocks from 3 minutes pre-exercise to 6 minutes post-exercise. Black bars indicate the period of exercise. Heart rate in young (**a**) and older (**c**) subjects rose above baseline within 30 seconds of exercise and remained there for the duration of exercise. MABP rose steadily over the period of exercise in both young (**b**) and older (**d**) subjects. Both HR and MABP returned to baseline within 30 seconds of ceasing exercise. Data are mean±SEM for 10 young and 4 older subjects, analysed using repeated measures ANOVA with special contrasts to group time points into baseline, exercise or recovery to allow statistical analysis. * signifies $p<0.01$ compared to baseline.

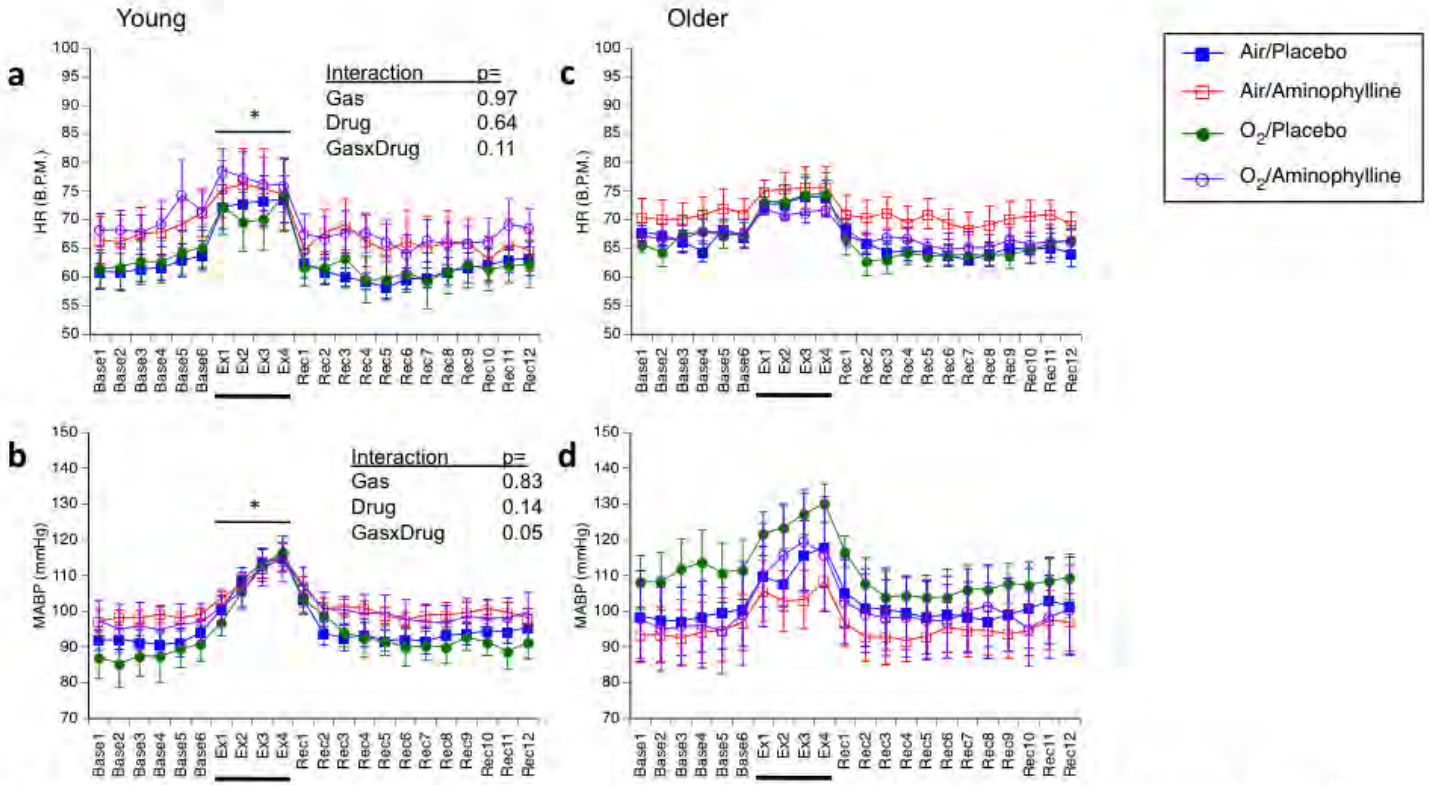

Figure 5.2. Heart rate and blood pressure before, during and after 2 minutes of dynamic handgrip exercise. Graphs show mean ± S.E.M. over 30 second blocks from 3 minutes pre-exercise to 6 minutes post-exercise. Solid black bars indicate the period of exercise. Heart rate in young (**a**) and older (**c**) subjects rose above baseline within 30 seconds of exercise and remained there for the duration of exercise. MABP rose steadily over the period of exercise in both young (**b**) and older (**d**) subjects. Both HR and MABP returned to baseline within 30 seconds of ceasing exercise. Data are mean±SEM for 10 young and 4 older subjects, analysed using repeated measures ANOVA with special contrasts to group time points into baseline, exercise or recovery to allow statistical analysis. * signifies $p<0.05$ compared to baseline.

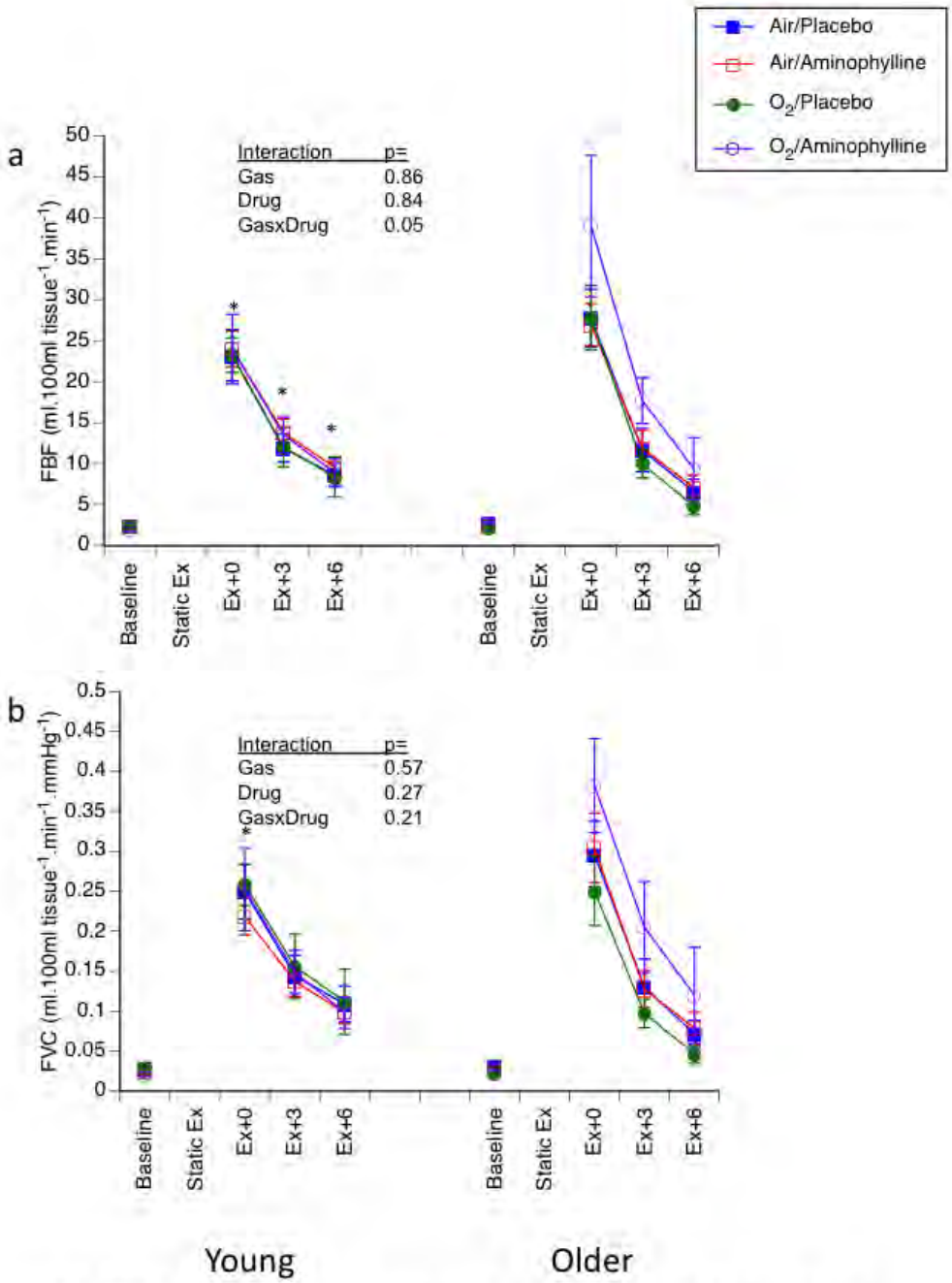

Figure 5.3. FBF (a) and FVC (b) in young and older subjects before and after 2 minutes of static handgrip exercise at 50% MVC. Both FBF and FVC increase significantly after exercise and then decline over the next 6 minutes, but remain elevated from baseline. Data are mean±SEM for (**a**) 7 young and 4 older subjects (**b**) 6 young and 4 older subjects. * = $p<0.05$ compared to baseline using repeated measures ANOVA.

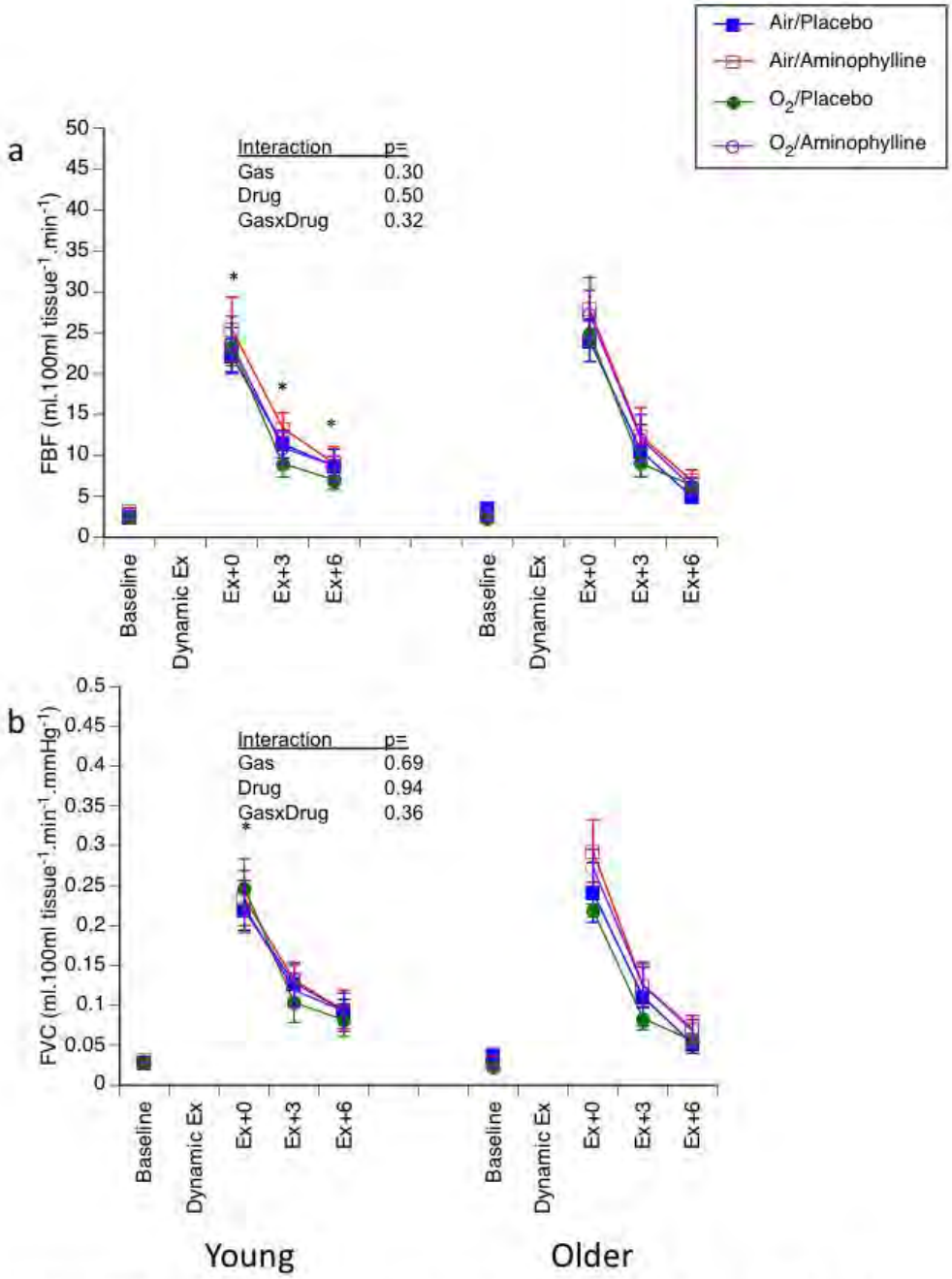

Figure 5.4. FBF and FVC in young and older subjects before and after 2 minutes of dynamic handgrip exercise at 50% MVC. Both FBF and FVC increase significantly after exercise and then decline over the next 6 minutes, but remain elevated from baseline. Data are mean±SEM for (**a**) 7 young and 4 older subjects (**b**) 5 young and 4 older subjects. * = $p<0.05$ compared to baseline using repeated measures ANOVA.

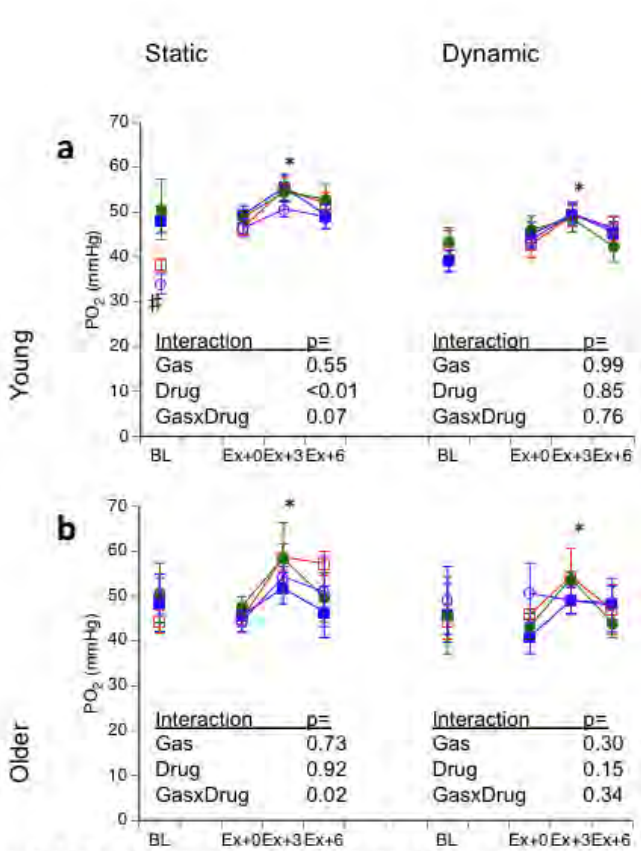

Figure 5.5a&b. Venous pO_2 before and after static and dynamic exercise in young (a) and older (b) subjects. Venous pO_2 was above baseline at all points post-exercise and peaked at 3 minutes post-exercise. Data are mean ±SEM for 10 young and 4 older subjects, analysed using GEE.* = p<0.001 compared to baseline. #= p< 0.001 aminophylline compared to control.

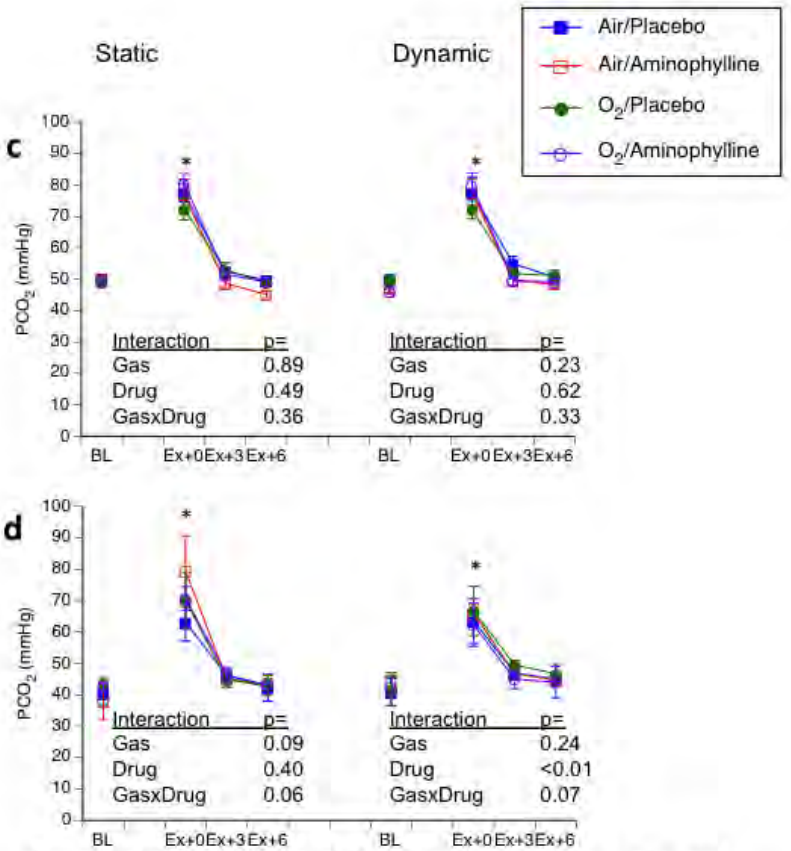

Figure 5.5c&d. Venous pCO_2 before and after static and dynamic exercise in young (c) and older (d) subjects. Venous pCO_2 was above baseline immediately post-exercise and had recovered by 3 minutes post-exercise. Data are mean ±SEM for 10 young and 4 older subjects, analysed using GEE.* = p<0.001 compared to baseline.

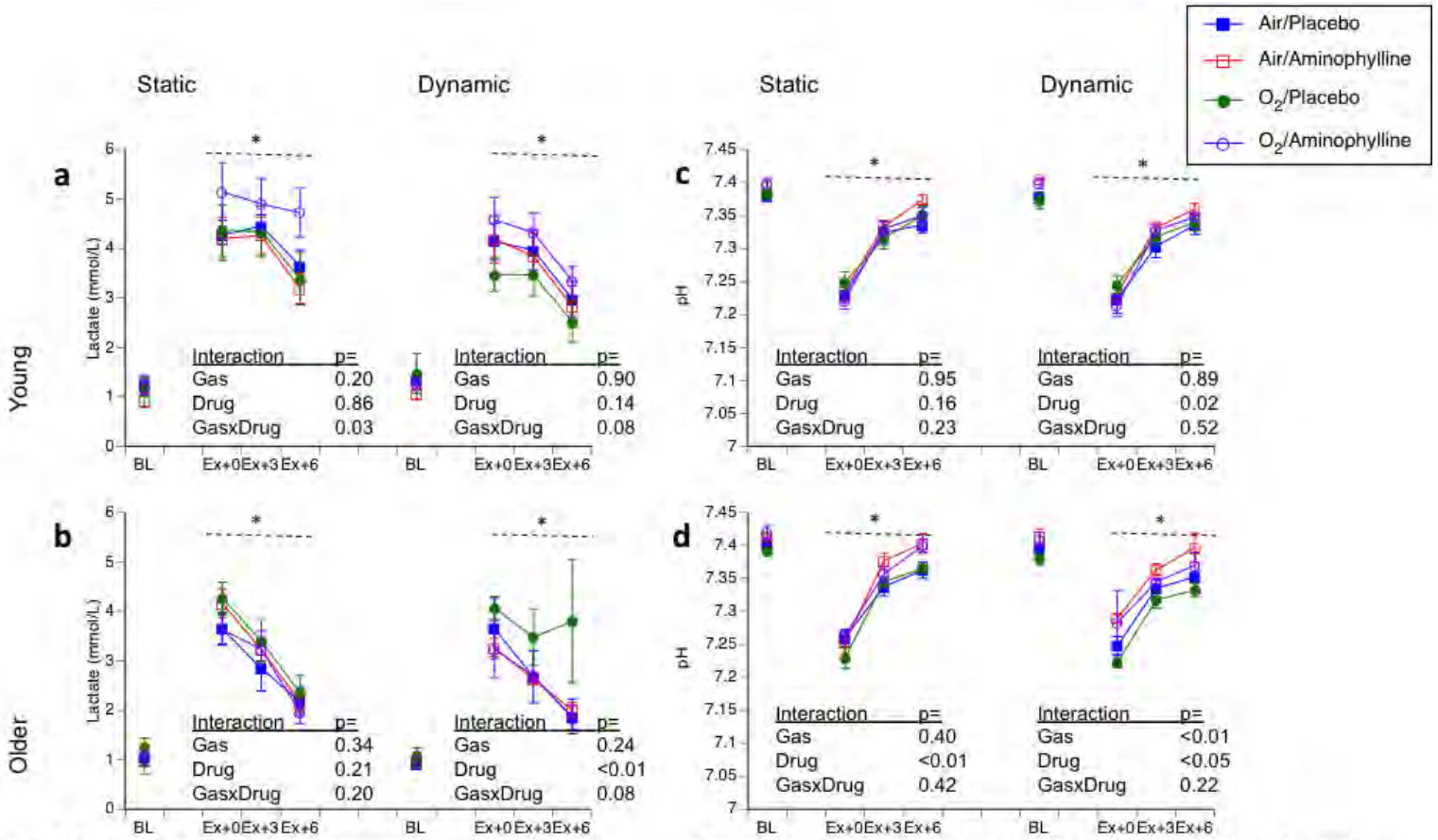

Figure 5.6a&b. Venous lactate concentrations before and after static and dynamic exercise in young (a) and older (b) subjects. Lactate was above baseline levels at all points post exercise. Data are mean±SEM for 10 young and 4 older subjects, analysed using GEE.* = p<0.001 compared to baseline.

Figure 5.6c&d. Venous pH before and after static and dynamic exercise in young (c) and older (d) subjects. Venous pH was below baseline at all points post-exercise and peaked immediately post-exercise. Data are mean±SEM for 10 young and 4 older subjects, analysed using GEE. * = p<0.001 compared to baseline.

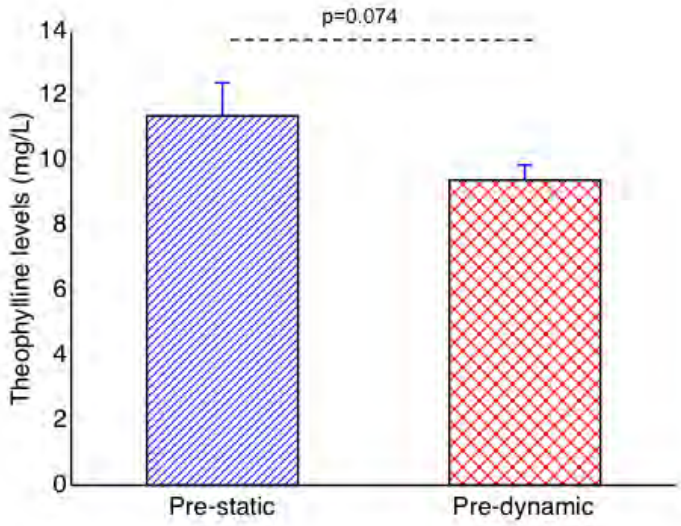

Figure 5.7. Theophylline levels measured before static and dynamic exercise. Time points are ~10 and ~40 minutes after completion of 5mg.kg^{-1} aminophylline respectively. There was a tendency for theophylline levels to drop over the course of the 30 minutes between measurements. = 6 subjects, analysed by paired t-test.

CHAPTER 6: THE EFFECT OF BREATHING 40% O_2 DURING RECOVERY ON FATIGUE

6.1 Introduction

Skeletal muscle fatigue is an important area of investigation due to the impact it has on human function in both health and disease. Despite this importance there is still no consensus as to how it is mediated. Section 1.3 gives a comprehensive overview of the substances that have been implicated, these being potassium ions (K^+), inorganic phosphate (Pi) and hydrogen ions (H^+). Despite the general view that lactate is involved in fatigue, there is no evidence to support this, as discussed in Section 1.3. Previous work from our laboratory investigated the effect of breathing supplementary 40% O_2 on fatigue development (Fordy & Marshall, 2004). They found that breathing O_2 only during the period of recovery between two periods of handgrip exercise at 100% MVE until exhaustion reduced the fatigue seen in the second period of exercise performed 7 minutes later. Importantly, neither pre-breathing supplementary O_2 nor breathing O_2 only during the period of exercise itself had any effect on fatigue, therefore it was concluded that an O_2 dependent mediator of fatigue acted during the period of recovery. However, venous blood samples were not collected to assay for any of the substances implicated in fatigue during this study and so we cannot say which mediator of fatigue is O_2 dependent. The discussion below considers the factors that are currently implicated as mediators of fatigue and whether they could be O_2 dependent mechanisms.

Resting levels of K^+ in plasma are typically 4-5mmol/L and levels rising to 8mmol/L having been demonstrated during exercise (Medbo & Sejersted, 1990). Evidence for a role of K^+ in fatigue has been found by a number of groups, as discussed in more detail in the General Introduction (Section 1.3.1).

Nielsen *et. al.* (2004) examined leg extension in subjects who trained one leg for 6 weeks with the untrained leg acting as a control. They demonstrated that the untrained leg fatigued faster than the trained leg and that this was associated with a more rapid increase in interstitial K^+. Further, it was demonstrated that venous K^+ was the same at the time of fatigue in two bouts of exhaustive exercise, even though fatigue was reached more rapidly in the second bout of exercise (Bangsbo *et al.*, 1992b). Finally, when leg interstitial concentrations of K^+ were increased by performing arm exercise prior to leg exercise, this was associated with a decreased time to leg fatigue compared to performing leg exercise alone (Nordsborg *et al.*, 2003). It has also been demonstrated in rat skeletal muscle that when the Na^+/K^+- ATPase was stimulated in various manners, which cleared K^+ from the extracellular space, the fatigue induced by exogenous K^+ administration was reduced (Clausen & Nielsen, 2007). Additionally, K^+ is released from muscle cells via K^+_{ATP} channels, which respond to decreased ATP by remaining open more frequently (Standen, 1992). Thus it is reasonable to hypothesise that the Na^+/K^+-ATPase or K^+_{ATP} channels are potential O_2 dependent mechanisms whereby increasing O_2 supply to the muscle by breathing supplementary O_2 could theoretically increase ATP production and therefore both reduce K^+ release and increase extracellular K^+ clearance (see Figure 1.3).

The second substance implicated in fatigue is Pi, concentrations of which increase in the muscle when production via ATP breakdown outstrips uptake by the mitochondria (Robergs *et al.*, 2004). Concentrations of plasma Pi in the venous efflux have been shown to increase following cycle exercise in healthy

young subjects (Yamamoto *et al.*, 1994). However, evidence for the involvement of Pi in fatigue is more indirect, with initial studies injecting exogenous Pi into animal muscle fibres and demonstrating a reduction in force generation (Cooke & Pate, 1985; Westerblad & Allen, 1996). Further evidence of a role comes from creatine kinase knockout mice who have a high resting myoplasmic Pi concentration but do not produce Pi in response to fatiguing contractions (Dahlstedt *et al.*, 2000). In keeping with a hypothesised role for Pi in mediating fatigue, initial force was lower in these mice than in wild type mice, but they did not show any fatigue over 100 contractions in contrast to the wild type mice who showed a 30% reduction in force (Dahlstedt *et al.*, 2000). In relation to O_2 dependency, Haseler and colleagues (1999; 2004) demonstrated that 100% O_2 increased phosphocreatine resynthesis in trained subjects but not in sedentary subjects, suggesting that sedentary subjects are limited by mitochondrial capacity and trained subjects limited by O_2 delivery. Given that phosphocreatine is a mechanism for removal of Pi within the muscle fibre (Robergs *et al.*, 2004), we can hypothesise that increasing delivery of O_2 will increase removal of Pi (see Figure 1.4).

Increases in H^+ (decreases in pH) have typically been assumed to be directly related to the development of muscle fatigue (Metzger & Moss, 1987; Cooke *et al.*, 1988). As discussed in Section 1.3.3, studies performed in human subjects have found no evidence of a direct effect of H^+, with the temporal relationship between fatigue and pH being out of sync (e.g. Cady *et al.*, 1989; Sahlin & Ren, 1989) or fatigue occurring at differing levels of pH (Bangsbo *et al.*, 1996). Currently it is thought that any effect of H^+ on fatigue is mediated indirectly via

stimulation of group III/IV afferent nerves (Westerblad *et al.*, 2002; Allen *et al.*, 2008). Again we can hypothesise a mechanism by which supplementary O_2 can reduce H^+ mediated fatigue. H^+ is produced when ATP exceeds mitochondrial respiration (Robergs *et al.*, 2004), therefore increasing oxidative phosphorylation by increasing O_2 delivery could potentially reduce H^+ production (see Figure 1.5).

The effect of ageing on fatigue development was discussed in full in section 1.3.4. Briefly, whilst there is a reduction in strength with ageing which is not accounted for by reduced activity and loss of muscle volume (Goodpaster *et al.*, 2006), the effect of ageing on fatigue is less clear. A recent meta-analysis concludes that ageing leads to an increase in fatigue resistance when performing static exercise but there is no difference compared to young subjects when dynamic exercise is performed (Avin & Frey Law, 2011). However, there has been little data collected as to the changes in muscle physiology, which may lead to this increased fatigue resistance in older subjects.

6.1.1 Hypotheses

1. In recreationally active young men, fatigue is mediated by a substance or substances that acts in an O_2-dependent manner during recovery. Candidates for an O_2-dependent mediator of fatigue are Pi, K^+ or H^+.
2. Healthy older subjects have a greater fatigue resistance than younger subjects.

We tested these hypotheses by measuring performance in two bouts of static handgrip exercise in healthy young and older subjects. Over two separate visits subjects were randomly assigned to breathe supplemental O_2 or room air during the recovery period between the two bouts of exercise. Venous blood samples were taken for measurement of venous blood gases, pH, K^+, lactate and Pi.

We anticipated that fatigue seen in the second bout of exercise would be reduced following supplemental O_2 breathing in recovery compared to air breathing. Supplemental O_2 would reduce the venous concentrations of one or more of Pi, H^+ or K^+. We anticipated that older subjects would be able to maintain static handgrip contraction for longer than young subjects. This would be associated with a reduced venous efflux of one or more of Pi, H^+ or K^+ compared to young subjects.

6.2 Methods

11 young subjects and 9 older subjects attended the laboratory on 2 separate occasions, at least a week apart. On the first visit to the laboratory, the subject was familiarised with the equipment and given an opportunity to ask any questions. The activity questionnaire (Appendix 1) was administered at this point. They were then set up and MVC calculated as described in Chapter 2.

6.2.1 Handgrip exercise

In the present protocol, each subject performed two periods of handgrip exercise at 100% maximum voluntary effort (MVE) until exhaustion, the two periods being separated by seven minutes of recovery. In performing the handgrip exercise, subjects were instructed to perform a handgrip at 100%

MVC and then maintain their grip; the force maintained decreased over time until they were no longer able to grip the handgrip dynamometer. This protocol was very dependent upon the motivation of the subject to maintain the contraction. Therefore, the importance of maintaining the contraction until exhaustion was explained to each subject and the subject was verbally encouraged to maintain maximum effort throughout all periods of exercise. This protocol was chosen as it was felt that it was more relevant to real life fatiguing contractions than electrically stimulated contractions and that fatigue was more measurable than holding a submaximal contraction. Static exercise was used as it was felt that this was again a more relevant exercise modality for handgrip than dynamic exercise.

6.2.2 Protocol

After all the equipment was attached, the subject rested for 10 minutes before a venous blood sample was taken and three recordings of baseline FBF were made by venous occlusion plethysmography. The facemask was then fitted to the subject although the gas was not switched on at this point. The subject then began the first period of exercise at 100% MVE, gripping the dynamometer as hard as possible until they were no longer able to maintain their grip. Immediately after the subject released the dynamometer the gas mixture was switched on (either air or 40% O_2) and another recording of FBF was taken. A venous blood sample was taken immediately following the measurement of FBF (approximately 10-15 seconds after completion of the exercise). The recovery period was 7 minutes long, further venous blood samples being taken at 3 and 6 minutes after the end of Exercise 1 and a further capillary sample at 3

minutes. At the end of the 7 minute recovery period the gas was switched off and the subject began a second period of exercise, again gripping as hard as possible until exhaustion. On completion of Exercise 2 FBF was recorded and a venous blood sample was taken with further venous blood samples being taken at 3 and 6 minutes, as after Exercise 1.

On the second experimental day subjects repeated the same protocol, breathing the gas that was not breathed during recovery on the first day. On each experimental day the subject was encouraged to try to reach the same 100% MVC in Exercise 1 as they had achieved on the first experimental day to control for any differences in effort.

6.2.3 Assays of venous efflux

All venous samples were prepared for blood gas analysis as described in the General Methods (Section 2.2.5). Blood gas analysis gave measurements of K^+, pH and lactate.

Pi was measured for 5 young subjects on completion of the whole study using plasma snap frozen immediately after each experimental protocol, as described above. Pi was measured as free plasma orthophosphate (PO_4^-) using a Malachite Green Phosphate Assay Kit (POMG-25H, BioAssay Systems, USA). Briefly, samples were diluted to 1:500 to bring them within the detection range of the assay. 80μl of the diluted sample was pipetted into a 96 well plate and 20μl of Malachite Green reagent was added to the well. Then plate was then incubated at room temperature for 10 minutes and then read using a plate reader (Synergy HT, BioTek) at 620nm. Each sample was analysed in duplicate and mean absorbance values were calculated for the samples and for

standards. Absorbance values were converted to [Pi] using a linear equation from the standard curve data.

6.2.3 Statistical analysis

In this chapter, the term “time to voluntary exhaustion” (TVE) is used to describe the duration of the period between initiation and release of the contraction. “Peak force” is the maximum force attained at the onset of the contraction. For analysis, the integral of the area under the curve of the whole contraction was also computed as a measure of the Tension-Time Index (TTI; the equivalent of workload when performing static exercise).

In the present study, due to changes in the calibration of the dynamometer, it was not possible to calculate peak force in the same units for young and old. As a result, peak force achieved in contraction 1 of that experimental day is taken as 100% MVC for that day and the peak force achieved in contraction 2 of that day is expressed as a percentage of that value.

All data are expressed as the mean±S.E.M. of the subjects. Statistical analysis was carried out using SPSS 17.0 for Mac. Force data, blood metabolite data, FBF and FVC were analysed using repeated measures ANOVA with within subjects factors for gas breathed and time. For force data, 1 comparison was made for time and 1 for gas breathed. For blood gases, 6 comparisons were made for time (post-exercise vs. baseline) and 1 for gas breathed. Blood metabolites were analysed as a change from baseline. Heart rate and blood pressure were analysed using repeated measures ANOVA.

6.3 Results

6.3.1 Subject characteristics

Subject characteristics are shown in Table 6.1. Young subjects were aged 20.89±0.45 (mean +/-SEM) years old compared to 60.89±1.9 years for the older subjects. There was no significant difference between young and older subjects on self-assessed activity levels or hours of activity per week. Both young and old subjects reported predominantly leg-based activity.

6.3.2 Peak force

There was no difference in the peak force obtained during exercise 1 on either experimental day in either young or old subjects. Figure 6.1 a and b show the percentage of peak force achieved in exercise 1 and 2 for young and older subjects respectively. Peak force achieved by both young and older subjects was lower in the second bout of handgrip exercise compared to that achieved in the first bout (Fig. 6.1 a&b; $p<0.001$). Breathing 40% O_2 or room air during recovery had no effect on the change in peak force between the two bouts of exercise in either the young or the older group ($p=0.98$; $p=0.51$ respectively). There was no significant difference in the decrease in peak force seen in exercise 2 with ageing. As mentioned in section 6.2.3, changes in the calibration of the dynamometer between young and older subjects mean we are unable to compare absolute levels of force between young and old.

6.3.3 Time to voluntary exhaustion (TVE)

Figure 6.2a shows the time to voluntary exhaustion achieved by a group of recreationally active young men. There was no significant reduction in TVE in

the second exercise bout compared to the first (p=0.47). As with the peak force, there was no significant main effect of breathing 40% O_2 during recovery (p=0.51).

The data for older subjects are shown in Fig. 6.2b. The TVE in exercise 2 was significantly shorter than in exercise 1 (p=0.02). As with the young subjects, there was no significant main effect of breathing 40% O_2 in recovery (p=0.58).

It should be noted that mean TVE was ~2 times greater for older subjects than younger subjects for every exercise bout, but the difference was not significant (p=0.29).

6.3.4 Tension-Time Index (TTI)

In young subjects, the TTI in the second exercise bout was significantly lower than that performed in the first bout of exercise (Fig. 6.3a; p<0.001). This decrease in TTI was unaffected by breathing supplemental O_2 compared to room air in the recovery period (p=0.65).

Older subjects (Fig. 6.3b) followed the same pattern, with a significant decrease in TTI in the second exercise period compared to the first exercise bout of that experimental day (p<0.001). The mean TTI in both exercise bouts was lower on the air breathing experimental day than the O_2 breathing day (p=0.04) but the decrease in TTI from exercise 1 to exercise 2 was not affected (p=0.4).

The TTI of older subjects was significantly greater than that achieved by younger subjects for all exercise bouts (p=0.03).

6.3.5 FBF, FVC and MABP

Table 6.2 shows the FBF, FVC and MABP at rest and immediately following both periods of exercise for young subjects. FBF was significantly raised from resting levels following both exercise 1 and 2, although there was no significant difference between the response following exercise 1 and 2. Breathing 40% O_2 during the 7 minute recovery period between the two bouts of exercise had no significant effect on the FBF after either period of exercise.

Older subjects had a similar response to young subjects, with a significant increase in FBF post exercise, but no difference between post-exercise 1 and 2 and no difference when 40% O_2 was breathed in recovery.

As there were no significant changes in MABP, changes in FVC for young and older subjects are comparable to that of FBF, demonstrating that changes in FBF are due to changes in vascular tone.

6.3.6 Lactate

In young subjects lactate was increased significantly immediately post-exercise compared to the resting baseline (Fig. 6.4a; $p<0.05$) and gradually decreased such that the levels at 6 minutes post-exercise showed a significant recovery relative to immediately post-exercise, whilst still being raised from baseline. Lactate concentrations following the second period of exercise followed the same pattern, achieving comparable levels initially and decreasing to a similar level. There was no significant main effect of breathing 40% O_2 during recovery on either the recovery of lactate concentrations following exercise 1 or on the lactate concentrations seen after exercise 2 ($p=0.907$).

The same pattern was seen in older subjects (Fig. 6.4b). There was no significant difference between the lactate concentrations measured at rest or post-exercise between young and older subjects (p=0.847).

6.3.7 pH

The venous pH measured in young subjects decreased significantly post-exercise compared to resting baseline (Fig 6.5a; $p<0.05$). There was a significant recovery at 3 minutes post-exercise that was still below baseline and then a total recovery by 6 minutes post-exercise. There was no significant difference in the response following exercise 1 and exercise 2. There was no effect of breathing 40% O_2 on venous pH at any timepoint (p=0.504).

Older subjects (Fig. 6.5b) showed a similar decrease in pH post-exercise followed by a recovery by 6 minutes post-exercise. Similarly there was no effect of breathing 40% O_2 during recovery. There was no significant difference in pH between young and older groups at any time point (p=0.367).

6.3.8 K^+

There was no clear pattern of response in K^+ in either young or older subjects (Fig. 6.6 a&b respectively). In fact, venous K^+ was significantly decreased from baseline at 6 minutes post-exercise 1 and at 3 and 6 minutes post-exercise 2 in both young and older subjects. There was no significant main effect of breathing 40% O_2 at any timepoint (p=0.484). There was no significant difference in response between young and older subjects (p=0.644).

6.3.9 Pi

Venous Pi was measured in a sub-group of 5 younger subjects (Fig. 6.7). Venous plasma concentrations increased significantly following exercise and then returned to baseline levels by 3 minutes post-exercise when air was breathed in recovery ($p=0.05$). The same pattern can be seen following exercise 2. There was no significant change in venous Pi at any time point when 40% O_2 was breathed in recovery ($p=0.49$).

6.4 Discussion

The study presented in this section is the first to use venous blood sampling to investigate the role of O_2-dependent substances in exercise. It was also the first to investigate the effect of ageing in this context. The main findings of the present study were:

1. In contrast to our first hypothesis, breathing supplemental 40% O_2 in the 7 minute recovery period following a period of handgrip exercise had no effect on the fatigue seen in a second period of exercise. This was the case regardless of whether fatigue was measured using peak force, TVE or TTI.
2. Supplemental O_2 had no effect on the levels of K^+, pH or lactate measured in the venous efflux post-exercise.
3. The rise in venous Pi measured after exercise when air was breathed in recovery was not present when 40% O_2 was breathed.
4. In agreement with our second hypothesis, older subjects had enhanced fatigue resistance compared to younger subjects, as demonstrated by

the significantly increased TTI produced in every period of exercise. There were no differences in the levels of venous metabolites measured compared to young subjects.

6.4.1 Effect of 40% O_2 on fatigue in young subjects

This study was only the second study to investigate the role of O_2-dependent substances on fatigue. Force data was measured for two periods of handgrip exercise at 100% MVE until exhaustion, separated by a seven minute recovery period. Comparison between the two periods of exercise was used to show fatigue development, with the specific variables measured being peak force, time until voluntary exhaustion (TVE) and the Tension-Time index (TTI). As expected, we observed the development of fatigue when room air was breathed in recovery, as demonstrated by a significant reduction in peak force and TTI in exercise 2 compared to exercise 1. There was no significant change in TVE between the two exercise bouts. Given that all subjects maintained contractions well and that the pattern of force decline was the same in both exercise bouts, the maintained TVE in exercise 2 is due to the lower peak force and TTI. In contrast to our first hypothesis, breathing supplemental O_2 during recovery had no effect on either peak force or TTI, demonstrating that an O_2-dependent mediator of fatigue was not playing a role. As when air was breathed during recovery, TVE was not significantly different in exercise 2 compared to exercise 1 when supplemental O_2 was breathed.

These findings contrast with the only previous study in the literature investigating the effect of supplemental O_2 on fatigue (Fordy 2004). Unlike in our data, fatigue in normoxia was demonstrated by a reduction in TVE in the

second bout of exercise, whilst peak force remained unchanged. In light of our present findings that TVE was maintained in exercise 2 due to a reduction in force and TTI, it seems likely that the maintained peak force demonstrated by Fordy is responsible for their observations of reduction in TVE.

The previous data concluded that supplemental O_2 reduced fatigue based on observations that the reduction in TVE had been ameliorated such that it was no longer significantly different to the TVE in exercise 1. Therefore, it seems likely that the differences in how fatigue manifests between the two studies may be partly responsible for the differences in effect of supplemental O_2. Based on these two studies it would seem that fatigue resulting in decreased force is not mediated by an O_2-dependent substance, but fatigue manifesting as reduced holding time is.

Apparent differences in fitness of subjects recruited to the two studies may also provide an explanation for differences in the observed effect of supplemental O_2. As has been discussed in Chapter 4, forearm circumference can be used as a representative index of total muscle mass (Section 4.4.2). The mean forearm circumference in the previous study was 28.3cm, which is greater than the 27cm in the present study, thus possibly supporting the assumption that participants in Fordy's study were fitter than the present group of subjects. Further evidence for a possible difference in fitness is that the TVE achieved by subjects in Fordy's study was greater than that achieved in the present study (~250 seconds vs. ~180 seconds). The notion that the effect of supplemental O_2 is dependent on the activity levels of the subjects is supported by the observations of Haseler *et al.* (1999; 2004), discussed in section 4.4.2. They

found that only trained subjects were able to utilise supplemental O_2, and that this was likely due to more efficient muscles.

Given the present results, there is no evidence that breathing supplemental O_2 had any effect on fatigue and thus O_2-dependent substances are not obligatory mediators of fatigue. Based on previous literature, we can suggest that the role of O_2-dependent substances is dependent on the manner in which fatigue is manifested and the activity levels of the subjects involved, such that they play a role in trained subjects but not in sedentary individuals.

6.4.2 Effect of ageing on fatigue

As was hypothesised, the present study has found evidence that fatigue resistance is increased in older subjects, as demonstrated by the significantly increased TTI. Also of note, TVE was nearly twice as long in older subjects as in younger subjects in exercise 1, despite this not reaching significance. This finding concurs with the results of a recent meta-analysis (Avin & Frey Law, 2011) which concluded that older subjects had reduced fatigue when performing static exercise. Specifically, the present data contributes to existing data by demonstrating fatigue resistance in the forearm, whereas previous studies have found similar results in the thumb (Chan *et al.*, 2000; Ditor & Hicks, 2000), ankle (Kent-Braun *et al.*, 2002; Lanza *et al.*, 2004) and elbow flexors (Hunter *et al.*, 2005).

In light of research by Lexell and colleagues (Lexell *et al.*, 1986; Lexell *et al.*, 1988) showing a reduction in Type II muscle fibres in vastus lateralis muscle with age, it seems possible that this fatigue resistance is due to a shift towards slow twitch Type 1 fibres that has been shown in ageing. It has been

demonstrated in vitro using mouse muscle fibres that slow twitch Type 1 fibres are more efficient at maintaining ATP levels (Crow & Kushmerick, 1982). Additionally, it has been shown in endurance-trained cyclists that those with the highest percentage of Type 1 fibres are the most energetically efficient, both in their trained sport of cycling and when performing a protocol of 2 legged knee extension that they had not previously been exposed to (Coyle *et al.*, 1992).

However, it must be noted that all of these findings have been made in leg muscle. Although we were unable to find any studies which directly looked at whether proportions of Type I and II muscle fibres change in the forearm with ageing, there is some evidence to suggest that arm and leg muscle ages differently. White and Carrington (1993) measured muscle twitch speeds in young and older subjects. They found that older subjects had slower twitch speeds than young subjects in the calf, but biceps twitch speed was unaffected by ageing. This suggests that fibre type may change in the leg but not in the arm. Therefore, although we can hypothesise that that the greater fatigue resistance seen in the older subjects is due to an increased efficiency resulting from a shift towards Type I fibres, this is still an area that needs more definitive research as there is no real evidence for this phenomenon in the forearm.

6.4.3 Effects of 40% O_2 on FBF and FVC

Both FBC and FVC significantly increased following both periods of exercise compared to resting levels but there was no significant difference between breathing either air or 40% O_2 during the recovery period between the two bouts of exercise. These results correlate with previous data that showed that 40% O_2 only had an effect on FBF or FVC when administered during the period of

exercise, not during recovery (Fordy & Marshall, 2004). This finding is significant for the interpretation of venous metabolites as they will be removed from the muscle at the same rate, regardless of whether air or 40% O_2 was breathed during the recovery period between the two periods of exercise.

6.4.4 Effects of 40% O_2 on venous metabolites

There were no significant differences between young and old subjects in the concentrations of venous metabolites measured or their response to breathing 40% O_2. Therefore young and old subjects will be considered together in the discussion below.

6.4.4.1 Venous pH and lactate concentrations

The resting levels of pH and lactate found in this study are within normal physiological levels and the measurements taken following exercise were similar both to those found in previous studies from this laboratory (Fordy & Marshall, 2004) and in other studies (Sjogaard *et al.*, 1988; Boushel *et al.*, 1998). It was hypothesised that pH would not decrease as much when O_2 was breathed during the recovery period as it may increase mitochondrial oxidative phosphorylation and so reduce H^+ formation (see Figure 1.5). It could also be expected that O_2 may reduce the lactate concentration as lactate is produced as a result of anaerobic metabolism (Robergs *et al.*, 2004). The results of the present study show no significant difference in either pH or lactate concentration between the two experimental days. This contrasts with previous data showing that breathing 40% O_2 during the period of contraction reduced both venous lactate concentration and the fall in pH by ~30% (Fordy & Marshall,

2004). Similarly, a study by Pedersen *et al.* (1999) found that supplementary O_2 breathed before and during maximum dynamic leg exercise reduced exercising venous lactate concentrations by a similar amount, although they observed no effect of supplementary O_2 on pH levels.

We can hypothesise that by giving the O_2 during the exercise, as opposed to during recovery as in this study, the supplementary O_2 had a greater effect on mitochondrial oxidative phosphorylation and so reduced the build-up of H^+ and lactate. An alternative explanation for the lack of effect of O_2 on venous pH may be increased phosphocreatine synthesis. During recovery creatine kinase phosphorylates creatine to form phosphocreatine and produces H^+ as a by-product (Robergs *et al.*, 2004). This concept is supported by studies by Haseler and colleagues (1999; 2004) in which supplementary O_2 increased phosphocreatine resynthesis in trained athletes but not in sedentary subjects. These findings are supported by observations in young subjects in the present study that the increase in Pi post-exercise was abolished by breathing 40% O_2. Given that phosphocreatine is a mechanism for removal of Pi within the muscle fibre (Robergs *et al.*, 2004), it is plausible that supplementary O_2 did increase oxidative phosphorylation, but that the reduction in H^+ was offset by an increased rate of H^+ production from phosphocreatine resynthesis.

6.4.4.2 Venous K^+

The resting values of venous K^+ found in this study were ~4.5mmol/L which correlates well with values found in previous studies (Medbo & Sejersted, 1990). Further, the peak concentrations found in this study were similar to values found by other groups using static (Sjogaard *et al.*, 1988) and dynamic

(Boushel *et al.*, 1998) hand-grip exercise, although the rise post-exercise in the present study was not significant. The peak values of 4.95mmol.L^{-1} shown post-exercise in the present study were lower than the 5.2mmol.L^{-1} shown in a similar handgrip protocol by Fordy (2004), despite similar resting levels. This may represent either lower fitness (therefore able to produce less effort) or reduced motivation to continue the contraction in the subjects in the present study, as discussed above (Section 6.4.1). Similarly, peak values found in the present study were significantly lower than the maximum concentrations of ~8mM found by Medbo and Sejersted (1990) in subjects performing exhaustive treadmill exercise. Given the non-significant rise in K^+ seen in the present study, it seems unlikely that K^+ is an obligatory mediator of fatigue. However, we can hypothesise that K^+ release from muscle cells is positively correlated with the intensity of the contraction and with the muscle mass used during the exercise, thus may play an increased role in mediating fatigue in these circumstances. This correlates well with a recent study showing that interstitial K^+ measured in leg muscle was higher when arm exercise was performed prior to leg exercise than when leg exercise was performed alone and that this was associated with an increased time to fatigue (Nordsborg *et al.*, 2003).

K^+ is released from contracting skeletal muscle fibres via K^+_{IR} and K^+_{ATP} and is taken back up into the muscle fibres by Na^+/K^+-ATPase (Standen, 1992; Clausen, 2003). We hypothesised that 40% O_2 would attenuate the increase in K^+ by increasing levels of ATP and therefore both reducing K^+ efflux via K^+_{ATP} channels and increasing the level of K^+ re-uptake by preventing inactivation of the Na^+/K^+-ATPase due to decreased levels of ATP (see Figure 1.3). However,

the results of this study showed no significant difference between venous K^+ concentrations in either condition, suggesting that this does not occur in the present exercise protocol.

In conclusion, we found no role for K^+ as a mediator of fatigue in the present data. A plausible explanation for the findings in the present study is that changes in the venous concentration of K^+ measured post-exercise were not great enough to be affected by breathing supplemental O_2. We can hypothesise that in more intense contractions, or in exercise using a greater portion of body muscle mass, K^+ may play a greater role in fatigue and that breathing supplementary O_2 may reduce the accumulation of K^+.

6.4.4.3 Venous plasma inorganic phosphate

This study was the first to attempt to quantify venous efflux of Pi following handgrip exercise. The results of the present study showed a significant increase in plasma [Pi] from baseline immediately post-exercise when air was breathed during recovery. Resting values of Pi found in this study are around expected values (Yamamoto *et al.*, 1994; Rhoades & Bell, 2009) and values post-exercise are similar to the 1.46mmol.L^{-1} shown by Yamamoto *et al.* (1994) following cycling exercise. It should be noted that the increase in venous [Pi] found in this study following fatiguing exercise may be lower than the increase in [Pi] occurring in the muscle interstitium, as concentrations between 3 and 41 mmol.kg^{-1} muscle dry weight have been found using muscle biopsies (review: Fitts, 1994).

This rise in Pi observed post-exercise would initially appear to support the view in the literature that Pi is involved in mediating fatigue. Many of the previous

studies which implicate Pi in the development of fatigue have been performed on single muscle fibres (e.g. Cooke & Pate, 1985) or in isolated muscles (e.g. Dahlstedt *et al.*, 2000; 2001) from animals. Studies investigating the role of Pi in fatigue development in humans have concentrated on using Nuclear Magnetic Resonance imaging (NMR) (Miller *et al.*, 1988) to measure the [Pi] within skeletal muscle and have found increased concentrations with fatiguing exercise. The present data significantly add to the literature by demonstrating a significant increase in venous [Pi] following fatiguing handgrip exercise, despite that fact that this methodology may underestimate the changes occurring within the muscle.

Further, the change in [Pi] may also be significantly underestimated due to the delay between completing exercise and sampling of venous blood. Measurement of forearm blood flow occurred immediately post-exercise, therefore the first venous blood sample taken after exercise was realistically taken at approximately 20 seconds after completion of exercise. Studies by Haseler et al. (1999; 2004) found that the time constant for phosphocreatine recovery when breathing normoxic air was ~25 seconds in trained athletes and ~30 seconds in sedentary subjects. Therefore it is possible that [Pi] had already decreased from peak levels as a result of Pi removal by PCr resynthesis at the time of venous blood sampling. This delay in sampling venous blood combined with a short time constant for phosphocreatine recovery means that it is biologically plausible to observe an effect of supplemental O_2 in the immediately post-exercise blood sample.

The previously mentioned studies by Haseler and colleagues found that the PCr recovery rate increased as a result of breathing an increased concentration of O_2 in trained athletes. Therefore, in the present study, it was hypothesised that breathing 40% supplemental O_2 during the recovery period between two periods of maximum handgrip exercise until exhaustion would reduce the concentration of Pi found in plasma by increasing phosphocreatine resynthesis (see Figure 1.4). Interestingly, the present findings showed that breathing 40% O_2 in recovery abolished the post-exercise peak in venous [Pi]. In light of the work by Haseler (1999; 2004), it is likely that this represents an increased clearance of extracellular Pi by increased PCr resynthesis. Alternatively, given that Pi accumulates when ATP breakdown exceeds resynthesis (Robergs *et al.*, 2004), we can hypothesise that supplementary O_2 increased oxidative phosphorylation during recovery, thus limiting accumulation of Pi. This may explain our findings that breathing 40% O_2 during recovery also abolished the peak in [Pi] following exercise 2. It is reasonable to hypothesise that increased oxidative phosphorylation occurs concurrently with increased phosphocreatine resynthesis.

6.4.5 Critical evaluation of the experimental protocol

Some limitations of the present protocol are in common with the experiments presented in previous chapters. Issues concerning recruitment of suitable subjects and the use of venous occlusion plethysmography have been covered in Section 4.4.6.

A limitation specific to this protocol was the use of 100% MVE until exhaustion. As discussed in section 6.2.1, this methodology is particularly susceptible to the

motivation of the subject to carry on as opposed to stopping when the discomfort starts and this may explain differences in TVE between the present study and previous work (Fordy & Marshall, 2004). Future work could consider using alternative methods to measure fatigue, such as measuring the time a subject is able to maintain a specific force for, or using repeated short MVC contractions and measuring the decrease in force output.

A limitation with the sampling of venous blood immediately after completion of exercise has been mentioned in Section 6.4.4, in that samples were taken approximately 20 seconds after release of the contraction as a result of obtaining venous occlusion plethysmography measurements. This may have led to an underestimation of values immediately after exercise as some recovery may already have taken place. Considering that both the present study and previous work from this laboratory (Fordy & Marshall, 2004) have demonstrated no effect of breathing supplementary O_2 in recovery on FBF or FVC, it is reasonable to suggest that future studies can omit plethysmography work when using a similar protocol.

A further limitation of using venous blood samples to measure the metabolites released by skeletal muscle fibres are that values are likely to be significantly diluted. An interesting alternative would be to perform microdialysis (as used by e.g. Hellsten *et al.*, 1998) to measure the concentrations within the skeletal muscle interstitium, as this would give a more accurate view of the concentrations of metabolites the muscle fibres are actually exposed to. Additionally it would be interesting to take arterial blood samples so that arterio-venous difference in concentrations of metabolites could be calculated. This

would give a more accurate picture of the uptake and release of substances by the active muscle.

6.4.6 Summary

In conclusion, the present study has found no evidence of an O_2-dependent mediator of fatigue in either young or older subjects. Breathing supplemental 40% O_2 did not alter the venous concentrations of pH, K^+ or lactate, but it did abolish the post-exercise rise in Pi. The present data also showed that older subjects were more fatigue resistant than younger subjects when using static forearm exercise, although concentrations of metabolites in the venous efflux were not significantly different from younger subjects suggesting that this is not the source of increased fatigue resistance.

	Young (Mean±S.E.M.)	Older (Mean±S.E.M.)
Age	20.89±0.45	60.89±1.9*
Height (m)	1.81±0.03	1.74±0.03*
Weight (kg)	75.8±3.44	74.1±3.9
Forearm Circ. (cm)	27±0.45	25.7±0.3
Peak force (kg)	N/A	33.3+2.7
Peak force (V)	7.99±0.32	N/A
Self reported activity	3.0	2.56±0.2
Hours activity/week	6.0±1.0	4.6±0.8

Table 6.1. Subject characteristics. * = $p<0.05$ vs. young

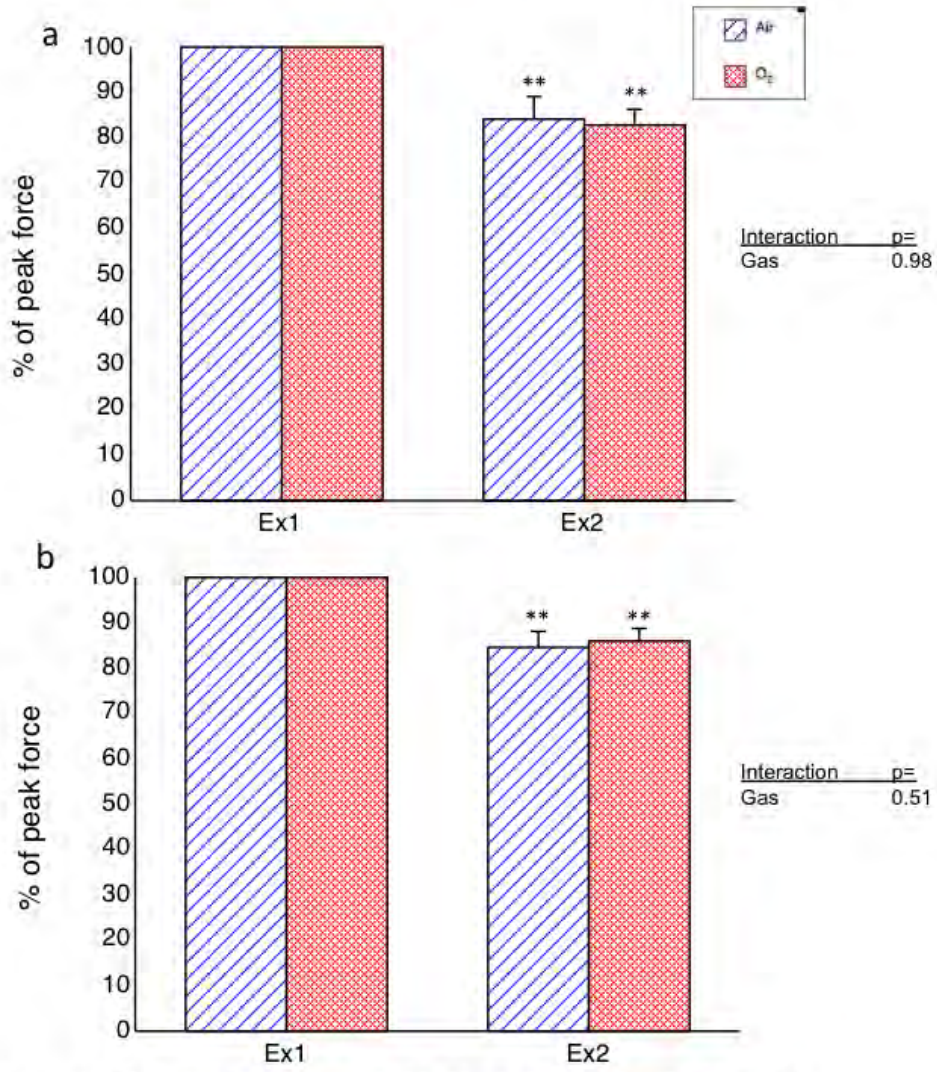

Figure 6.1. Peak handgrip forces achieved during 2 bouts of static handgrip exercise at 100% MVE until exhaustion separated by 7 minutes recovery in young (a) and older (b) subjects. Peak force in both young and old subjects was decreased in exercise 2 compared to exercise 1. There was no significant difference in the change in peak force between Ex1 and 2 whether air or 40% O_2 was breathed during the recovery period. n = 11 young, 9 older. ** = $p<0.001$ vs. Ex1 peak force for that gas breathed.

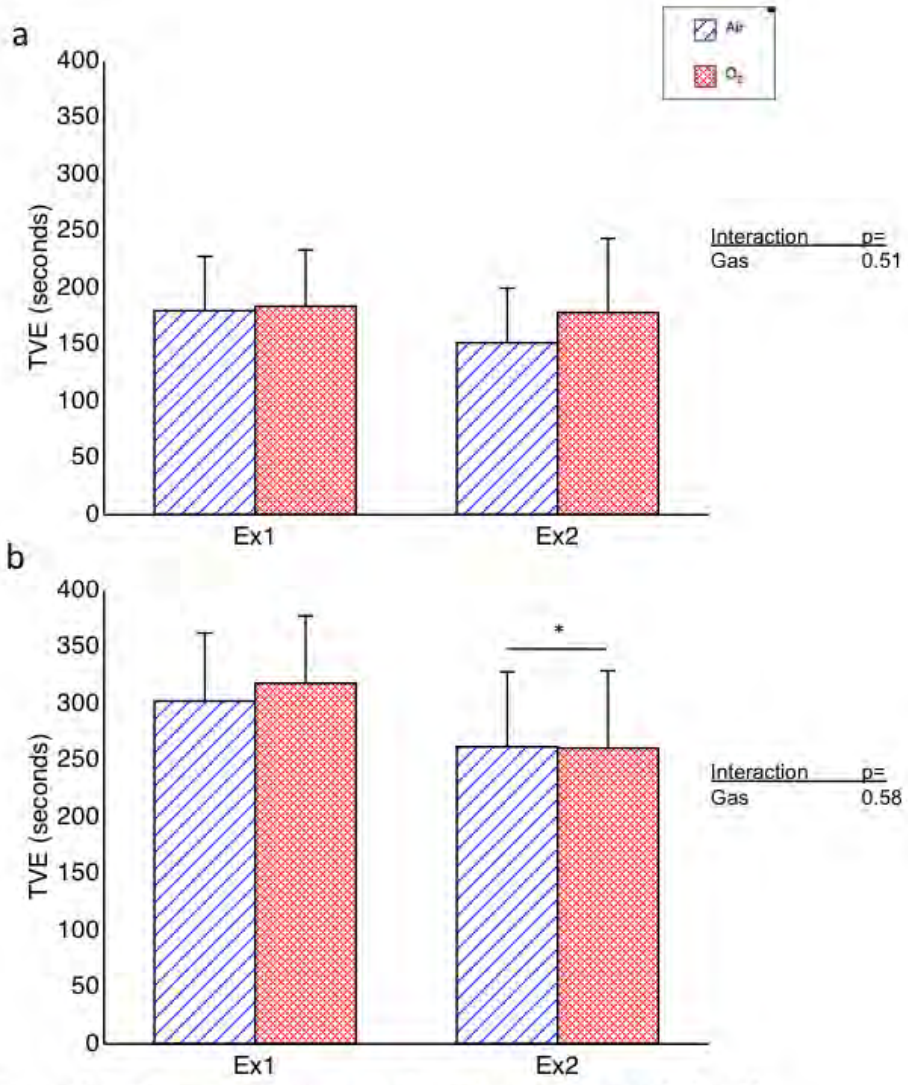

Figure 6.2. Time to voluntary exhaustion achieved during 2 bouts of static handgrip exercise at 100% MVE until exhaustion separated by 7 minutes recovery in young (a) and older (b) subjects. In young subjects TVE decreased in exercise 2 following room air breathing, but not following supplemental O_2. In older subjects, TVE fell following both room air and supplemental O_2. n = 11 young, 9 older. * = $p<0.05$ vs. Ex1 peak force for that gas breathed. There was no significant difference in the change in peak force between Ex1 and 2 whether air or 40% O_2 was breathed during the recovery period.

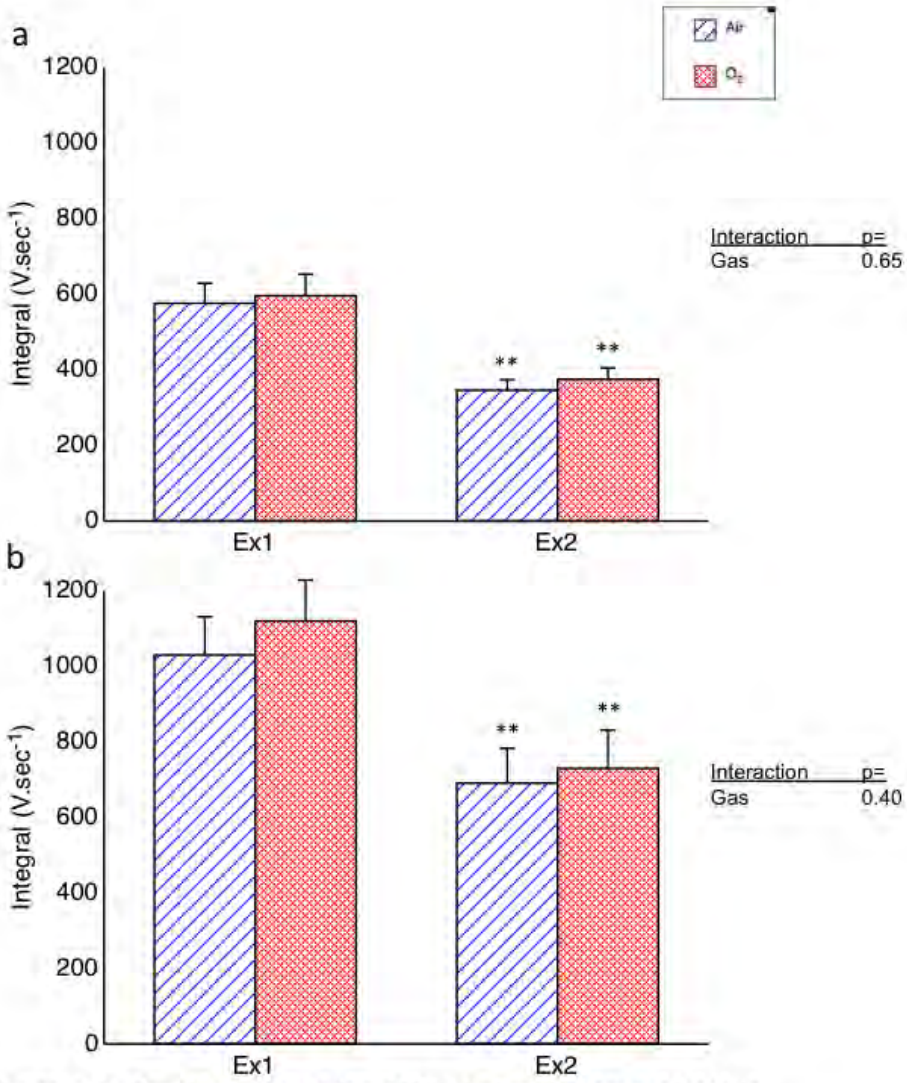

Figure 6.3. Tension-Time index (TTI) during 2 bouts of static handgrip exercise at 100% MVE until exhaustion separated by 7 minutes recovery in young (a) and older (b) subjects. TTI was assessed by calculating the integral of area under the curve for each period of exercise. TTI fell in exercise 2 compared to exercise 1 during both experimental days, with no significant difference in the change between Ex1 and 2 whether air or 40% O_2 was breathed during the recovery period. Older subjects achieved a significantly greater workload on all exercise bouts. n = 11 young, 9 older. ** = $p<0.001$ vs. Ex1 peak force for that gas breathed.

		Young (Mean±S.E.M.)		Older (Mean±S.E.M.)	
		Air	40% O_2	Air	40% O_2
FBF	Baseline	2.09±0.23	2.18±0.45	2.75±0.55	2.88±0.26
	Ex1+0	23.4±6.09*	20.2+4.12*	24.75±3.98*	25.61±4.15*
	Ex2+0	15.6±2.90*	20.3±4.49*	26.22±1.94*	25.61±10.46*
FVC	Baseline	0.03±0.002	0.03±0.005	0.03±0.006	0.03±0.003
	Ex1+0	0.27±0.09*	0.22±0.049*	0.26±0.04*6	0.27±0.03*5
	Ex2+0	0.15±0.03*	0.22±0.05*	0.27±0.026*	0.27±0.033*
MABP	Baseline	80.1±4.2	89.2±5.5	89.6±4.8	90.3±5.5
	Ex1+0	86.1±4.5	88.0±3.2	97.5±4.6	95.8±5.5
	Ex2+0	94.6±6.2	89.7±3.9	99.4±3.8	97.0±5.5

Table 6.2. FBF, FVC and MABP in young and older subjects at rest and immediately following 2 periods of handgrip exercise. Either air or 40% O_2 was breathed during the recovery period between the two periods of exercise. * = $p<0.05$ vs baseline for that condition.

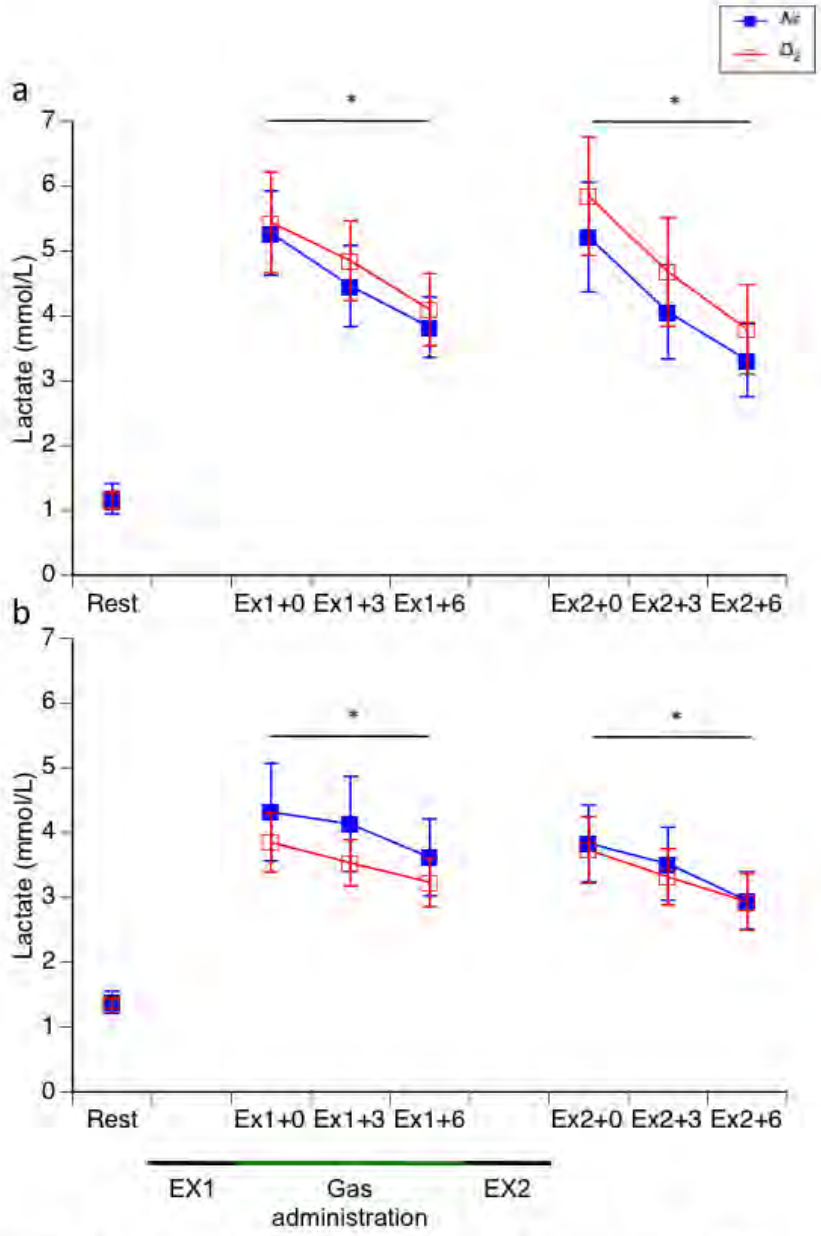

Figure 6.4. Venous lactate concentrations before and after 2 bouts of static handgrip exercise at 100% MVE until exhaustion in young (a) and older (b) subjects. For all exercise bouts lactate rose significantly from baseline after exercise and remained high for the 6 minutes recovery. Levels at 6 minutes post-exercise were significantly lower than immediately post-exercise. There was no difference between young and older subjects (p=0.85). There was no effect of breathing O_2 in recovery (p=0.91). n = 11 young, 6 older. * = p<0.05 vs. all time points post-exercise.

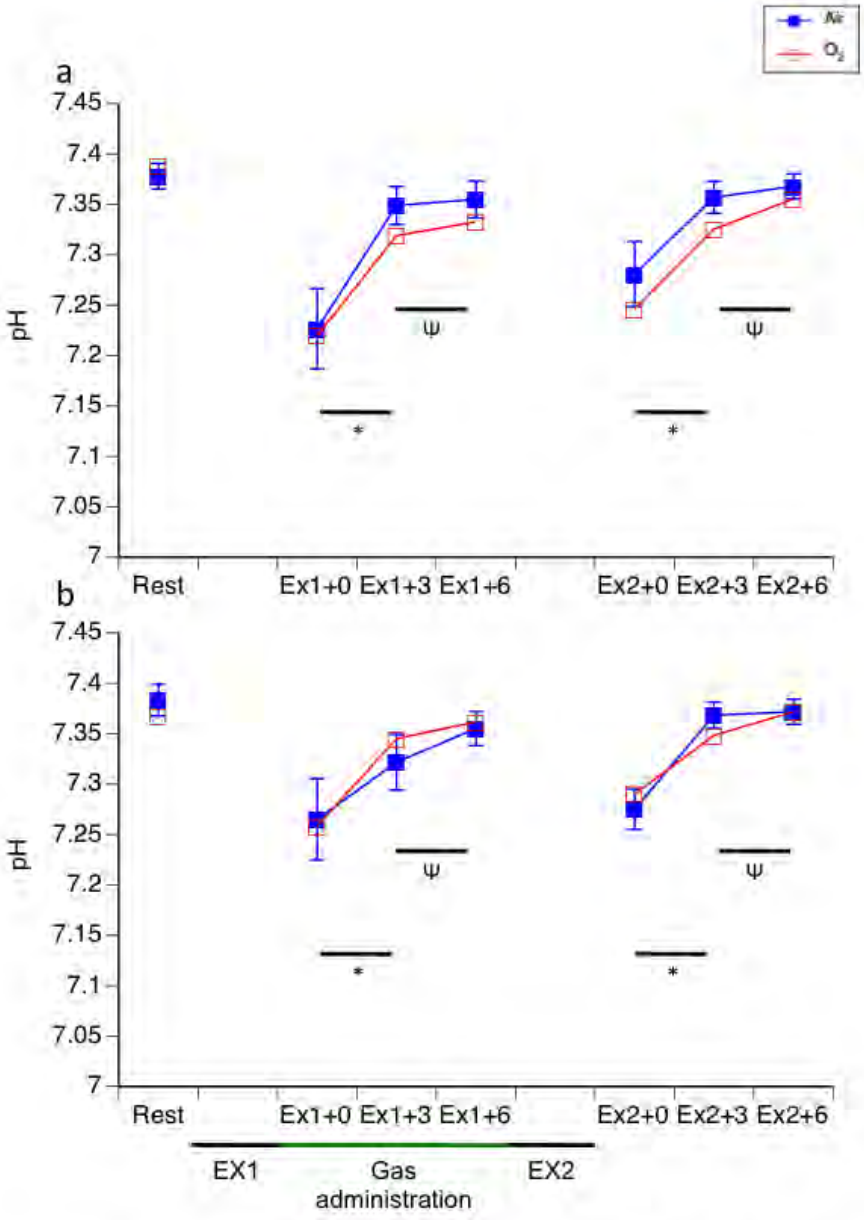

Figure 6.5. Venous pH before and after 2 bouts of static handgrip exercise at 100% MVE until exhaustion in young (a) and older (b) subjects. pH decreased significantly from resting levels after exercise. There was a significant recovery at 3 and 6 minutes post-exercise, with levels at 6 minutes post-exercise not significantly different to rest. There was no significant difference in pH measurements between young and older subjects (p=0.37). There was no effect of breathing O_2 in recovery (p=0.50). n = 11 young, 6 older. * = p<0.05 vs. rest.

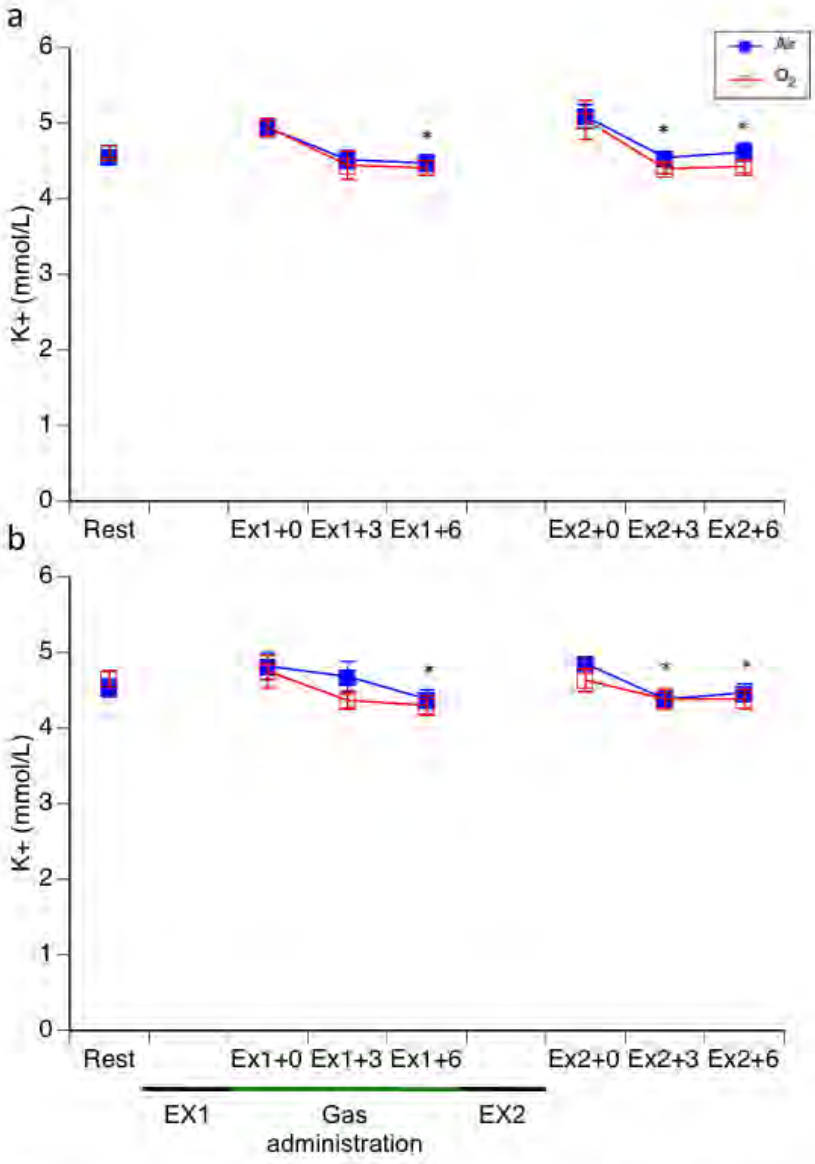

Figure 6.6. Venous K+ concentrations before and after 2 bouts of static handgrip exercise at 100% MVE until exhaustion in young (a) and older (b) subjects. Any changes in K^+ were not significant apart from a decrease from baseline at Ex1+6 and Ex2+3 and+6. There was no significant difference in K^+ between young and older subjects (p=0.64). There was no effect of breathing O_2 in recovery (p=0.48). n = 11 young, 6 older. * = p<0.05 vs. rest.

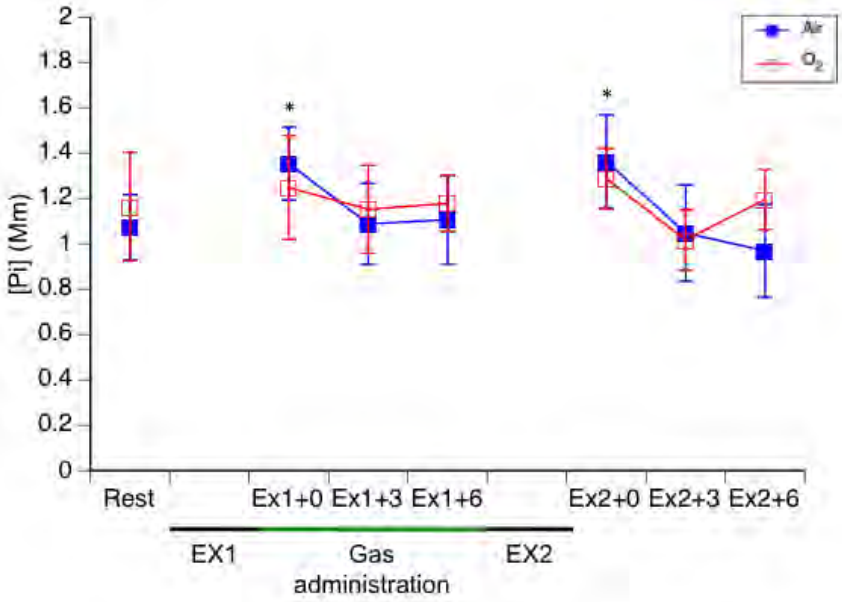

Figure 6.7. Venous Pi concentrations before and after 2 bouts of static handgrip exercise at 100% MVE until exhaustion in young subjects. Pi was significantly raised after exercise when room air was breathed during recovery, before returning to baseline levels by 3 minutes post-exercise. When supplemental O_2 was breathed there were no significant changes in Pi concentration. n = 5. * = $p<0.05$ vs. pre-exercise value for air breathing only. There were no significant differences when 40% O_2 was breathed during the recovery period ($p=0.49$).

CHAPTER 7: GENERAL DISCUSSION

The global aims of the studies presented in this thesis were to investigate the role of O_2-dependent substances in exercise. Specifically, we hypothesised that O_2-dependent substances produced *during exercise* mediate a component of the vasodilatation seen immediately following handgrip exercise, and that O_2-dependent substances acting *during recovery* mediate a portion of fatigue. Novel aspects of the studies presented in this thesis were the use both static and dynamic exercise protocols to investigate post-exercise hyperaemia. Venous blood sampling was utilised to measure the concentration of various metabolites from the forearm and monitor any changes that occur as a result of breathing supplementary O_2 or pharmacological intervention. Finally, we added new insights into the changes in post-exercise hyperaemia and fatigue development that occur with healthy human ageing. The studies included in this thesis were the first to investigate the effect of ageing on the role of O_2-dependent substances in exercise.

The NIRS study (Chapter 3) used a novel protocol to address the question of whether occlusion occurs within active muscle. It expanded on previous literature by using a technique to specifically investigate active muscle rather than the whole limb. This was important in the validity of the protocol in subsequent studies, as if blood flow were mechanically occluded by the contraction force, O_2 would not be able to reach the active muscle to effect vasodilatation. If blood flow occurs in active muscle contracting maximally it is reasonable to assume that significant blood flow occurs during exercise at 50% MVC.

Using NIRS we monitored levels of oxyhaemoglobin and deoxyhaemoglobin within the active flexor digitorum superficialis muscle during handgrip exercise. By comparing values measured during exercise with values obtained during complete arterial occlusion, we concluded that significant blood flow still occurs within contracting muscle during maximal static contraction.

Many substances have been postulated to mediate a component of exercise and post-exercise hyperaemia, including NO, prostaglandins, adenosine and EDHF. Based on the literature, we hypothesised that a portion of post-exercise hyperaemia is mediated by one of these substances produced during exercise in an O_2-dependent manner. We focussed on the roles of prostaglandins and adenosine. Contrary to our hypotheses however, our data in both Chapter 4 and 5 showed no effect of supplementary O_2 on the magnitude of post-exercise hyperaemia following either static or dynamic exercise. It appears that differences in the literature concerning the effect of supplemental O_2 may be due to differences in activity levels of subjects studied. This notion is consistent with the findings of Haseler and colleagues (Haseler *et al.*, 1999; Haseler *et al.*, 2004), who found that phosphocreatine resynthesis is limited by mitochondrial efficiency in sedentary subjects, not by O_2 delivery. Thus we can hypothesise that in active subjects with more efficient muscles, supplementary O_2 can be utilised to reduce production of vasodilator metabolites which are produced during normoxic exercise. By contrast, in less active or sedentary subjects, the reduced efficiency of the muscle means that the supplementary O_2 can not be utilised and so the magnitude of hyperaemia is not affected. Differences in methodology between studies also allow for the possibility that the intensity of

the stimulus may play a role in the effect of supplementary O_2, with no O_2-dependence seen at lower intensities and O_2-dependence during more intense exercise protocols.

Secondly, we observed no role for prostaglandins in mediating post-exercise hyperaemia. This finding supports observations made by other groups (Shoemaker *et al.*, 1996; Mortensen *et al.*, 2007), although there is some contrasting data in the literature (Wilson & Kapoor, 1993; Duffy *et al.*, 1999; Win & Marshall, 2005). Our findings are consistent with the fact that we observed no change in the forearm venous efflux of PGE metabolites. Whilst previous studies have found an increased levels of PGE_2 post-exercise in the venous efflux (Wilson & Kapoor, 1993) and in the muscle interstitium (Karamouzis *et al.*, 2001a; Karamouzis *et al.*, 2001b), it is of interest that two of the above studies have observed that this is only the case above a certain threshold of intensity (Karamouzis *et al.*, 2001b; Boushel *et al.*, 2002). We therefore propose that the differences in the literature represent evidence that prostaglandins mediate a component of exercise and post-exercise hyperaemia above a certain threshold but are not obligatory below this threshold.

Concerning the role of adenosine in mediating post-exercise hyperaemia, we show no effects of adenosine receptor blockade on the vasodilatation post-exercise. This can be partly explained by the methodological constraints of using aminophylline. As discussed, aminophylline is not a complete adenosine receptor antagonist, with values of between 30% and 60% inhibition being reported (Leuenberger *et al.*, 1999; Casey *et al.*, 2009). Despite these technical challenges, our data agree with findings that adenosine is not an obligatory

mediator of hyperaemia in the forearm (Martin *et al.*, 2006; 2007). An alternative hypothesis fits with the notion discussed above of a threshold of intensity above which prostaglandins play a role in exercise hyperaemia. It has recently been shown that the actions of infused adenosine are dependent on prostaglandins and NO (Mortensen *et al.*, 2009; Nyberg *et al.*, 2010). Therefore we can hypothesise that a similar interdependency occurs in exercise hyperaemia, in which both adenosine and prostaglandins play an interdependent role above a certain intensity threshold.

The post-exercise hyperaemia that we observed in the present studies is not explained by prostaglandins or adenosine individually. An interesting topic for future work is to assess what substance is causing this vasodilatation. A plausible candidate is NO, based on previous studies which show that NO is responsible for mediating a portion of post-exercise hyperaemia (Shoemaker *et al.,* 1997; Radegran & Saltin, 1999). An alternative theory may be the effect of interdependency and redundancy, such that single blockade as utilised in our studies results in increased importance of another substance to compensate.

An interesting finding following aminophylline infusion was the presence of a transient decrease in venous pO_2 at rest, approximately 10 minutes following aminophylline infusion, which had recovered by 40 minutes post-infusion. To our knowledge, this was a novel finding in the human forearm, although it supports previous literature that methylxanthines (the family to which aminophylline belongs) increase metabolic rate (Dulloo *et al.*, 1989; Ide *et al.*, 1995). We conclude that this transient decrease in venous pO_2 is due to an increase in aerobic metabolism resulting in a greater O_2 uptake.

We hypothesised that a component of the fatigue associated with sustained static handgrip contraction was O_2-dependent and concentrated on the roles of K^+, pH and Pi. Contrary to our hypothesis, we observed no effect of breathing 40% O_2 on the fatigue seen in a second period of handgrip exercise in young subjects. Differences between these findings and the only previous literature on the subject (Fordy & Marshall, 2004) appear to be partly due to differences in how fatigue was manifested. Thus, we concluded that supplemental O_2 does not play a role in ameliorating fatigue associated with a decrease in force, but may play more of a role when fatigue appears as a reduction in holding time. As with post-exercise hyperaemia, differences in the literature appear to be partly due to activity levels of the subjects studied, with O_2-dependent substances only playing a role in trained subjects and not in sedentary individuals.

As far as we are aware this is the first study to measure changes in venous concentration of Pi in an attempt to investigate any role in fatigue production. We found that venous Pi increased post-exercise when air was breathed during recovery in the subgroup of young subjects in whom Pi was assayed. This finding supports a role for Pi in fatigue production, which to date has only been demonstrated in experiments using isolated muscle (Cooke & Pate, 1985; Dahlstedt *et al.*, 2000; Dahlstedt *et al.*, 2001) or using indirect observations in humans (Miller *et al.*, 1988). Interestingly, the present findings support the concept that [Pi] can be altered by increasing inspired O_2, where increased O_2 delivery results in an accelerated removal of Pi via conversion to phosphocreatine.

We observed role for K^+ in mediating fatigue, which contrasts with the previous literature (Medbo & Sejersted, 1990). This may be due to the changes in venous K^+ being of insufficient magnitude or may represent K^+ not playing a role in fatigue. Our results for pH show a significant increase in H^+ following exercise. In light of the literature on pH (discussed in section 1.3.3) it seems most likely that if pH is playing a role, it is via indirect mechanisms (Westerblad & Allen, 2002).

The literature reports opposing data concerning the effect of ageing on the magnitude of exercise and post-exercise hyperaemia, with studies showing both a decrease (Carlson *et al.*, 2008) and no change (Jasperse *et al.*, 1994; Donato *et al.*, 2006). We demonstrated no effect of ageing on the magnitude of post-exercise hyperaemia following either static or dynamic handgrip exercise. Based on these and previous findings, we conclude that in healthy older subjects there is no impairment of exercise hyperaemia when exercise is prolonged past the single 1-second contractions used by Carlson *et al.* (2008). Additionally we observed no role for prostaglandins in mediating post-exercise hyperaemia in older subjects, a finding that is consistent with previous literature (Schrage *et al.*, 2004; 2007). Admittedly, the number of older subjects in the adenosine study was low, but in light of the data reported in young subjects and in the prostaglandin study, it seems a reasonable assumption that adenosine receptor antagonism would have had no effect if a larger number of older subjects had been tested. Finally, we showed that fatigue resistance is increased in older subjects compared to young subjects. These data support the consensus in the present literature. As with young subjects we found no

effect of supplemental O_2 on the magnitude of fatigue. Based on literature looking at leg muscle, we propose that changes in muscle fatiguability with age are due to a shift in muscle fibre type towards a slow twitch Type I phenotype.

In summary, the studies presented in this thesis have aimed to investigate the role of O_2-dependent substances in exercise. We found no evidence for a role of O_2-dependent substances in mediating post-exercise hyperaemia, with neither prostaglandins nor adenosine appearing to play an obligatory role. Based on previous literature, we can hypothesise that candidates for mediating the post-exercise hyperaemia seen in these studies may be either NO or EDHF. We also observed no effect of supplemental O_2 on the magnitude of fatigue observed following static exercise, demonstrating that O_2 –dependent substances are not obligatory mediators of fatigue. However, we propose that O_2 plays more of a role in mediating both post-exercise hyperaemia and fatigue in individuals who are trained, as opposed to the sedentary subjects tested in this thesis. Ageing had no effect on the magnitude of post-exercise hyperaemia following static or dynamic handgrip exercise, but older subjects were more fatigue resistant.

7.1 Future directions

The studies described in this thesis have raised some interesting questions for future research, especially with regard to the effect of the role of activity levels in determining factors contributing to exercise hyperaemia. Of particular interest for future study is the apparent difference in mechanisms of exercise hyperaemia between active subjects and less active subjects. This question could be answered by repeating the protocol in Chapter 4 in groups of young

men classified by their activity status, calculated either by using a more rigorous activity questionnaire or by using peak grip strength as a proxy.

A fundamental question is that if exercise hyperaemia is not being mediated by O_2-dependent substances such as prostaglandins or adenosine, what substance is taking this role? It may be that inhibition of prostaglandins, or more likely adenosine, is not sufficient to totally block their actions. EDHF is a possible contributor, which would be worth investigating. In addition, NO has been shown to contribute to recovery blood flow, and may contribute to exercise hyperaemia in more intense exercise, as was described in section 1.2.3.2. Therefore, it is possible that NO is playing a role in mediating the hyperaemia seen in Chapters 4 and 5, especially given that blood flow is measured post-exercise.

An alternative direction would be the use of animal models. This would have the advantage of being easier to control for lifetime activity levels. Additionally, more effective adenosine receptor antagonists are available for use in animals (Marshall, 2007), thus this model may be better suited to test hypotheses regarding any O_2-dependent role of adenosine.

References

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Appendix 1: Screening questionnaire

Name: _____ D.O.B.: _____

Activity level:

How would you describe your present level of activity?

Sedentary Moderate Active Highly Active

How many hours per week do you spend doing exercise? _____ hours

What form of exercise do you undertake?

_____
_____
_____

Medical status:

Do you have any history of cardiovascular or respiratory disorder? Y/N

Do you have any history of high blood pressure? Y/N

Do you suffer from dizziness? Y/N

Have you ever suffered from Diabetes / Asthma / Epilepsy? Y/N

Do you have any previous or current injury which may affect your ability to exercise? Y/N

Have you seen a doctor in the last 6 months for exercise related reasons? Y/N

Are you currently on any medication? (Including non-prescription). Y/N

Smoking habits:

Never

Used to smoke How many/day?_____

Occasionally smoke How many/day?_____

Regularly smoke How many/day?_____

Declaration

I declare that all information given above has been read, understood and is correct to the best of my knowledge.

Signature: _____ Date: _____

Appendix 2: Missing data for blood gases in Chapter 4 and 5

Chapter 3: Prostaglandin study

Test	Group	Total data points	Missing data points	% missing
pO_2	Young static	192	26	13.5
	Young dynamic	192	31	16.1
	Older static	192	27	14.1
	Older dynamic	192	25	13
pCO_2	Young static	192	25	13
	Young dynamic	192	30	15.6
	Older static	192	20	10.4
	Older dynamic	192	24	12.5
Lactate	Young static	192	27	14.1
	Young dynamic	192	32	16.7
	Older static	192	20	10.4
	Older dynamic	192	21	10.9
pH	Young static	192	25	13
	Young dynamic	192	30	15.6
	Older static	192	20	10.4
	Older dynamic	192	20	10.4

Chapter 4: Adenosine study

Test	Group	Total data points	Missing data points	% missing
pO_2	Young static	160	27	16.9
	Young dynamic	160	22	13.8
	Older static	/	/	/
	Older dynamic	/	/	/
pCO_2	Young static	160	25	15.6
	Young dynamic	160	20	12.5
	Older static	/	/	/
	Older dynamic	/	/	/
Lactate	Young static	160	25	15.6
	Young dynamic	160	20	12.5
	Older static	/	/	/
	Older dynamic	/	/	/
pH	Young static	160	25	15.6
	Young dynamic	160	20	12.5
	Older static	/	/	/
	Older dynamic	/	/	/

Appendix 3: Mean±S.E.M. values for Chapter 4 data

Values for FBF

		Air / Placebo		Air / Aspirin		O_2 / Placebo		O_2 / Aspirin	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	2.8	0.3	2.3	0.2	2.3	0.2	2.2	0.2
	Ex+0	24.4	3.0	23.8	1.9	21.8	1.7	21.8	2.0
	Ex+3	13.1	2.1	14.0	1.6	15.0	2.1	13.1	1.4
	Ex+6	9.0	1.9	10.3	1.5	10.8	1.5	9.8	1.2
Older Static	Baseline	2.6	0.2	2.4	0.2	2.6	0.3	2.8	0.2
	Ex+0	27.8	4.8	24.2	3.3	22.7	2.2	26.1	2.6
	Ex+3	18.1	2.0	15.6	1.9	14.6	2.0	13.0	2.1
	Ex+6	9.6	2.3	9.2	2.1	8.4	1.2	7.7	1.6
Young Dynamic	Baseline	2.7	0.3	2.9	0.2	2.7	0.3	2.7	0.4
	Ex+0	22.4	3.4	24.1	2.7	20.0	2.0	23.5	1.5
	Ex+3	10.1	1.6	11.9	1.3	9.3	1.4	11.9	1.9
	Ex+6	6.6	1.2	8.0	1.2	7.4	0.9	8.4	1.9
Older Dynamic	Baseline	3.1	0.3	2.7	0.3	2.8	0.2	2.9	0.2
	Ex+0	28.1	5.1	30.9	3.5	26.6	2.9	28.0	3.2
	Ex+3	15.2	3.4	13.5	1.9	12.9	1.5	10.8	2.1
	Ex+6	9.7	1.6	8.7	1.5	7.3	0.9	8.3	1.4

Values for FVC

		Air / Placebo		Air / Aspirin		O_2 / Placebo		O_2 / Aspirin	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	0.032	0.004	0.027	0.003	0.027	0.003	0.026	0.003
	Ex+0	0.245	0.039	0.241	0.024	0.217	0.019	0.216	0.021
	Ex+3	0.142	0.024	0.157	0.019	0.165	0.024	0.147	0.017
	Ex+6	0.099	0.021	0.112	0.019	0.119	0.015	0.113	0.015
Older Static	Baseline	0.027	0.002	0.027	0.002	0.029	0.003	0.032	0.003
	Ex+0	0.260	0.049	0.260	0.039	0.237	0.024	0.270	0.030
	Ex+3	0.177	0.022	0.178	0.025	0.167	0.027	0.147	0.028
	Ex+6	0.087	0.024	0.101	0.023	0.087	0.017	0.085	0.018
Young Dynamic	Baseline	0.029	0.003	0.029	0.002	0.029	0.003	0.031	0.003
	Ex+0	0.216	0.031	0.239	0.040	0.199	0.022	0.239	0.015
	Ex+3	0.103	0.015	0.114	0.012	0.097	0.014	0.128	0.020
	Ex+6	0.069	0.012	0.069	0.010	0.076	0.009	0.087	0.016
Older Dynamic	Baseline	0.031	0.003	0.029	0.003	0.030	0.002	0.032	0.003
	Ex+0	0.283	0.067	0.313	0.038	0.272	0.032	0.283	0.032
	Ex+3	0.165	0.045	0.140	0.020	0.136	0.016	0.119	0.025
	Ex+6	0.098	0.019	0.093	0.018	0.076	0.010	0.085	0.018

Values for pO_2

		Air / Placebo		Air / Aspirin		O_2 / Placebo		O_2 / Aspirin	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	41.18	4.717	42.73	3.464	48.44	6.388	43.3	4.238
	Ex+0	50.45	2.225	48.64	1.429	50.78	2.222	48.55	1.631
	Ex+3	54.2	4.005	57.82	2.11	55.89	3.075	53.73	2.848
	Ex+6	45.45	3.659	51	2.244	50.44	3.141	49.18	2.614
Young Dynamic	Baseline	37.18	4.031	41.91	2.956	39.9	4.135	41.8	4.242
	Ex+0	43.36	1.608	44.73	2.566	43.6	3.067	42.89	2.771
	Ex+3	46.78	3.027	46.5	2.794	47	3.902	50	2.934
	Ex+6	42.45	3.599	42.36	2.715	44.3	3.856	45.38	2.982
Older Static	Baseline	45	4.698	43.36	3.473	36.75	3.139	41.27	4.328
	Ex+0	50.5	2.113	50.09	1.866	49.25	1.955	47.4	1.485
	Ex+3	55.86	2.923	55.36	2.245	54.45	2.383	53.27	2.864
	Ex+6	49.62	3.348	49.73	2.265	47.17	3.233	46.64	3.449
Older Dynamic	Baseline	38.5	5.392	41.73	3.456	39.83	3.823	41.36	5.468
	Ex+0	47.63	1.647	45.45	1.631	44.5	1.525	45.64	1.448
	Ex+3	51.13	3.085	49.73	2.324	48.45	2.262	50.18	2.834
	Ex+6	48.88	3.232	47.36	2.051	45.33	3.016	46.36	3.031

Values for pCO_2

		Air / Placebo		Air / Aspirin		O_2 / Placebo		O_2 / Aspirin	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	53.73	2.059	51.27	1.674	49.33	2.034	50.36	1.416
	Ex+0	81.64	5.542	77.91	4.473	88	9.023	80.55	6.547
	Ex+3	54.1	1.779	51	1.25	51.33	1.958	57.18	2.869
	Ex+6	50.91	1.676	49.91	0.939	49.22	1.73	51.09	1.224
Young Dynamic	Baseline	51.36	1.473	49.64	1.073	50.2	1.413	47.6	3.718
	Ex+0	80.18	4.101	77.27	3.89	77.7	4.331	80.5	8.168
	Ex+3	54.33	0.799	53.4	0.98	51.8	2.102	55.11	2.182
	Ex+6	51.91	1.455	51.27	0.776	50.3	2.281	51.5	1.547
Older Static	Baseline	51.25	2.274	49.09	2.069	51.08	1.852	53.42	2.601
	Ex+0	80.38	6.153	80.91	5.838	82.67	3.117	78.42	4.926
	Ex+3	51.63	2.322	53.36	1.562	52.42	1.786	51.67	1.345
	Ex+6	50.75	2.119	50.27	1.573	49.92	1.869	50.17	1.392
Older Dynamic	Baseline	50	1.871	48.45	1.681	48.25	1.754	49.92	1.362
	Ex+0	77.88	4.498	75.360	4.642	72.73	2.905	78.5	4.08
	Ex+3	54.25	1.473	54.45	1.97	52.1	1.1	51.83	1.98
	Ex+6	50.75	1.688	49	1.779	49.91	1.304	51	1.619

Values for lactate

		Air / Placebo		Air / Aspirin		O_2 / Placebo		O_2 / Aspirin	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	1.4	0.2	1.1	0.1	1.2	0.1	1.1	0.1
	Ex+0	4.4	0.3	4.6	0.5	4.7	0.4	4.6	0.5
	Ex+3	4.7	0.4	4.2	0.4	4.6	0.3	4.6	0.5
	Ex+6	3.8	0.3	3.5	0.4	3.4	0.3	3.9	0.4
Young Dynamic	Baseline	1.3	0.1	1.1	0.1	1.3	0.1	1.2	0.1
	Ex+0	4.6	0.3	4.2	0.3	3.7	0.3	4.5	0.4
	Ex+3	4.7	0.4	4.1	0.4	3.5	0.4	4.3	0.5
	Ex+6	3.6	0.4	2.9	0.3	2.8	0.5	3.3	0.5
Older Static	Baseline	1.1	0.1	1.2	0.2	1.0	0.1	1.3	0.2
	Ex+0	4.3	0.4	4.2	0.3	4.2	0.2	4.1	0.3
	Ex+3	4.2	0.4	4.2	0.3	4.1	0.3	3.9	0.4
	Ex+6	3.3	0.3	3.4	0.4	3.1	0.3	3.3	0.4
Older Dynamic	Baseline	1.0	0.1	1.3	0.2	1.1	0.1	1.2	0.1
	Ex+0	4.3	0.4	3.9	0.4	3.9	0.2	4.0	0.4
	Ex+3	3.9	0.3	3.7	0.4	3.5	0.3	3.5	0.4
	Ex+6	2.9	0.4	2.7	0.4	2.8	0.3	2.6	0.4

Values for pH

		Air / Placebo		Air / Aspirin		O_2 / Placebo		O_2 / Aspirin	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	7.36	0.01	7.38	0.01	7.38	0.01	7.38	0.01
	Ex+0	7.21	0.02	7.22	0.02	7.19	0.03	7.22	0.02
	Ex+3	7.30	0.01	7.32	0.01	7.31	0.01	7.29	0.01
	Ex+6	7.33	0.01	7.33	0.01	7.34	0.01	7.33	0.01
Young Dynamic	Baseline	7.37	0.01	7.38	0.01	7.38	0.01	7.39	0.01
	Ex+0	7.20	0.02	7.22	0.01	7.23	0.02	7.21	0.02
	Ex+3	7.28	0.01	7.30	0.01	7.32	0.01	7.30	0.01
	Ex+6	7.32	0.01	7.34	0.01	7.35	0.01	7.33	0.01
Older Static	Baseline	7.37	0.01	7.39	0.01	7.38	0.01	7.38	0.01
	Ex+0	7.22	0.03	7.22	0.03	7.21	0.01	7.24	0.02
	Ex+3	7.32	0.01	7.31	0.01	7.31	0.01	7.33	0.01
	Ex+6	7.34	0.01	7.34	0.01	7.34	0.01	7.36	0.01
Older Dynamic	Baseline	7.38	0.01	7.39	0.01	7.38	0.01	7.39	0.01
	Ex+0	7.22	0.02	7.24	0.02	7.23	0.01	7.23	0.02
	Ex+3	7.30	0.01	7.31	0.02	7.32	0.01	7.33	0.01
	Ex+6	7.34	0.01	7.35	0.01	7.34	0.01	7.36	0.01

Values for PGE metabolite assay

		Air / Placebo		Air / Aspirin		O_2 / Placebo		O_2 / Aspirin	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young	Rest	13.80	3.78	9.35	2.04	11.74	3.22	10.30	2.47
	Post-exercise	12.75	3.28	9.48	2.34	12.58	3.79	11.68	3.22
Older	Rest	8.88	2.09	6.56	1.11	9.01	1.77	6.39	1.11
	Post-exercise	9.17	1.84	6.00	0.98	6.97	1.09	6.60	0.90

Appendix 4: Mean±S.E.M. values for Chapter 5 data

FBF

		Air		Air / Aminophylline		O_2		O_2 / Aminophylline	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	2.3	0.2	2.4	0.4	2.3	0.4	2.0	0.2
	Ex+0	23.2	3.1	24.0	2.2	23.2	2.1	24.0	4.2
	Ex+3	11.9	1.7	13.8	1.7	12.0	2.4	13.5	2.2
	Ex+6	8.6	1.4	9.6	1.3	8.4	2.4	8.9	1.6
Older Static	Baseline	2.7	0.3	2.5	0.1	2.3	0.4	2.8	0.1
	Ex+0	27.7	3.5	26.9	2.6	27.8	4.0	39.0	8.6
	Ex+3	11.7	2.6	11.9	2.2	10.0	1.7	17.7	2.8
	Ex+6	6.6	1.5	7.1	1.5	4.8	1.0	9.4	3.8
Young Dynamic	Baseline	2.6	0.3	3.2	0.3	2.5	0.4	2.9	0.3
	Ex+0	22.2	2.1	25.4	3.9	23.3	2.3	23.6	3.4
	Ex+3	11.4	1.7	13.3	1.9	9.0	1.6	10.9	1.9
	Ex+6	8.7	1.2	9.1	2.0	7.0	1.2	8.8	2.0
Older Dynamic	Baseline	3.6	0.6	2.7	0.3	2.4	0.3	2.6	0.5
	Ex+0	24.1	2.6	27.9	3.9	25.0	1.6	27.3	2.9
	Ex+3	10.6	3.2	12.4	3.5	9.1	1.7	12.1	2.9
	Ex+6	5.0	0.8	6.9	1.3	6.3	0.9	6.1	1.1

FVC

		Air		Air / Aminophylline		O_2		O_2 / Aminophylline	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	0.029	0.003	0.024	0.004	0.029	0.007	0.021	0.003
	Ex+0	0.250	0.034	0.219	0.023	0.258	0.025	0.252	0.052
	Ex+3	0.144	0.026	0.137	0.019	0.156	0.040	0.149	0.027
	Ex+6	0.109	0.022	0.098	0.014	0.112	0.041	0.098	0.020
Older Static	Baseline	0.031	0.005	0.027	0.003	0.022	0.004	0.030	0.004
	Ex+0	0.296	0.041	0.304	0.044	0.251	0.043	0.382	0.059
	Ex+3	0.130	0.035	0.127	0.023	0.097	0.018	0.205	0.057
	Ex+6	0.072	0.017	0.079	0.020	0.047	0.012	0.120	0.060
Young Dynamic	Baseline	0.028	0.003	0.032	0.003	0.028	0.006	0.030	0.004
	Ex+0	0.219	0.024	0.235	0.034	0.248	0.036	0.224	0.033
	Ex+3	0.128	0.023	0.132	0.022	0.104	0.025	0.119	0.021
	Ex+6	0.094	0.014	0.093	0.026	0.082	0.021	0.093	0.023
Older Dynamic	Baseline	0.038	0.008	0.030	0.004	0.023	0.005	0.030	0.008
	Ex+0	0.242	0.037	0.294	0.039	0.219	0.008	0.273	0.023
	Ex+3	0.111	0.037	0.125	0.026	0.084	0.014	0.126	0.029
	Ex+6	0.052	0.011	0.071	0.011	0.056	0.009	0.068	0.019

pO_2

		Air		Air / Aminophylline		O_2		O_2 / Aminophylline	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	48.33	2.986	38.29	1.409	50.57	6.747	34	2.228
	Ex+0	49.44	1.973	46.57	1.616	48.89	1.359	46.44	2.021
	Ex+3	55.56	2.911	55.13	2.741	54.5	2.236	50.67	1.803
	Ex+6	49.33	3.082	51.88	2.531	52.89	3.259	49	2.693
Young Dynamic	Baseline	39.56	2.887	42.88	3.056	43.44	3.06	39	2.427
	Ex+0	45	2.728	42.75	2.858	46.11	3.002	43.78	1.935
	Ex+3	49.56	2.583	49.38	2.556	48.5	3.012	49.63	2.236
	Ex+6	45.56	3.363	46.38	2.611	42.33	3.371	46.33	1.581
Older Static	Baseline	48.5	6.436	44.33	2.603	50.75	6.638	50	4.041
	Ex+0	45.75	2.496	45	2.887	47.75	2.175	44.33	2.404
	Ex+3	51.75	3.449	58.67	2.963	58.5	7.9	54.33	2.333
	Ex+6	46.5	5.737	57.33	2.667	49.75	5.452	51	7.81
Older Dynamic	Baseline	46.25	6.511	44.33	3.93	45.75	8.616	49	7.572
	Ex+0	41	3.851	46.000	1.155	43.25	2.869	50.67	6.692
	Ex+3	49	2.944	54.33	6.36	54	1.472	49	3
	Ex+6	48.5	3.797	47	5.508	44	3.189	48	6

pCO_2

		Air		Air / Aminophylline		O_2		O_2 / Aminophylline	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	49.78	1.024	49.63	1.954	49.29	1.426	49.25	0.796
	Ex+0	77.44	2.784	76.88	5.051	72.33	3.416	80.5	3.14
	Ex+3	52.56	1.375	48.75	1.306	53	2.309	51.56	1.538
	Ex+6	49.67	1.354	45.13	1.043	49	0.957	49.13	1.457
Young Dynamic	Baseline	49.78	1.422	46.37	0.596	49.44	1.827	46.33	1.093
	Ex+0	77.56	4.953	77.63	4.127	72.11	2.884	80	3.912
	Ex+3	55	2.285	49.5	1.389	51.78	1.597	49.56	1.375
	Ex+6	50.67	1.633	48.5	1.165	51.22	1.69	49.22	0.909
Older Static	Baseline	40.25	3.521	38	6.028	43.5	1.936	42.67	1.202
	Ex+0	63	5.874	79.33	11.333	70	9.055	70.67	3.756
	Ex+3	46.25	1.652	45	0.577	44.75	2.358	46.33	2.333
	Ex+6	42.25	4.27	43.33	1.764	43	3.189	43	1
Older Dynamic	Baseline	41	4.453	44.67	2.333	41.25	4.82	42.67	2.603
	Ex+0	63.5	7.03	66	2.646	66.75	7.857	62.33	6.888
	Ex+3	47	1.581	46.67	1.856	49.5	1.443	45	3
	Ex+6	45	2.273	44.67	1.764	46.75	3.038	44	5

Lactate

		Air		Air / Aminophylline		O_2		O_2 / Aminophylline	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	1.28	0.15	0.93	0.14	1.20	0.19	1.18	0.14
	Ex+0	4.27	0.17	4.20	0.43	4.36	0.52	5.15	0.58
	Ex+3	4.46	0.23	4.26	0.37	4.34	0.52	4.90	0.52
	Ex+6	3.64	0.33	3.19	0.30	3.39	0.53	4.73	0.49
Young Dynamic	Baseline	1.32	0.18	1.08	0.13	1.48	0.40	1.27	0.11
	Ex+0	4.14	0.37	4.19	0.48	3.48	0.35	4.58	0.46
	Ex+3	3.97	0.40	3.85	0.46	3.47	0.42	4.32	0.40
	Ex+6	2.98	0.36	2.83	0.42	2.52	0.43	3.34	0.29
Older Static	Baseline	1.05	0.16	0.97	0.26	1.25	0.18	1.03	0.03
	Ex+0	3.65	0.33	4.17	0.29	4.28	0.31	3.63	0.28
	Ex+3	2.85	0.46	3.23	0.23	3.40	0.42	3.23	0.37
	Ex+6	2.18	0.29	2.17	0.22	2.35	0.35	1.97	0.23
Older Dynamic	Baseline	0.93	0.11	0.95	0.05	1.10	0.14	1.00	0.06
	Ex+0	3.65	0.61	3.27	0.19	4.08	0.23	3.23	0.57
	Ex+3	2.68	0.53	2.63	0.09	3.48	0.57	2.70	0.00
	Ex+6	1.88	0.35	2.00	0.15	3.80	1.25	1.85	0.25

pH

		Air		Air / Aminophylline		O_2		O_2 / Aminophylline	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Young Static	Baseline	7.3811	0.00964	7.3925	0.00818	7.3829	0.00837	7.3988	0.00789
	Ex+0	7.23	0.01202	7.2363	0.01523	7.2489	0.01541	7.2212	0.01329
	Ex+3	7.3244	0.01725	7.3337	0.00844	7.3156	0.01676	7.33	0.01027
	Ex+6	7.3356	0.01168	7.3738	0.00706	7.35	0.01414	7.35	0.01268
Young Dynamic	Baseline	7.3767	0.00866	7.405	0.00189	7.3722	0.01152	7.3978	0.00662
	Ex+0	7.2233	0.02154	7.2312	0.01381	7.2456	0.01345	7.2133	0.01658
	Ex+3	7.3033	0.01708	7.3313	0.00811	7.3178	0.0131	7.3278	0.00894
	Ex+6	7.3356	0.01425	7.36	0.00845	7.34	0.0128	7.3478	0.00596
Older Static	Baseline	7.4	0.0108	7.4133	0.00333	7.3925	0.00479	7.42	0.01
	Ex+0	7.26	0.01291	7.2533	0.01667	7.23	0.01633	7.2633	0.00667
	Ex+3	7.3375	0.01377	7.3767	0.01202	7.345	0.00645	7.3567	0.01202
	Ex+6	7.3625	0.0125	7.4033	0.01453	7.365	0.00645	7.4	0.01
Older Dynamic	Baseline	7.395	0.00645	7.4133	0.01202	7.38	0.00913	7.41	0.01
	Ex+0	7.2475	0.01436	7.29	0.00577	7.2225	0.0075	7.2833	0.04842
	Ex+3	7.335	0.01323	7.3633	0.00882	7.3175	0.0125	7.345	0.005
	Ex+6	7.3525	0.00854	7.3967	0.02186	7.3325	0.01031	7.37	0.02

Theophylline levels

	Mean	SEM
Pre-static	11.4	1.0208
Pre-dynamic	9.417	0.4665