# CONTRIBUTION OF OXYGEN-DEPENDENT MECHANISMS TO VASCULAR RESPONSES OF EXERCISE IN YOUNG AND OLDER MEN: THE ROLE OF PROSTAGLANDINS AND ADENOSINE

By

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## ABSTRACT

Previous work suggests vasodilating prostaglandins (PGs) are released during isometric handgrip exercise in an $O_2$-dependent manner in young men. This project investigates their contribution to the exercise hyperaemia of isometric and rhythmic handgrip contraction performed by healthy, recreationally-active young and older men. Hyperoxia (40% $O_2$), aspirin, and their combination equally attenuated exercise and *post*-exercise hyperaemia, and venous efflux of $PGE_2$ and $PGI_2$ in both age groups: efflux of these PGs was not attenuated with age, but their contribution to the hyperaemic response was. Further, the release of COX products evoked reflex vasoconstriction in an $O_2$-dependent manner. Moreover, 40% $O_2$, aspirin, and their combination equally inhibited the exercise-evoked vasoconstriction in both age groups. However, both the exercise-evoked attenuation in perfusion of resting skeletal muscles and the contribution of COX products were attenuated with age. Additional experiments showed that adenosine contributes to the hyperaemia of electrically evoked isometric twitch contractions in an $O_2$-dependent manner; adenosine may contribute to the increase in the concentrations of vasodilating PGs. Importantly, unlike 60% and 100% $O_2$, 40% $O_2$ did not attenuate acetylcholine-evoked endothelium-dependent dilatation in either age group, supporting the argument that the effect of 40% $O_2$ during exercise is independent of hyperoxia-related oxidative stress.

## DEDICATION

*To my grandparents, parents, siblings,*

*and dearest wife.*

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## AUTHOR'S DECLEARATION

I hereby explicitly state that I personally undertook all of the experimental work presented in this thesis; including human and animal experimental work. To this end, UK Home Office license training (Animals (Scientific Procedures) Act 1986 – Accredited Training Scheme for Licensees) was undertaken and a personal license was acquired. All of the experimental design, data extraction, statistical analysis, presentation of Figures, and Tables is also my own work. The only exception is the young subject data presented in Chapter 8: these experiments were undertaken by Florentyna D'Souza for her BMedSc (3rd year) project. Nonetheless, data from her experiments were independently extracted and analyzed by me, and are presented here. Lastly, it is also explicitly acknowledged that I am the author of this thesis. Guidance, feedback, and supervision during the course of this entire project and during the preparation of this thesis were provided by Prof. Janice M Marshall and Dr. Clare J Ray.

Rehan T Junejo

# TABLE OF CONTENTS

## INDEX TO FIGURES / ILLUSTRATIONS

# INDEX TO TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ~ | Approximately |
| $\Delta$ | Delta |
| A | Aspirin |
| AA | Arachidonic Acid |
| ABP | Arterial Blood Pressure |
| ACh | Acetylcholine |
| ADP | Adenosine Di-phosphate |
| AMP | Adenosine Mono-phosphate |
| ANOVA | Analysis of Variance |
| ATP | Adenosine Tri-phosphate |
| AU | Arbitrary Unit(s) |
| AUC | Area under the Curve |
| BMI | Body Mass Index |
| $Ca^{2+}$ | Calcium |
| CBF | Calf Blood Flow |
| CO | Carbon-monoxide |

| | |
|---|---|
| $CO_2$ | Carbon-dioxide |
| COX | Cyclooxygenase |
| CU | Conductance Units |
| CVR | Calf Vascular Resistance |
| EDHF | Endothelium Derived Hyperpolarizing Factor |
| ELISA | Enzyme linked immune-sorbent assay |
| eNOS | Endothelial Nitric Oxide Synthase |
| FABF | Femoral Artery Blood Flow |
| FAC | Forearm Circumference |
| FAVC | Femoral Artery Vascular Conductance |
| FBF | Forearm Blood Flow |
| FMD | Flow Mediated Dilatation |
| FVC | Forearm Vascular Conductance |
| $H^+$ | Hydrogen / Proton |
| HHb | Deoxy-haemoglobin |
| HR | Heart Rate |
| iNOS | Inducible Nitric Oxide Synthase |
| $K^+$ | Potassium |

| | |
|---|---|
| $K_{ATP}$ | Adenosine Tri-phosphate Sensitive Potassium Channel(s) |
| $K_{Ca}$ | Calcium Activated Potassium Channel(s) |
| KCl | Potassium-Chloride |
| L-NAME | $N^{\omega}$-nitro-$_L$-arginine |
| L-NMMA | $N^{\omega}$-monomethyl-$_L$-arginine |
| L-NNA | $N^{\omega}$-nitro-$_L$-arginine |
| MABP | Mean Arterial Blood Pressure |
| MBF | Muscle Blood Flow |
| min | Minute(s) |
| MSNA | Muscle Sympathetic Nerve Activity |
| MVC | Maximum Voluntary Contraction |
| NADPH | Nicotinamide Adenine Di-nucleotide Phosphate |
| NIRS | Near-Infrared Spectroscopy |
| NO | Nitric Oxide |
| nNOS | Neuronal Nitric Oxide Synthase |
| NOS | Nitric Oxide Synthase |
| NSAID(s) | Non Steroidal Anti-Inflammatory Drug(s) |
| $O_2$ | Oxygen |

| | |
|---|---|
| O | Older |
| P | Placebo |
| PECO | Post Exercise Circulatory Occlusion |
| PET | Positron Emission Tomography |
| PG(s) | Prostaglandin(s) |
| $PGE_2$ | Prostaglandin-$E_2$ |
| $PGI_2$ | Prostacyclin / Prostaglandin-$I_2$ |
| Pi | Inorganic Phosphate |
| $PO_2$ | Partial Pressure of Oxygen |
| RBC(s) | Red Blood Cell(s) |
| RCF | Red Cell Flux |
| ROS | Reactive Oxygen Species |
| RU | Resistance Units |
| s | Second(s) |
| SNP | Sodium-Nitroprusside |
| $SO_2$ | Oxygen Saturation |
| THI | Tissue Haemoglobin Index |
| TOI | Tissue Oxygenation Index |

| | |
|---|---|
| TPR | Total Peripheral Resistance |
| TTI | Tension Time Integral |
| TX(s) | Thromboxane(s) |
| V | Volt |
| $VO_2$ | Oxygen Uptake |
| $VO_2$-max | Maximal Oxygen Uptake / Maximal Aerobic Capacity |
| W | Watt |
| Y | Young |
| 8-PT | 8-Phenyltheophylline |
| 8-SPT | 8-Sulphophenylthrophylline |

# Chapter 1

# General Introduction

## 1.1 Overview

Humans perform some form of exercise every day; whether it is sitting up in a bed or high performance training. In order to maintain any level of activity, exercising body requires harmony between respiratory, circulatory, and metabolic aspects, along with the neural control of musculoskeletal system. The unabated continuation of exercise causes profound metabolic alterations in the working muscles leading to a painfully exhausting feeling: fatigue. An increase in cardiac output with a parallel decrease in vascular resistance are parts of an integrated response that helps to preserve the balance between supply and demand of nutrients (328). The phenomena of increased limb / muscle blood flow (MBF) and parallel the decrease in vascular resistance (i.e., vasodilatation) is termed “Active, Functional, or Exercise Hyperaemia”. To help meet the metabolic demands of active musculature, blood vessels supplying inactive muscles and viscera are known to constrict.

Blood flow is regulated by mechanistic pump-action of the heart, elastic recoil within arteries, mechanical compression of veins, and negative pressure within the thoracic cavity (135). From the left ventricle, it enters systemic circulation through the aorta which divides to form ever smaller arteries and arterioles, eventually turning primarily into a single layer of endothelial cells approximately (~) 1 µm thick (135). Although exchange of gases and nutrients takes place throughout the vasculature, these thin vessels termed capillaries remain the primary site for equilibration with peri-vascular interstitium (135). Blood is then collected into venules, draining into veins, and is returned to the right side of the heart to pass through the pulmonary circulation. A parallel closed lymphatic transport system also exists which transports tissue fluids into the venous circulation (135). Resistance to blood flow is provided by the luminal diameter of the feed arteries and arterioles; terminal arterioles regulate blood flow through the capillary bed (135).

These vessels make the major contribution to total peripheral resistance (TPR), and within skeletal muscle, changes in their diameter influence local vascular resistance and MBF.

As mentioned above, continuation of exercise is dependent on the metabolic environment of the musculature: hyperaemia's function is to match the supply to demand of nutrients and oxygen ($O_2$) (135). Pathological examples where the supply and demand of nutrients and $O_2$ remains mismatched include anaemia, chronic obstructive pulmonary disease (COPD), and chronic heart failure. Alternatively, environmental factors (for example, high-altitude) can also induce a state of hypoxia (reduced $O_2$) that requires supplementary $O_2$ for relief (313). In all these cases, hypoxia affects $O_2$ equilibrium all the way down to the mitochondria. Therefore, its contribution to exercise and by extension exercise hyperaemia has been the subject of scientific enquiry. For example, dynamic and isometric exercise studies with supplementary $O_2$ have revealed delayed fatigue onset (2, 236), reduced time to complete a required task (317, 319), and decreased blood flow to the vessels supplying the exercising muscles (123, 437).

The work described in this thesis was carried out to investigate the role of $O_2$ and its dependent products in the regulation of blood flow during exercise in recreationally active, healthy, young and older individuals. The discussion below primarily focuses on the potential mediators of exercise / *post*-exercise hyperaemia and briefly, the role of some of these metabolites to exercise-evoked vasoconstriction. The results Chapters focus on the role of $O_2$ and its dependent products to exercise / *post*-exercise hyperaemia of young and older individuals, their influence on exercise-evoked / reflex vasoconstriction, and the contribution of adenosine and its receptors to the $O_2$-dependent regulation of exercise hyperaemia. Moreover, the production of radical $O_2$ species (ROS) with different supplementary $O_2$ concentrations and their effect on endothelial dilatation was also investigated in both age groups.

## 1.2 Exercise hyperaemia

In late 1700s, Hunter must have used good intuition to state, "*blood goes where it is needed*" (351). However, the first observation of change in vessel diameter was recorded by Jones during his work on bat wings (33). Gaskell's work provides a detailed record of early experiments conducted to appreciate the effect of muscle contraction on local MBF. He observed a marked change in the vessel diameter supplying frog's mylohyoid muscle during the contractile stimulus (138). Similarly, he also observed a massive increase in blood flow of vessels supplying the contracting muscles of dogs (136, 138). These studies led him to propose that the metabolic products of muscle contraction were the primary reason for the observed vasodilatation (136, 137). Since then, experiments involving differing techniques have been employed to better understand this hyperaemic response. Neural, mechanical, metabolic, and shear stress related factors are now the most popular ideas explaining these changes. All of these are briefly discussed below; particular attention is paid to the metabolic aspects.

### 1.2.1 Neural control

Bernard, in the mid 1800s observed that transection of the cervical spine caused a significant fall in arterial blood pressure (ABP) (139); and Gaskell while working on frog's mylohyoid muscles observed that sectioning of nerve always caused muscle reddening (138). Gaskell's examinations revealed rapid dilatation of the arteries peaking 20 – 30 seconds (s) after sectioning, but by 4 – 5 minutes (min), dilatation waned until the original diameter was reached (138). These observations pointed towards the neural control of local circulation and showed an active basal sympathetic vascular tone. These early experiments paved way for the neurological understanding of the cardiovascular system.

Exercise hyperaemia occurs almost instantaneously with the onset of exercise (192). Following is the discussion regarding the three main ideas that explain neural control of exercise hyperaemia. Firstly, it is hypothesized that active dilator fibres in the neural pathway cause vasodilatation of exercise hyperaemia (192). In agreement with this hypothesis is evidence which shows that mammalian skeletal muscle in some species is innervated by sympathetic vasodilator nerves (1, 38, 41, 85, 121). Wilkins and Eichna showed that the emotional stress induced by performing arithmetic led to an increase in forearm blood flow (FBF) (26). It was suggested that similar to animals (1, 38, 41, 85, 121), sympathetic cholinergic vasodilator nerves were responsible for this observation in humans. Later, Barcroft and Edholm's work on post-haemorrhagic syncope in normal and sympathectomised individuals led to a similar conclusion (21). However, the lack of any anatomical / histochemical evidence of these nerves in humans has cast doubt on this hypothesis (191, 192). Evidence also shows that FBF responses to mental stress in humans are nitric oxide (NO) dependent; not the result of an active neural dilatation (89, 191, 336). Simultaneous measurement of FBF and muscle sympathetic nerve activity (MSNA) during mental stress in humans has revealed a decrease in vascular resistance that is associated with a parallel decrease in MSNA; dilatation is attenuated after selective β-adrenergic blockade (151). Separately however, Carter and Ray's work revealed that during five min of mental stress, arm vascular resistance decreased while no such change occurred in the calf vascular resistance; MSNA activity during this time failed to change in either limb (53). Therefore, collectively these data do not implicate sympathetic influences as a primary cause of active vasodilatation during mental stress.

The second theory is that the motor pathways responsible for muscle contraction "spill-over" acetylcholine (ACh), and thereby contribute to exercise hyperaemia (195). This pathway would ensure vasodilatation is localized to the active musculature and would be in accord with the near-

instantaneous exercise hyperaemia. Work on hamster retractor muscle preparations by Segal et al. showed that arterioles dilated in response to micro-iontophoresis of ACh, and this dilatation travelled into the feed artery (195, 429). More importantly, their work also showed that when muscle contractions were abolished by tubocurarine (nicotinic-receptor antagonist), stimulation of the motor nerve still resulted in dilatation of feed arteries and arterioles; muscarinic antagonist atropine inhibited this phenomenon (429). However, Buckwalter et al. failed to observe any change in exercise hyperaemia of dogs with atropine infusion, including the response time to peak hyperaemia (38, 40). The same group also showed that ganglionic blockade with hexamethonium (nicotinic-receptor antagonist) or atropine did not alter the rate and magnitude of exercise hyperaemia (36). Moreover, when muscle contractions are inhibited by the use of neuromuscular inhibitors in anesthetized dogs, electrical stimulation of nerves does not lead to any change in blood flow (9, 296). These results are consistent with the lack of any observable difference in exercise hyperaemia in conscious rats following atropine infusion (11). Similarly, intra-arterial infusion of atropine did not attenuate exercise hyperaemia induced by single handgrip contraction at 20% maximum voluntary contraction (MVC) (32). Moreover no change occurred in blood flow of the arm when subjects attempted to exercise following post-synaptic neuromuscular blockade with pipecuronium (105). Thus, it appears from these studies that ACh spill-over is not a major contributor to exercise hyperaemia in humans. The disparity between the findings of Welsh and Segal and others might lie between methods of motor nerve stimulation (field vs. remote or voluntary stimulation; i.e., very few arterioles are in the close proximity of neuromuscular junction for ACh to have a significant effect).

The third neurogenic theory of exercise hyperaemia relies on the withdrawal of sympathetic vasoconstrictor influences during exercise. However, in both active and inactive muscle mass, MSNA is known to increase during exercise in an intensity-dependent manner (158, 159); i.e., an

increase in the vasoconstrictor influence. Indeed, although exercise hyperaemia may well be mainly due to local metabolic vasodilator influences (See below) (92, 93, 158, 343, 414), sympatho-excitation does impose vasoconstriction during exercise. An early study observed that local administration of phentolamine (non-selective α-adrenoceptor antagonist) during isometric exercise led to an increase in FBF (432). Further, in dynamically exercising dogs, iliac artery injection of selective $\alpha_1$-adrenergic antagonist prazosin increased local vascular conductance (39). Since these studies, $\alpha_1$ and $\alpha_2$ adrenergic-receptors have been identified as important mediators of sympatho-excitation related vasoconstriction (37, 154). However, inhibition of sympathetic vasoconstriction due to vasodilatory influence of metabolites (i.e., functional-sympatholysis) also appears to be intensity-dependent, beginning at exercise intensities of 10 – 20% MVC (158, 414). This important response helps maintain perfusion through the exercising musculature. Separately, increased central venous pressure leads baro-receptor loading that is shown to consequently reduce exercise associated increase in MSNA (330).

Therefore considering afore mentioned studies, it is to reasonable assume lack of a significantly active neural contribution to exercise hyperaemia.

### 1.2.2 Mechanical control

The “Muscle-pump” as a mechanical phenomenon that regulates blood flow was initially mentioned by Hunter who described its contribution in reducing orthostatic oedema (351). Put simply, the hypothesis proposes that muscle contraction mechanically expels blood from the vasculature towards the heart, and the subsequent relaxation assists flow into the muscles due to the change in perfusion pressure (i.e., arterio-venous gradient) (86, 227). In theory this would result in increased limb / MBF with or without vasodilatation.

The extent to which this mechanical action on vasculature contributes to initial and steady-state functional hyperaemia is controversial. Sheriff et al. showed that functional hyperaemia is initiated within 1 – 2 s of contraction (364). A single, passive knee-extension also causes an increase in leg blood flow at a similar rate, consistent with a mechanical component to initial part of functional hyperaemia (328). Further, Wunsch et al. individually tested potassium-chloride (KCl), adenosine, ACh, and NO donor sodium-nitroprusside (SNP) and concluded that it took at least 4 s for any of these substances to cause arteriolar vasodilatation (441). Studies have also shown that the initial rise in blood flow during the first 10 s of exercise is dependent on stride frequency, rather than the contraction intensity (363-365). For example, Sheriff et al. changed treadmill speed and/or inclination for exercising rats and observed an immediate (within 1 s) hyperaemic response, even at -10° incline (363, 365). In humans, Gotshall et al. observed that despite a constant work rate (200 W), an increase in cycling frequency was found to result in an increase in vascular conductance (144). However, $O_2$ uptake measured as arterio-venous difference also increased with the increase in pedal frequency (144).

Another way of investigating the contribution of the muscle-pump to the hyperaemic response is to investigate the role of venous pressure. Shiotani et al. observed that when subjects performed low-intensity (5 Watt; W) cycling exercise for 10 s in an upright position, mean ankle venous pressure decreased, parallel with a 5.3 fold increase in femoral artery blood flow (FABF) (367). However, subjects with venous return incompetence due to heart failure showed a reduced drop in mean ankle venous pressure and only a 1.7 fold increase in FABF. These observations led them to propose that in healthy individuals ~ 67% of hyperaemia was due to the effect of muscle-pump. Recently, Nådland et al. showed a greater rise in FABF of healthy individuals following leg exercise in 30° head-up tilted rather than supine position (294). In agreement, removal of the great saphenous vein from symptomatic varicose vein patients results in a modest accentuation

of the increase in leg blood flow at the onset of exercise; seemingly due to the greater effectiveness of muscle-pump (293). However, it is important to note here that similar to the observations of Gotshall et al. (144), Shiotani et al. (367) observed increased muscle $O_2$ uptake ($VO_2$) during the low intensity 10 s exercise.

Alternatively, evidence for concomitant rapid metabolic vasodilatation at the onset of exercise is also present. Tschakovsky et al. observed that a single handgrip contraction elicited a greater increase in FBF than that evoked by mechanical compression of the forearm to a similar intensity by inflation of a cuff (410). The same research group also showed that the immediate hyperaemic response following single handgrip contractions was intensity-dependent (5 – 70% MVC); venous emptying was already maximum at 5% MVC (412). Further, Shoemaker et al. found the early component of the hyperaemia to be related to handgrip contraction intensity rather than frequency (369).

Pharmacological investigations have also revealed some interesting results. For example, Dobson and Gladden observed that if muscle blood vessels were already dilated due to a combination of localized hypoxia along with adenosine and SNP infusions, electrically stimulated isometric tetanic contractions of gastrocnemius plantaris muscles caused a decrease in blood flow (95). In agreement, when Hamann et al. infused their dogs with adenosine in one hind leg and saline in the other, treadmill exercise did not further increase blood flow in the hind limb with adenosine infusion; control leg showed normal hyperaemic response (155). Such results indicate that muscle-pump does not play a major role in the regulation of exercise hyperaemia. In a different study, continuous intra-arterial infusion of potassium ($K^+$) used as an *in-vivo* clamp for membrane depolarization virtually abolished all of the hind limb exercise hyperaemia elicited by 1 s tetanic contraction in dogs (153). Nonetheless, ~ 10% increase in blood flow was still recorded.

It is important to mention here that studies which utilize electrical stimulation of the motor nerve to evoke muscle contraction can be criticised because they cause un-physiological fibre recruitment patterns (228, 413). Further, use of conscious quadrupedal animals might fail to reveal a significant muscle-pump effect because positioning of limb below the heart increases limb volume and venous pressure, making it more difficult for muscle contraction to increase the pressure gradient across muscle (228, 369, 410, 412, 413).

On balance however, it is likely that both muscle-pump and metabolic factors contribute to exercise hyperaemia, including the initial hyperaemic response; but the part played by the muscle-pump is likely to be minor under most circumstances and unlikely to be $O_2$-dependent.

### 1.2.3 Metabolic control

The idea that metabolic products of muscle contraction influence vascular tone was proposed in 1870s by Gaskell (136, 137). This implies that not only is hyperaemia proportional to the influence of vasoactive metabolites, but its magnitude is proportional to the metabolic demands of the working muscles. Since Gaskell's early work, our understanding of the vasoactive metabolites has evolved considerably to include substances produced from muscles, vascular endothelium, red blood cells (RBCs), and those carried and delivered by blood (193). In 2007, Joyner and Wilkins republished the criteria describing a potential vasodilator substance; originally written by Shepherd in Handbook of Physiology (193). These are:

1. *The substance(s) or its precursor(s) should be present in skeletal muscle (or perhaps blood or nerves).*
2. *The substance(s) should have access to the muscle resistance vessels.*
3. *The concentration in the interstitial fluid (or at the endothelium) must be sufficient to cause dilatation and the concentration should be proportional to the contractile activity.*

4. *Exogenous administration of the substance(s) should be capable of causing prolonged dilatation without sensation in humans.*
5. *Pharmacological agents or physiological manoeuvres which modify the blood flow responses to exercise should also modify the dilator responses to any putative substance given exogenously.*

Considering these criteria, the following discussion systematically addresses each major vasoactive metabolite's contribution to hyperaemia, their interdependency, and the issue of redundancy. Furthermore, the recent evidence that the synthesis, release, and/or action of certain vasoactive metabolites changes with age is also addressed.

#### 1.2.3.1 Oxygen ($O_2$)

Exercise is not only accompanied by a hyperaemic response but $O_2$ uptake of the working muscles also increases; unsurprising considering its importance to metabolism. Early studies into the effect of exercise on partial pressure of $O_2$ ($PO_2$) concentrations used $O_2$-electrodes. Gorczynski and Duling observed that the twitch contractions of hamster cremaster muscle were accompanied by arteriolar dilatation that was restricted to the region of muscle that contracted, while tissue $PO_2$ declined (143). However, peri-arteriolar $PO_2$ recorded with an $O_2$-electrode placed immediately adjacent to the arteriole, remained unchanged. Superfusion of the exposed area with hyperoxic solution decreased resting arteriolar diameter, and increased tissue $PO_2$, but also largely prevented the fall in tissue $PO_2$ during twitch contractions and attenuated the arteriolar dilatation. These observations led Gorczynski and Duling to propose that ~ 38 – 55% of the vasodilator response was due the decline in tissue $PO_2$. They suggested an indirect $O_2$-dependent mechanism was responsible for exercise hyperaemia but did not propose where the substance/s might be released from (143).

Subsequently, Marshall and Tandon reported that venules and arterioles in the spinotrapezius muscle of the rat, showed active dilatator responses, which were graded with twitch frequency, and were localised to the region of muscle that was made to contract (250). Further, Lash and Bohlen again using an *in-vivo* preparation of rat spinotrapezius muscle showed that periarteriolar $PO_2$ remained unchanged with exercise but perivenular $PO_2$ decreased significantly (226). This led them to suggest that the change in $PO_2$ on the venous side was the cause of the observed vasodilatation. In humans, Bylund-Fellenius et al. showed by inserting an $O_2$-electrode in the medial-gastrocnemius that exercise at 8 kg workload caused ~ 70% decline in muscle $PO_2$, which was even more pronounced at the higher workload (12 kg) (47).

Since these early observations, studies have utilized proton nuclear magnetic resonance spectroscopy ($^3$H-NMR) to detect myoglobin de-oxygenation. This technique sheds important light not only on intramuscular $PO_2$, but also on the potential source of $O_2$-dependent factors that contribute to hyperaemia. Richardson et al. observed that at rest, human skeletal muscle intracellular $PO_2$ under normoxic conditions is ~ 34 mmHg (339). When competitive cyclists performed graded knee-extension exercise at 25 to 100% MVC, venous $PO_2$ decreased progressively with each increment in exercise intensity (338); mean capillary $PO_2$ declined from ~ 44 mmHg at rest to ~ 37 mmHg at maximal exercise (338, 339). However, quadriceps muscle myoglobin $PO_2$ decreased to 3.1 mmHg within the first 20 s and remained as such until exercise ceased (338). In a subsequent study, comparable decrease in intracellular $PO_2$ (~ 4 mmHg) occurred in recreationally active men performing a similar exercise protocol (337). Further, in a study performed by Soller et al. rhythmic handgrip contractions performed at 15, 30, and 45% MVC by untrained subjects caused a fall in interstitial $PO_2$ within flexor digitorum-profundus to ~ 8 mmHg at 15% MVC, and to a near zero at 30 and 45% MVC (376). Collectively, these studies show that intramuscular $PO_2$ declines profoundly during exercise, raising the possibility that $O_2$-

dependent substances that contribute to exercise hyperaemia are released from skeletal muscle fibres.

Such pronounced changes in tissue $PO_2$, particularly towards the venous end of the capillaries and in the intramuscular $PO_2$, in both in animals and humans, suggest that substance/s are generated and/or released at these locations when $PO_2$ falls; i.e., $O_2$-dependent substances may contribute to the hyperaemic response. In fact, Saito et al. evoked twitch contractions of hamster cremaster muscle by electrical stimulation with and without disruption of venular endothelium and showed that this significantly attenuated the dilator response of the arteriole that was running alongside the venule (354). The most likely explanation of this observation is the diffusion of vasodilator substances from venules towards arterioles (169).

Given the evidence that decline in tissue and/or venular $PO_2$ results in vasodilatation during exercise, several studies have changed inspired $O_2$ content as a way of investigating the influence of $PO_2$ on vasodilatation and hyperaemia. For example Rowell et al. showed that the increase in leg blood flow and vascular conductance were higher when dynamic quadriceps exercise was performed during hypoxia (10 – 11% inspired $O_2$), than normoxia (352). Similarly, Koskolou et al. found that breathing 11% $O_2$ increased leg blood flow and conductance during sub-maximal exercise (221). By contrast, Richardson et al. showed no difference in leg blood flow between normoxia and hypoxia (12% $O_2$) during single leg dynamic quadriceps exercise; although there was an increase in $O_2$ extraction during hypoxia (340) Thus, collectively these studies suggest that the magnitude of exercise hyperaemia is enhanced when the fall in tissue $PO_2$ is accentuated.

Supplementary $O_2$ has also been administered to healthy subjects as an alternative way of investigating the role of $O_2$ in exercise hyperaemia. Welch et al. observed that hyperoxia (100%

$O_2$) during cycling exercise at 55 – 70% of maximal aerobic capacity ($VO_2$-max) caused an 11% decline in the exercise-induced increase in leg blood flow (428). As ABP did not differ between conditions, this reflected a smaller fall in vascular resistance. A similar attenuation of exercise hyperaemia (~ 12%) was also recorded when 60% $O_2$ was used during knee-extensor exercise (316). Alternatively, Macdonald et al. did not find any significant changes in leg vascular resistance when subjects breathed 14, 21, or 70% $O_2$ during dynamic leg exercise (242). However, their data did show the expected changes in blood flow, femoral artery diameter, cardiac output, and heart rate (HR): i.e., both during rest and exercise, hypoxia and hyperoxia induced an increase and decrease in these responses respectively. However, their data are not conclusive as the group size was only 6 subjects and the variability of the data was high.

The effect of modest hyperoxia on *post*-exercise hyperaemia has been investigated by our group. Win and Marshall who used isometric handgrip exercise at 60% MVC found that 40% $O_2$ had no effect on baseline FBF or forearm vascular conductance (FVC), but caused ~ 30% reduction in *post*-exercise hyperaemia; similar to that observed with lone cyclooxygenase (COX) blockade (See Section 1.2.3.4 for further discussion) (437). Further, in a separate study, Fordy and Marshall asked subjects to perform two exhaustive isometric handgrip contractions at 100% MVC separated by 7 min of recovery (123). Subjects either breathed 40% $O_2$ during the handgrip contractions or during the recovery period between them while normoxia was used as a control. They observed that breathing 40% $O_2$ during contraction attenuated the increase in FVC by ~ 25%. By contrast, when 40% $O_2$ was breathed during the 7 min between two exercise bouts, this had no effect on the hyperaemia. These studies suggested that exercise hyperaemia is mediated by $O_2$-dependent vasoactive metabolites that are generated and released *during* the period of exercise and continue to act when contraction ceases. Based on the preceding discussion, it is possible that during isometric contraction at least, $O_2$-dependent metabolites may be released

from the blood vessels and/or in the contracting muscle fibres. Comparable experiments using a relatively modest concentration of supplementary $O_2$ have not been done during rhythmic contractions when $O_2$ delivery would be compromised less by the mechanical effect of muscle contraction. Further, no attempt has yet been made to determine whether breathing 40% $O_2$ does in fact raise the $PO_2$ in exercising muscle, or prevent it from falling to such low levels during exercise.

### 1.2.3.2 Adenosine and adenine nucleotides

The idea of adenosine and adenine nucleotides contributing towards exercise hyperaemia comes from the "adenosine hypothesis". Berne proposed that the mismatch between supply and demand of $O_2$ led to adenosine tri-phosphate (ATP) breakdown in working cardiac muscles (25). Additional breakdown of adenosine di-phosphate (ADP) and adenosine mono-phosphate (AMP) augmented adenosine concentrations, which moved out of the cardiac cells and caused vasodilatation. The subsequent increase in blood flow alleviated the decline in $O_2$, thereby prevented further nucleotide breakdown. In 1971, Dobson et al. extended this hypothesis to propose a similar role for adenosine in exercise hyperaemia in skeletal muscles (94).

#### 1.2.3.2.1 Adenosine

Interstitial adenosine is mainly produced by skeletal and smooth muscle cell membrane bound 5'ectonucleotidase (164, 314, 315). However, adenosine and its nucleotides are also released into the interstitium from motor nerves upon their activation (80). On the other hand, intravascular adenosine is most likely produced by soluble and endothelial 5'ectonucleotidase from ATP that is released from deoxygenated RBCs (112, 167, 314, 315) and from endothelial cells when they become hypoxic (407). Adenosine is also released from endothelial cells when $PO_2$ falls as a

consequence of the competition between $O_2$ and NO for the same binding site on cytochrome-oxidase; ATP synthesis is attenuated and adenosine is generated (109).

Venous adenosine concentrations increase substantially following electrical muscle stimulation (18, 130). Comparison of the vasodilatation associated with muscle contractions, and that with adenosine infusion, with the arterio-venous difference in adenosine concentrations showed that ~ 15% of vasodilatation at 1 min and 40% between 5 and 20 min were due to the contribution of adenosine (18). Similarly in rats, Ray and Marshall have also observed an increased contribution of adenosine towards hyperaemia in the later part of 5 min exercise (333). In humans, adenosine concentrations increase both in skeletal muscle and the peritendinous connective tissue in response to planter-flexion exercise (223).

Measurements made with microdialysis showed that exercise causes an increase in interstitial adenosine and nucleotide concentrations (70, 165, 223). Thus, following 10 W knee-extension exercise, Hellsten et al. observed a near 5 fold increase in the concentrations of vastus-lateralis interstitial adenosine and its precursor nucleotides (AMP, ADP, and ATP): only modest further increases being observed with greater workload (165). Nonetheless, the rate of increase was proportional to the exercise-intensity and a good correlation ($r = 0.98$) was found between interstitial adenosine and blood flow; consistent with adenosine being causally implicated in the vasodilatation. Interestingly, Costa et al. measured flexor digitorum-superficialis interstitium concentrations while their subjects performed low-intensity (15% MVC) rhythmic handgrip contractions without or with ischemia (70). Consistent with the idea that tissue hypoxia is causally important in the release of adenosine during muscle contraction, adenosine concentrations were significantly higher during ischemic exercise.

Early studies showed that adenosine-deaminase reduced exercise hyperaemia in dog and cat oxidative muscles (210, 360). On the other hand, increasing adenosine concentrations by either preventing adenosine reuptake or its breakdown with dipyridamole and erythro-9(2-hydroxy-3-nonyl)adenine respectively, augmented the hyperaemic response (210, 229). In humans, even theophylline or aminophylline (theophylline-ethylenediamine) which are rather weak adenosine-receptor antagonists that have no selectivity between adenosine-receptor subtypes, and that inhibit phosphodiesterase activity (249), significantly attenuate adenosine-induced vasodilatation (392) and exercise hyperaemia (327). However, their weak receptor antagonism makes it impossible to deduce the full extent of adenosine's involvement in the exercise hyperaemia in people.

Adenosine-receptor antagonists that are selective between the receptor subtypes are not licensed for use in humans; however they have been used in studies on cats and rats. Thus, Poucher observed in the cat that exercise hyperaemia was attenuated by ~ 30% following selective $A_{2A}$-adenosine receptor blockade with ZM241385 and also following the non-selective adenosine-receptor blockers theophylline and 8-phenyltheophylline (8-PT) (324). This indicates that the adenosine component of exercise hyperaemia is mainly due to stimulation of $A_{2A}$-receptors. Similarly, Ray and Marshall (333) showed that ZM241385 and 8-sulphophenylthrophylline (8-SPT; non-selective adenosine-receptor blocker) had comparable attenuating effects on exercise hyperaemia of twitch and tetanic hindlimb contractions by 14 and 25% respectively (333). In humans, immnunohistochemical examination of skeletal muscle biopsy samples by Lynge and Hellsten showed that $A_1$, $A_{2A}$, and $A_{2B}$ receptors are all localized to endothelial and vascular smooth muscle cells (241). However, skeletal muscle cell plasma membrane and cytosol are devoid of $A_1$-receptors. Thus, it may be that the $A_{2A}$-receptor is functionally important in exercise hyperaemia in humans as well, but as yet this cannot be tested.

It should be noted here that exogenous adenosine does not produce vasodilatation in all individuals (251-253). Martin et al. investigated exercise hyperaemia of handgrip exercise performed at 3 different intensities (7, 14, and 21% MVC) with intra-arterial adenosine infusions (252). In some individuals (responders), both adenosine infusion and exercise resulted in similar hyperaemic response. However in others (non-responders), adenosine infusion was accompanied by blunted FBF responses compared to exercise hyperaemia. Nitric oxide synthase (NOS) inhibition however attenuated exercise hyperaemia in both groups. In a later study, the same group also observed that aminophylline attenuated adenosine infusion hyperaemia only in responders (253). Moreover while lower intensity exercise hyperaemia was not attenuated by aminophylline in some individuals, this was not the case at higher workload (21% MVC); aminophylline equally attenuated exercise hyperaemia in both responders and non-responders. This suggests an intensity-dependent contribution of vasoactive adenosine in exercise hyperaemia regulation. When dipyridamole was used to block adenosine transport across cell membrane, adenosine infusion hyperaemia was similar in both groups; suggesting increased activity of nucleoside transporters in non-responders (251).

Consistent with the idea of adenosine being a metabolite that is released in muscle as a consequence of a fall in $PO_2$, adenosine has been implicated in the muscle vasodilator responses evoked by systemic hypoxia (71, 237, 265, 274, 297). Using intra-vital microscopy, Mian and Marshall showed that during systemic hypoxia (6% $O_2$) some arterioles and venules supplying rat spinotrapezius dilated while others constricted (265). Topical application of 8-PT attenuated dilator responses in arterioles and venules, but had no effect on the constricting vessels. The authors therefore suggested that the vasodilating adenosine was produced locally by vascular 5'nucleotidase. In agreement with this, Mo and Ballard showed in dogs that under systemic hypoxia, venous adenosine concentrations increased while interstitial adenosine

remained unchanged (274). However, during muscle contraction, adenosine concentrations increased both in venous blood and interstitium. Similar observations were made by Lo et al. in rat muscle (237), while Costa et al. showed that 3 min of forearm ischemia had no effect on interstitial adenosine concentrations but increased venous adenosine by two-fold (71).

Studies performed with selective adenosine-receptor antagonists in the rat have shown that during systemic hypoxia, adenosine's dilatation is largely due to $A_1$-receptor stimulation with some input from $A_{2A}$-receptors during severe hypoxia (34, 35, 243, 331, 334, 425). These results are consistent with the adenosine-receptor localization study performed by Lynge and Hellsten (241). Although adenosine may act preferentially from the interstitium to induce dilatation during muscle contraction as discussed above, the evidence that adenosine can also be released by vascular endothelium when $PO_2$ falls, leaves the possibility that adenosine may also be released from the endothelium during exercise; probably from the capillaries and venules, given that peri-arteriolar $PO_2$ does not fall during muscle contraction (143, 226). Indeed, Hester has shown that application of adenosine in a venule leads to the dilatation of a paired arteriole (168). Clearly, these ideas cannot be tested in humans at the current time due to the lack of $A_1$- or $A_{2A}$-receptor antagonists for human use. However, they could be more fully tested in rats.

#### 1.2.3.2.2 ATP

In 1969 Forrester and Lind measured arterial and venous ATP concentrations during and following sustained handgrip exercise and noticed a significant increase in plasma ATP particularly in the veins (124) In 1992 Bergfeld and Forrester showed that human RBCs release ATP under hypoxic / hypercapnic conditions (24). These findings were later confirmed by Ellsworth et al. who by using hamster cheek pouch muscle also observed significant vasodilatation in response to the application of intra-luminal ATP (112). Therefore, it was suggested that RBCs not only deliver $O_2$ to working muscles but also act as indirect sensors /

modulators of vascular tone (24, 112). Work done by Jagger et al. showed that ATP release from RBCs was due to the change in the state of the haemoglobin; i.e., from relaxed liganded oxygenated to deoxygenated (186). They lowered $O_2$ saturation ($SO_2$), $PO_2$, and used carbon-monoxide (CO), which has a higher affinity for haemoglobin than $O_2$ to prevent any change in haemoglobin conformation. The results revealed that CO lowered the ATP release from RBCs when $PO_2$ was decreased. Furthermore, ATP concentrations correlated better with changes in $SO_2$ than $PO_2$ ($r^2$ = 0.88 vs. 0.54), confirming the release of ATP from erythrocytes was due to the change in their oxygenated state.

Subsequently, Gonzalez-Alonso et al. investigated the contribution of human erythrocytes and their release of ATP to exercise hyperaemia evoked by incremental knee-extension during normoxia, hypoxia (10%), hyperoxia (100%), and CO with normoxia (142). They showed that venous ATP efflux increased with incremental exercise, but ATP concentrations and muscle vascular conductance were significantly higher when exercise was performed during hypoxia than during normoxia. Whereas both hyperoxia, and CO with normoxia decreased plasma ATP release during exercise. These findings are entirely consistent with the role of RBCs as $O_2$ sensors / vascular modulators, and consistent with ATP contributing to exercise hyperaemia (142).

Recently, it has been suggested that mechanical deformation / shear stress also act as stimuli for ATP release. Mortensen et al. investigated this using passive leg-movement without and with rapid sphygmomanometer cuff compressions (60/min) around the thigh (290). Despite an increase in vascular conductance, venous ATP concentration increased only when vascular compression was applied.

Extra-luminal application of ATP is known to result in vasoconstriction, whereas intra-luminal application causes concentration-dependent vasodilatation (44, 254). Thus, the ATP that is released into interstitium during exercise (165) is unlikely to *directly* contribute to exercise hyperaemia; it must act from inside the luminal side. Indeed, intravascular ATP acts on endothelial and smooth muscle $P_{2Y}$-receptors to cause vasodilatation (44, 286, 344, 444). However, Mortensen et al. showed that in humans these receptors are actually present on endothelium, smooth, and skeletal muscle cells (286). Moreover, they showed that the vasodilatation induced by ATP infusion was not attenuated following theophylline; indicating that dilatation by ATP is not solely by its degradation to adenosine (286). The contribution of ATP to exercise hyperaemia is difficult to test directly because there are no ATP-receptor antagonists that are selective between the P2-receptor subtypes. However, Mortensen et al. did show that reactive blue, a putative P2-receptor antagonist, attenuated exercise hyperaemia in miniature swine during treadmill exercise (288).

In summary, it is clear that the interstitial and plasma adenosine and adenine nucleotide concentrations increase in response to exercise. Moreover, adenosine-receptor blockade attenuates exercise hyperaemia, and the evidence suggests it acts largely from the extra-luminal surface of arterioles but may also be released and act from endothelial cells on the venous side of the capillary bed. Although ATP is also released into plasma from RBCs when haemoglobin off-loads $O_2$; whether it plays a significant role in exercise hyperaemia has yet to be finally established but it seems very likely. A number of studies have suggested that adenosine and ATP work interdependently with other vasoactive substances, including NO and prostacyclin ($PGI_2$) (65, 101, 156, 254, 286, 289, 304, 306, 332, 333, 444). This evidence is considered below in the relevant sections.

### 1.2.3.3 Vasoactive ions

#### 1.2.3.3.1 Lactic acid

Lactate is produced due to anaerobic glycolysis; its efflux into the vasculature is linearly correlated with the concentrations in the muscle (20, 63). The increase in interstitial and circulating lactate during exercise has led to consideration of whether it plays an active role in exercise hyperaemia. Its exact mechanism of action is not clear, but the cyclic guanosine mono-phosphate pathway, $K^+$-channels, and neuronal NOS (nNOS) have all been implicated. In the study performed by Chen et al., lactate application to isolated rat cremaster arterioles led to their dose-dependent dilatation which was not affected by COX blockade (indomethacin), NOS blockade ($N^G$-nitro-L-arginine; L-NNA), their combination, or by the removal of endothelium (60). However, it was inhibited by the cyclic guanosine mono-phosphate blockers methylene-blue and 6-anilino-5,8-quinolinedione. Further, Mori et al. showed that KCl contracted pig coronary arteries dilated in a dose-dependent manner in response to lactate; calcium ($Ca^{2+}$) activated $K^+$-channel ($K_{Ca}$) blockade with tetraethylammonium reversed the vasodilatation, whereas the ATP-sensitive $K^+$-channel ($K_{ATP}$) blocker (glibenclamide) did not (282). $K_{Ca}$-channel activation was also induced by lactate in coronary smooth muscle cells under patch-clamp technique (282). On the other hand, Mendrinos et al. used NOS antagonist $N^{\omega}$-nitro-$_L$-arginine (L-NAME) in an *in-vivo* porcine model to investigate the lactate induced retinal arteriolar dilatation (260). Some pigs received an intra-venous L-NAME infusion for an hour followed by lactate infusion into the vitreous-humour using a micropipette. 10 min later, a juxta-arteriolar intra-vitreous infusion delivered L-NAME into the eyes. Pigs without NOS blockade only received lactate injection which resulted in reproducible retinal arteriolar dilatation, a response similar to those receiving intra-venous L-NAME. However, intra-vitreous L-NAME infusion reversed the vasodilatation. This implicates nNOS in the lactate induced vasodilatory response.

However, the major problem with lactate as an important contributor to exercise hyperaemia is the lack of any temporal relationship between its concentrations and blood flow (205, 239). By using microdialysis, Lott et al. showed that quadriceps exercise performed at 30 and 60% MVC resulted in similar increases in interstitial lactate concentration, and during recovery limb blood flow declined whereas interstitial lactate concentrations continued to increase (239). Separately, although Fordy and Marshall observed that 40% $O_2$ attenuated venous lactate concentrations of 100% MVC but the increase in these concentrations was completely unrelated to FBF; i.e., by 7 min *post*-exercise, FBF returned to baseline while venous lactate still remained significantly elevated (123). Additionally, McArdle's syndrome patients who do not produce much lactate have exercise hyperaemic responses that are similar in magnitude to those observed in normal individuals (205). Therefore, even though lactate can induce dilatation, it seems unlikely that lactic acid makes an essential contribution to exercise hyperaemia in an $O_2$-dependent or independent manner.

#### 1.2.3.3.2 Hydrogen ions ($H^+$)

Exercise leads to a decrease in interstitial and intravascular pH (20, 239); raising the possibility that $H^+$ may be involved in the active regulation of the vascular tone. Indeed, hypercapnic and normocapnic acidosis evoke dilatation of the isolated rat cerebral arteries with a concomitant decrease in smooth muscle intracellular $Ca^{2+}$ (318). This vasodilatation is understood to involve $K_{Ca}$, $K_{ATP}$, and NO (58, 235). For example, when Lindauer et al. decreased extra-luminal pH of isolated rat cerebral arteries from 7.4 to 7.0, their diameter increased ~ 35% (235). $K_{Ca}$-antagonist etraethylammonium attenuated this dilatation and it was then abolished by $K_{ATP}$-channel blockade with glibenclamide. Recently, Celotto et al. observed attenuation of acidosis induced dilatation of rat aortic segment with L-NAME; glibenclamide and small conductance $K_{Ca}$-

channel inhibitor apamine on their own or in combination with L-NAME nearly abolished the dilator response whereas indomethacin had no such affect (58)

Despite these observations, *in-vivo* evidence for a direct correlation between pH and the magnitude or pattern of exercise hyperaemia has not been produced. Lott et al. observed that despite a clear difference in blood flow when leg exercise was performed at 30 or 60% MVC, vastus-lateralis interstitial pH concentrations were fairly similar (239). In a different study, interstitial pH concentrations declined progressively with incremental leg exercise but this decline continued even after the end of exercise; peak acidification occurring 1 min after cessation of exercise (388). The inescapable conclusion of these studies is that although pH changes do modify vascular tone, a direct / significant role for $H^+$ in exercise hyperaemia is unlikely.

#### 1.2.3.3.3 Inorganic phosphate (Pi)

Pi has also been implicated in exercise hyperaemia. In a series of elegant experiments performed by Hilton et al. in 1978, they observed that contraction evoked stimulation of the motor nerve to cat gastrocnemius muscle resulted in increased venous efflux of Pi; concentrations of venous Pi were closely related to the changes in blood flow (176). Further, when Pi was applied topically on the spinotrapezius muscle, this evoked arteriolar dilatation with a latency of ~ 5 s.

However, the study of Lott et al. mentioned above also measured interstitial Pi in quadriceps during 5 min single leg knee-extension exercise performed at 30 and 60% MVC (239). They found that although the hyperaemia was greater at the higher workload, the interstitial Pi concentrations were similar. Moreover when exercise hyperaemia was decreasing at the end of exercise, interstitial Pi was still increasing. This dyssynchrony between exercise hyperaemia and interstitial Pi concentrations argues against an important role for Pi in exercise hyperaemia. However, its contribution is difficult to test as there is no obvious antagonist.

#### 1.2.3.3.4 Potassium ($K^+$)

Active vasodilatation induced by $K^+$ has been proposed for a long time. The earliest unambiguous experiments were published by Dawes in 1941 (84). He showed that KCl infusion into the external iliac artery resulted in cat and dog hindlimb vasodilatation. Later Kjellmer observed $K^+$ release from working muscles into venous blood; concentrations being accompanied by a proportionate vasodilator response (216). This correlation between increased interstitial and venous $K^+$ with appropriate vasodilatation has been confirmed by a number of other studies (10, 208, 239). Evidence has also been presented that $K^+$ concentrations in interstitium and venous blood are related to the amount of work done (194, 239).

Importantly, when Kiens et al. measured venous concentrations of a number of metabolites during quadriceps contraction performed at 10% MVC for 5 or 30 s, it was shown that shorter 5 s contraction significantly increased venous $K^+$ only, but 30 s contraction increased concentrations of lactate, Pi, and $K^+$ (208). As hyperaemia accompanied both contractions, $K^+$ was suggested as a potential mediator of initial increase in blood flow. This case is strengthened by the observations of Armstrong et al. (10). They stimulated hamster cremaster muscle preparations at multiple frequencies (4 to 80 Hz; 250 ms) and showed that the rapid dilatation of arterioles (at 4 s) was attenuated by ~ 60%, by either 3,4-diaminopyridine (voltage-dependent $K^+$-channel antagonist) which was expected to prevent release of $K^+$ from the muscle fibres, or by ouabain ($Na^+/K^+$-ATPase antagonist), or barium-chloride (inward-rectifying $K^+$-channel antagonist) which were expected to interfere with the action of $K^+$ on the vascular smooth muscle (10, 43). Perhaps the role of inward-rectifying $K^+$-channels in $K^+$ induced vasodilatation may be more important as mice deficient of these channels lack a $K^+$ induced vasodilator response (445). However, the release of $K^+$ from muscles during action potentials does seem unlikely to be $O_2$-dependent.

### 1.2.3.4 Prostaglandin (PG)

Arachidonic acid (AA) is released from the membrane phospholipids by phospholipase enzymes; acted on by COX to produce vasoconstricting endoperoxides, prostaglandin $G_2$ ($PGG_2$), and the subsequent $H_2$ ($PGH_2$) (63, 277-279). These intermediates are further acted upon by specific synthases to produce an array of vasodilators and constrictors; including prostaglandins $D_2$ ($PGD_2$), $E_2$ ($PGE_2$), $F_{2\alpha}$ ($PGF_{2\alpha}$), $PGI_2$, and thromboxane $A_2$ (TX; $TXA_2$) (279, 397). The idea that the potent vasodilators $PGE_2$ and $PGI_2$ contribute to exercise hyperaemia has been in the literature for many years.

In 1976, Moncada et al. published their observations describing the anticoagulant and potent vasodilating properties of a certain PG which they termed X (277). The following year, while describing the production of this compound within the blood vessels, they named it as prostacyclin or $PGI_2$ (278). The mechanism by which it causes vasorelaxation involves adenylyl-cyclase / cyclic AMP transduction system and smooth muscle hyperpolarization (63, 312). Its release in the intravascular compartment occurs from the endothelial cells (266, 306) and is linked to exercise (198, 390), tissue hypoxia (45, 264), presence of the other vasodilators (i.e., adenosine and ATP; See Section 1.2.3.7) (304, 306), and shear stress (See Section 1.2.4) (127). However as $PGI_2$ concentrations also increase in the interstitial compartment in response to exercise and the presence of other exogenous vasoactive metabolites (304, 306); $PGI_2$ release may also occur from the abluminal surface of capillaries and/or from non-endothelial structures (306). Skeletal muscle fibres are understood to predominantly release the vasodilator $PGE_2$ (400): exercise-intensity and tissue hypoxia have been linked to its release (198, 199, 390).

The first study to investigate the role of COX derived vasodilating PGs in human exercise hyperaemia was performed 40 years ago by Kilbom and Wennmalm (209). Using venous

occlusion plethysmography, they showed that rectal administration of indomethacin substantially attenuated peak and total *post*-exercise hyperaemia evoked by 5 min of isometric (15% MVC) and rhythmic handgrip contractions (0.83 and 1.67 W for women and men, respectively). Since this initial study, knowledge of the role of vasodilator PGs in exercise hyperaemia has increased considerably. Cowley et al. showed that COX inhibition with aspirin attenuated *post*-exercise calf blood flow (CBF) following cycling exercise (73, 74) and similar attenuation was observed when COX blockade with aspirin and indomethacin were compared (74). Later, Duffy et al. showed that intra-arterial infusion of aspisol (COX inhibitor) decreased exercise hyperaemia evoked by rhythmic wrist-flexion exercise; this effect was not added to by simultaneous NOS inhibition (100). Similarly Schrage et al. who used Doppler-ultrasound to measure FBF showed that COX inhibition with Ketorolac infusion decreased exercise hyperaemia of relatively weak rhythmic handgrip exercise (20 min, 10% MVC) by ~ 12% (359). Moreover our group reported that COX inhibition attenuated *post*-exercise hyperaemia evoked by isometric handgrip contraction at 60% MVC (437).

By analysing biopsies of cat triceps-surae, which would have included vascular tissue as well as muscle fibres, it was shown that isometric contractions increased the concentrations of both $PGE_2$ and 6-keto-$PGF_{1\alpha}$, the stable metabolite of $PGI_2$, (by 45 and 53%, respectively) (390). The study also observed that a separate low intensity isometric contraction with arterial occlusion increased $PGE_2$ by ~ 70%, whereas similar intensity contraction without occlusion failed to change the muscle $PGE_2$ concentration. These results not only suggest an important relationship between $O_2$ availability (tissue hypoxia) and PG production, but also suggest that the release of PGs could be exercise intensity dependent (See below).

In humans, Nowak and Wennmalm observed that exercise performed at 75% $VO_2$-max significantly increased venous concentrations of PGE (302). In a subsequent study, Wilson and

Kapoor showed that rhythmic wrist-flexion exercise increased both, FBF and venous concentrations of $PGE_2$ and $PGI_2$; indomethacin attenuated exercise hyperaemia by ~ 20% and virtually abolished the release of PGs (435). Further, using microdialysis Karamouzis et al. showed that 60 min of 20 W knee-extensor exercise-induced a significant increase in the interstitial concentrations of $PGE_2$ (199) In their second study, dynamic leg exercise at 100 and 150 W induced an intensity-dependent increase in interstitial $PGE_2$ and 6-keto-$PGF_{1\alpha}$ concentrations, but a parallel decrease in the concentration of the stable derivative of the vasoconstrictor $TXA_2$; $TXB_2$ (198).

The observation of a relationship between exercise intensity and PG release is supported by a number of other studies (29, 447, 448). Boushel et al. measured quadriceps micro-vascular blood flow using near-infrared spectroscopy (NIRS) and found that combined NOS and COX blockade attenuated exercise hyperaemia evoked by graded dynamic knee-extension at 30, 45, and 60 W, but not at 15 W (29). Further, increases in interstitial $PGE_2$ concentrations were measured only at the higher workloads (45 and 60 W). Regarding $PGI_2$, Zoladz et al. recently showed that venous plasma 6-keto-$PGF_{1\alpha}$ concentrations were positively correlated to an individual's $VO_2$-max (447, 448). In their first study, venous plasma 6-keto-$PGF_{1\alpha}$ concentrations of subjects performing exhaustive cycling exercise were higher in subjects who had a higher $VO_2$-max (447). Their subsequent cycling study found that five weeks of endurance training led to an overall increase in $VO_2$-max, power output, and the venous 6-keto-$PGF_{1\alpha}$ efflux of their subjects (448). These observations indicate that the release of both $PGE_2$ and $PGI_2$ during exercise is positively related to the intensity of muscle contractions.

Evidence also suggests that systemic and localized hypoxia increase the synthesis of vasodilator PGs (45, 46, 264, 266), while severe hypoxia / anoxia may prevent $PGI_2$ production by endothelial cells (375). Since extreme intravascular hypoxia / anoxia is not likely during exercise

under normal conditions, the discussion here focuses on PG synthesis in physiological levels of hypoxia. Michiels et al. showed that hypoxia increased PG production in cultured human endothelial cells (266). Moreover, Busse et al. showed that the intra-luminal hypoxia (20 – 40 mmHg) in perfused rat tail and dog femoral artery preparations resulted in their dilatation; response that was abolished by either endothelium removal or COX blockade (45, 46). In a subsequent study, they showed that this hypoxia induced vasodilatation was accompanied by a 2 – 3 fold increase in 6-keto-$PGF_{1\alpha}$ concentrations (45). Similarly, Messina et al. showed that isolated rat cremaster arteries dilated when bath $PO_2$ was reduced from 150 to 15 mmHg, and this dilatation was abolished by either endothelium removal or COX inhibition with indomethacin (264). In accord with these findings, Win and Marshall showed that breathing 40% $O_2$ during isometric handgrip exercise attenuated *post*-contraction hyperaemia to the same extent as COX inhibition (437). Moreover, combined COX inhibition and 40% $O_2$ had no further effect on the response. Based on these observations our group therefore proposed that the contribution of vasodilator PGs to exercise hyperaemia induced by isometric handgrip contractions is $O_2$-dependent.

Despite these observations, a number of studies on the role of PGs in exercise hyperaemia have revealed conflicting results. For example, Mortensen et al. showed that indomethacin infusion had no effect on hyperaemia evoked by knee-extensor exercise at 20% MVC for 5 min (287). Schrage et al. observed 12% attenuation in hyperaemia evoked by 10% MVC handgrip contractions when ketorolac was infused during the contractions, but this reduction was transient in nature (359). By contrast, when the NOS inhibitor L-NAME was infused during the contractions, exercise hyperaemia was attenuated by ~ 20%. Co-infusion of NOS and COX inhibitor decreased FBF by ~ 30%, but again only transiently. The authors therefore proposed that although vasodilator PGs participate in exercise hyperaemia, their contribution is not

essential, and other substances can restore / replace their contribution. Indeed, Shoemaker et al. who also used a similar exercise protocol and methodology failed to observe any attenuation in exercise hyperaemia when ibuprofen was used for COX inhibition (368). The results of both these studies may be explained by the observations that exercise-evoked release of PGs does not occur in all individuals, even when they are performing the same amount of exercise (responders and non-responders) (448), and is minimal or non-existent at light levels of exercise (29). Interestingly, Zoldaz et al. showed that the concentrations of $PGI_2$ released during exercise are increased by endurance training (448). The effect of training on PG production may therefore complicate the outcome of many studies in this field.

In summary, the exact contribution of PGs to exercise hyperaemia in humans is unclear. However, results of most studies indicate that in most individuals, PGs, probably $PGE_2$ and $PGI_2$ contribute significantly, especially during moderate to intense exercise, and that at least one of these PGs makes a contribution that is $O_2$-dependent.

### 1.2.3.5 Nitric oxide (NO)

Ever since the discovery of NO in 1980s (311), its importance as a possible regulator of the vascular tone has increased tremendously. NO is produced from L-arginine by the enzyme NOS, which is present in three different isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (116, 384). In humans the constitutive NOS isoforms, eNOS and nNOS are localized to the vascular endothelium, and to skeletal muscles, neurones, and vascular smooth muscle, respectively (126). Their activation has been attributed to $Ca^{2+}$ concentrations (63, 248, 332). The process of NO production is a two-step procedure requiring NADPH and $O_2$; L-arginine is converted to $N^{\omega}$-hydroxy-L-arginine which is then converted to citrulline and NO (248). Moreover, NO can also bind to haemoglobin as S-nitrosohaemoglobin

which is released in response to RBCs offloading $O_2$ (383). However, deoxyhaemoglobin molecules also produce NO from the nitrate present in the blood (68).

Despite the evidence that NOS activity is increased by acute and chronic exercise (19, 341), studies into the role of NO in active exercise hyperaemia regulation have yielded conflicting results. In humans, Wilson and Kapoor found that despite the decrease in resting FBF induced by $N^{\omega}$-monomethyl-L-arginie (L-NMMA; NOS inhibitor), the magnitude of the exercise hyperaemia of wrist flexion exercise (0.2 and 0.4 W) did not change (434). This suggested no role for NO in exercise hyperaemia. Endo et al. also came to similar conclusions when they asked subjects to perform isometric handgrip contractions for 3 min without and with L-NMMA infusion: the resting and *post*-exercise FBF was decreased, but the magnitude of change in FBF during exercise was similar between conditions (113).

Shoemaker et al. who used Doppler-ultrasound showed that atropine and L-NMMA given together had no effect on exercise hyperaemia evoked by rhythmic handgrip contractions at 10% MVC, but the time course of *post*-exercise hyperaemia was shortened (370). Similarly, Radegran and Saltin discovered that L-NMMA infusion did not attenuate exercise hyperaemia evoked by graded knee-extension exercise, but attenuated the *post*-exercise hyperaemia by ~ 34% (329). These findings suggest that NO is important in regulating resting and *post*-exercise blood flow.

By contrast, a number of older and more recent studies suggest that NO does actively participate in exercise hyperaemia. Gilligan and colleagues who asked their subjects to perform rhythmic handgrip contractions at 15, 30, and 45% MVC (5 s contraction: 15 s relaxation – 5 min each intensity) and measured FBF during relaxation periods between contractions using venous occlusion plethysmography concluded that NOS inhibition by L-NMMA infusion attenuated the increase in vascular conductance by ~ 18% (140). Similarly, Dyke et al. found that FBF was

reduced by 20 – 30% if L-NMMA was infused in the brachial artery of subjects while they performed rhythmic handgrip contractions (1s contraction: 1s relaxation; 15% MVC) (106). However, it could be argued that both studies were reporting on the effects of NOS inhibition on *post*-exercise hyperaemia, for both recorded FBF between rhythmic contractions because they used venous occlusion plethysmography (106, 140).

More recent studies in which Doppler-ultrasound was used to measure blood flow have also suggested an active role of NO in exercise hyperaemia. Schrage et al. reported that L-NAME infused during rhythmic handgrip contractions at 10% MVC attenuated steady-state hyperaemia by ~ 17% (359). Similarly, Casey et al. showed that NOS blockade with L-NMMA attenuated the increase in FVC evoked by rhythmic handgrip contractions at 20% MVC by ~ 20%, and increased the time to reach steady-state hyperaemia; but had no affect on hyperaemia of 10% MVC contractions (55). Further, Wray et al. showed that NOS blockade with L-NMMA attenuated exercise hyperaemia evoked by rhythmic handgrip contractions at 8, 12, 16, 20, and 24 kg; but not 4 kg (440). However, when these data were normalized to take account of the NOS-induced decrease in resting flow, NOS blockade attenuated hyperaemia only at higher (20 and 24 kg) workloads. This suggests an intensity-dependent contribution of NO and raises the possibility that in some of the other studies, exercise intensity was not high enough to engage NO. However, this would contradict with the evidence showing that even low-intensity exercise's hyperaemia is regulated by NO (358, 359).

In conclusion, collectively these results show that NO does contribute towards resting blood flow and active exercise hyperaemia regulation. However, it is interesting to note that no one has so far shown that NO concentrations actually increase in the interstitium or in the venous blood during muscle contraction; i.e. the question whether exercise hyperaemia is NO-dependent or whether NO plays more of a mediating role remains for now unanswered.

### 1.2.3.6 Endothelium derived hyperpolarizing factor (EDHF)

The concept of EDHF originated from the observations that bradykinin and ACh induce endothelium-dependent hyperpolarization and relaxation of porcine coronary artery smooth muscles in the presence of NOS and COX blockade (63, 295). Current consensus is that a number of substances / factors may contribute to the endothelium-dependent hyperpolarization of smooth muscle cells (for review see Feletou and Vanhoutte, 2009) (116). The products of cytochrome-P450 enzyme are discussed here due to their proposed relevance in exercise hyperaemia.

Rosolowsky and Campbell observed that AA induced vasodilatation of bovine coronary arteries was partially inhibited by indomethacin and partially by SKF-525a (cytochrome-P450 inhibitor) (345). Combination of COX and cytochrome-P450 inhibitors completely abolished the response. This implicated cytochrome-P450 in AA-mediated dilatation of vessels. It is also known that epoxyeicosatrienoic-acids are produced from AA metabolism by the cytochrome-P450 pathway (50, 116, 345). Campbell et al. showed that the cytochrome-P450 inhibitors SKF-525a and miconazole attenuated metacholine (muscarinic-receptor agonist) induced dilatation of bovine coronary arteries, and completely abolished smooth muscle hyperpolarization (50). Further, vessels pre-treated with AA released epoxyeicosatrienoic-acids and showed relaxation; a response that was abolished in response to $K_{Ca}$-channel inhibition. Isolated human coronary artery preparations also showed similar smooth muscle cell hyperpolarization involving cytochrome-P450 and $K_{Ca}$-channels (272). Later Halcox and colleagues showed that arterial infusion of bradykinin in the presence of NOS and COX blockade induced an increase in FBF that was inhibited by KCl infusion or miconazole (150). Thus, the results of these studies show that in the absence of the NOS and COX pathways, human forearm smooth muscle cells can show endothelium-dependent hyperpolarization via cytochrome-P450 pathway.

Regarding exercise hyperaemia, Hillig et al. found that when the cytochrome-P450-2C9 inhibitor sulfaphenazole was infused alone during single leg knee-extensor exercise (10 min; near-maximal workload), there was no change in the exercise hyperaemia (175). However, when sulfaphenazole was co-infused with L-NMMA, at a dose that was ineffective in attenuating exercise hyperaemia in a similar protocol (329), then exercise hyperaemia was attenuated by ~ 16% (175). Thus, they proposed that the products of the cytochrome-P450-2C9 and NOS contribute to exercise hyperaemia in an interdependent manner during sub-maximal exercise such that NOS inhibition up-regulates the contribution of epoxyeicosatrienoic-acids. Mortensen et al. reached a different conclusion when they investigated relative weak intensity exercise (287). They found that COX inhibition alone failed to attenuate exercise hyperaemia evoked by knee-extensor exercise at ~ 20% of maximum, combined COX and NOS inhibition attenuated exercise hyperaemia by ~ 36%, and that addition of the $K_{Ca}$-channel tetraethylammonium-chloride inhibitor reduced the hyperaemia by ~ 28% compared to control. These results indicated that at light workloads, epoxyeicosatrienoic-acids do not contribute towards exercise hyperaemia; i.e., their production is not up-regulated by COX and NOS inhibition.

#### 1.2.3.7 Interdependency and redundancy

The complexity of the vasodilators that contribute to exercise hyperaemia becomes clear from the discussion above. Lack of a single essential mediator of exercise hyperaemia means that pharmacological blockade of a single vasodilator during exercise can apparently lead to an increase in the contribution of another. Therefore, due to its importance, the interdependent / redundant relationship that exists between these metabolites is considered separately here, but in brief.

Mortensen et al. noticed that independent NOS or COX blockade failed to attenuate exercise hyperaemia evoked by 5 min of one leg knee-extension exercise, but their combined use attenuated the hyperaemia by ~ 36% (287, 329). Similar results were obtained in a recent study performed by Heinonen et al. (162). NOS blockade alone reduced resting blood flow (measured using positron emission tomography), but exercise hyperaemia evoked by single leg knee-extension exercise with an average workload of 4.5 kg was unaffected. By contrast, combined NOS and COX inhibition attenuated exercise hyperaemia by ~ 13%. These studies exemplify the complex relationship that exists between vasodilator metabolites.

Current understanding regarding the interdependency between vasodilators and their contribution towards exercise hyperaemia focuses on adenosine and ATP. In 2010, Nyberg et al. reported that exogenous adenosine infused intra-arterially into the leg or into the interstitium of vastus-lateralis increased muscle NO and $PGI_2$ concentrations (306). They also showed that adenosine stimulated endothelial cells release both NO and $PGI_2$, while skeletal muscle cell cultures released NO. Thus, their study showed that adenosine can increase NO and $PGI_2$ production on either side of the blood vessel in humans. The same group obtained similar results upon exposure of rat endothelial and skeleto-muscle cells to ATP (304). Further, in that study, ATP superfusion over rat gluteus-maximus evoked ~ 400% increase in blood flow that was attenuated by NOS inhibition, and abolished by combined NOS and COX inhibition. In humans, ATP infusion in vastus-lateralis increased local interstitial NO and $PGI_2$ concentrations by 60 and 40% respectively (304). By contrast, Crecelius et al. showed that dilatation induced by intra-arterial ATP was unaffected by NOS blockade with L-NMMA (5 mg/min) (77). However, a study performed by Mortensen et al. used a higher dose of L-NMMA (12 mg/min) which attenuated ATP induced vasodilatation by ~ 14%; similar to that observed with COX inhibition (286).

However, both studies showed higher attenuation in ATP induced vasodilatation when NOS and COX (ketorolac or indomethacin: ~ 600 μg/min) inhibitions were combined (77, 286).

Moreover, Ray and Marshall reported that L-NAME infusion attenuated exercise hyperaemia of twitch contraction by ~ 32% with a further 28% reduction due to subsequent $A_{2A}$-blockade (335). Following restoration of vascular conductance with NO-donor SNP, L-NAME failed to attenuate increase in blood flow, but $A_{2A}$-blockade still reduced hyperaemia by ~ 25%. Together these results suggest that adenosine influences the release / action of NO; while NO itself does not contribute towards the release / action of adenosine. Regarding human exercise hyperaemia, Mortensen et al. observed that theophylline attenuated the exercise hyperaemia by ~ 14%, while combined NOS and COX inhibition (L-NMMA+indomethacin) attenuated the increase in flow by ~ 29% (289). However, combination of all three drugs did not result in any added attenuation suggesting that adenosine contributes towards the release / action of NO and vasodilating COX products. Recently Casey et al. failed to observe attenuation in exercise hyperaemia with aminophylline, while combined use of L-NMMA and aminophylline reduced limb blood flow (56).

In conclusion, collectively these studies suggests that not only do adenosine and ATP mediate exercise hyperaemia, but they also influence the production of NO and PGs (286, 289, 304, 306, 332, 333). However, the exact extent to which this interdependent relationship between metabolites dictates limb / MBF is still not clear.

### 1.2.4 Shear stress

Shear stress can be defined as the physical / mechanical force applied on the vascular walls due to their deformation during exercise or due to an increase in blood flow. This mechanical force is a stimulus for the release of vasodilator metabolites; making it an important regulator of vascular

tone at rest and that may have an increased role during exercise. Indeed, both PGs and NO are released due to an increase in shear stress.

When Frangos et al. exposed a cultured monolayer of human endothelial cells to steady (10 dyne/cm$^2$ for 7 – 8 hr) and pulsatile (8-12 dyne/cm$^2$ at 1 Hz) shear stress, there was an immediate increase in $PGI_2$ concentration with flow onset, which declined steadily towards a stable concentration; ~ 16 times greater than those observed in stationary culture (127). A pulsatile increase in shear stress to mimic normal blood flow patterns doubled the steady concentrations of $PGI_2$. These observations were later confirmed on rat aortic smooth muscle cells subjected to four different shear stress levels: even a small increase of 0.5 dyne/cm$^2$ caused a significant increase in $PGI_2$ release which remained elevated for 3 hours, but $PGE_2$ release did not increase until shear stress was increased to 5 dyne/cm$^2$ (7). In line with these results, Koller et al. showed that shear stress related dilatation of isolated rat gracilis muscle arterioles and venules was attenuated by COX inhibition with indomethacin (217, 219). Moreover, in rat cremaster muscle, the arteriolar dilatation caused by release of arterial occlusion was attenuated by COX inhibition (218).

Similarly, NO synthesis is increased by shear stress. For example, Koller et al. also observed that NOS inhibition attenuated shear stress related vasodilatation in gracilis arterioles, and venules (217, 219). Moreover, combined NOS and COX inhibition nearly abolished the flow-induced dilatation in both studies (217, 219). The mechanism by which increased shear stress leads to NO synthesis involves activation of phosphatidylinositol-3-kinase, which acts on protein-kinase-B, leading to serine phosphorylation and eNOS activation (90, 120). In humans, NO has received important consideration as a modulator of reactive hyperaemia; a number of studies have observed an attenuation in blood flow following NOS inhibition (98, 146, 220). It has been argued that NO synthesis increases in response to increased shear stress secondary to the

increase in blood flow that results from myogenic and metabolic vasodilatation caused by circulatory occlusion.

Recently, shear stress has directly been linked to the exercise-related modulation of the production of vasodilator metabolites and endothelial function (90, 403, 405, 406). Tinken et al. tested the effects of 8 week bilateral handgrip training on endothelial function (406). In order to prevent the increase in FBF induced by handgrip, and any associated increase in shear stress from affecting endothelial function in one arm, the change in FBF was attenuated during each period of handgrip throughout the 8 weeks by inflating a cuff around this (control) arm to 60 mmHg. By measuring brachial artery diameter and blood flow velocity, they established that brachial artery shear stress did indeed increase during handgrip in the un-cuffed arm but not in the cuffed arm. Although similar increases in girth, strength, and volume were observed in both arms, flow mediated dilatation (FMD) of the brachial artery improved only in the un-cuffed arm. This selective improvement also extended to peak reactive hyperaemia.

## 1.3 Effect of aging on exercise hyperaemia

Muscle wasting, weakness, change in the fibre type, skeletal motor unit decline, and reduced exercise capacity are all hallmarks of the aging process (42, 49, 160). Moreover, a general decline in $VO_2$, $VO_2$-max, and cardiac output also accompany aging: effects which are attenuated in healthy, active (trained) elderly individuals (307). In recent years, the effect of ageing on exercise hyperaemia has also been the subject of investigation. The discussion below focuses on this topic because a question arises as to whether the age-related changes to exercise hyperaemia may be related to its $O_2$-dependency.

In 1987, Irion et al. showed that exercise hyperaemia was attenuated by ~ 30% in older (24 months) Fischer rats relative to young (12 months), when tetanic planter flexor contractions were

evoked by stimulating the sciatic nerve at the frequency of 2 Hz, and hind-limb blood flow was measured using microspheres (185). In humans, the effects of age on exercise hyperaemia are equivocal. Jasperse et al. showed that when young (19 – 29 years) and older (60 – 74 years) individuals were matched for the chronic physical activity and forearm size, exercise hyperaemia evoked by brief or sustained handgrip exercise was not different between them (188). Moreover, peak FBF and FVC of these individuals was also very similar, indicating that healthy active aging does not attenuate human exercise hyperaemia. In contrast, Proctor et al. noticed that exercise hyperaemia of incremental cycling exercise was attenuated by ~ 20 – 30% in older men (326). However, this study failed to measure baseline blood flow, therefore the percentage change in flow could not be compared between age groups. In agreement, Poole et al. found that hyperaemic response evoked by intense exercise was significantly attenuated in sedentary old compared to sedentary young (322). However, when work rate and $VO_2$-max were matched between age groups, vasodilatation evoked by knee-extensor exercise was more pronounced in the older individuals (230).

Investigating the effects of aging on exercise hyperaemia, Donato et al. employed rhythmic handgrip and knee-extension exercise model and noticed that FBF and FVC were not affected by aging; either when expressed as absolute workloads or as relative to the muscle mass at a given exercise intensity (97). However leg blood flow was attenuated, indicating a limb specific age-related decline in exercise hyperaemia. However, Carlson et al. noticed that exercise hyperaemia of 1 s handgrip contractions was attenuated in older individuals (52). It is interesting to note that despite similar muscle mass between young and old in both studies, the conclusions regarding forearm exercise hyperaemia contrast (52, 97), perhaps reflecting the activity level of subjects in each study (See below).

In relation to exercise and the factors affecting hyperaemia, Schrage et al. observed that aging significantly attenuated the hyperaemic response when subjects (55 – 81 years) performed rhythmic handgrip contractions at 10% MVC for 20 min (358). Further, when L-NAME and ketorolac were infused during the rhythmic contractions, and the results were compared to their previous study on young subjects (359), it was concluded that the contribution of NO to exercise hyperaemia was attenuated by ~ 45% and the contribution of PGs was completely abolished in the older individuals. Subsequently, Kirby et al. asked older subjects to perform either short single handgrip contractions at 10, 20, and 40% MVC, or rhythmic contractions at 10% MVC for 10 – 15 min without and with infusion of the antioxidant ascorbic-acid into the brachial artery (214). Although ascorbic-acid did not improve the hyperaemia of single contractions, responses to rhythmic handgrip contraction were improved by ~ 30% leading them to propose that oxidative stress blunted the endothelium-dependent components of exercise hyperaemia in older individuals. Subsequently, Crecelius et al. used a similar rhythmic handgrip exercise protocol (10% MVC) and showed ascorbic-acid infusion restored the effect of NOS infusion on exercise hyperaemia, but not that of COX inhibition (78). Thus, they proposed that oxidative stress attenuated NO availability, but did not explain loss of the dilator contribution of PGs to exercise hyperaemia.

In agreement with the hypothesis that the decline in endothelial function is a main cause of the age-related attenuation of vasodilatation, Woodman et al. showed an age-related decline in flow and ACh-induced dilatation of rat soleus feed arteries; response to SNP remained intact (438). Moreover, combined NOS and COX inhibition in young arteries attenuated the dilatation to such an extent that the response was similar to that observed in old arteries. This is in agreement with the findings of Spier et al. (380). In humans, Taddei et al. showed a negative correlation ($r = -0.86$) between the increase in age and intra-brachial ACh-induced dilatation (393). Further,

Nicholson et al. showed that the dilatation of intra-arterial $PGI_2$ administered is attenuated with age, reflecting perhaps altered responsiveness of vessels to vasodilator PGs (300). Similar results were found recently by Eisenach et al. (110). These results indicate that despite no obvious direct functional impairment in the vascular smooth muscles, endothelial function is compromised with aging.

Interestingly, the expression of $PGI_2$-synthase in endothelial and vascular smooth muscle cells is unaffected by aging, at least in 36 and 72 week old rats; thromboxane-synthase expression is significantly increased with aging (396). Further, Woodman et al. showed that COX inhibition improved flow-mediated dilatation in isolated arteries taken from older rats (438), and Taddei et al. showed that ACh-induced forearm dilatation was improved in older subjects by COX inhibition (394). Collectively, this indicates an age-related increase in the role of vasoconstrictor COX products and/or the action of $PGI_2$ on the thromboxane-prostanoid receptors (418).

Recently, a decline in plasma ATP concentrations has also been implicated in age associated attenuation in exercise hyperaemia (213). Kirby et al. showed that the venous efflux of ATP was not increased during either systemic hypoxia (~ 80% $SO_2$; 5 min), or graded rhythmic handgrip contractions (5, 15 and 25% MVC; 5 min each) in older subjects, in contrast to the substantial increases in young (213). This reflected a near complete loss of ATP release from deoxygenated RBCs in the older subjects, rather than any age-related differences in ATP hydrolysis.

The age-related decline in exercise hyperaemia may also be affected by, or dependent on a relatively sedentary life style; i.e., lifelong activity prevents this decline. Spier et al. showed treadmill exercise training of Fisher-344 rats prevented age-related decline in endothelial function of isolated soleus and gastrocnemius arterioles; ACh-induced dilatation was not different between young and old (380). Additionally, training elevated eNOS mRNA and protein

expression in old. Independent NOS blockade, or combined NOS and COX inhibition attenuated the ACh's dilatation to values that were comparable with younger rats. Separately, the same group have shown that exercise training preserves FMD, predominantly due to improved function of NOS pathway (381). Although age and training did not affect COX pathway's contribution towards FMD, nonetheless exercise did increase basal and stimulated increases in $PGI_2$ concentrations in older rats.

The effect of lifelong activity on human vasodilatory responses was the subject of interest in two recent studies (285, 305). Nyberg et al. showed that arterial infusion of N-acetylcysteine (antioxidant) increased the concentrations of metabolites of NO ($NO_2^-$ and $NO_3^-$; NOx) measured during one leg knee-extension exercise in older sedentary individuals, but not in young sedentary, or older lifelong active individuals (305). Leg biopsy samples also showed that eNOS and nNOS protein expression were 32% and 24% higher in older active subjects than sedentary old subjects. The blood flow during exercise was also significantly higher in the older active individuals and similar to those observed in young individuals. In a separate study they showed that leg blood flow and vascular conductance were lower during exercise while lactate concentrations were higher in the sedentary old than in the young, and active old (285). Moreover, interstitial ATP concentrations and $P_{2Y2}$-receptor expression were significantly higher in the active old. Lastly, arterial infusions of ACh and ATP were used to examine endothelial function; their vasodilatory responses were lowest in the sedentary old. Although the exact mechanisms by which exercise prevents the age associated decline in vascular function is not known, nonetheless exercise is associated with increased shear stress (403, 405, 406) which itself has been shown to improve mRNA expression of eNOS (439).

In conclusion, evidence does suggest that in less active individuals, aging does lead to attenuated exercise hyperaemia responses; reduction in NO- and ATP-mediated vasodilatation

contribute towards this decline. However, it is not clear whether or not exercise hyperaemia is blunted in older subjects who are recreationally active, rather than involved in regular intense training. Further, it is not known whether the contribution of PGs to exercise hyperaemia induced by moderate levels of exercise is attenuated by ageing, and/or whether the $O_2$-dependency of PGs persists.

## 1.4 Exercise-evoked vasoconstriction

Exercise, particularly strenuous exercise is accompanied by localized hyperaemia along with certain systemic changes; increase in HR, mean ABP (MABP), MSNA, and peripheral vasoconstriction (5, 72, 119, 172, 246, 371, 421); mechanisms responsible for them remain poorly understood. Nonetheless, mainly two theories are presented to explain these responses which are not mutually exclusive in nature (200, 433). The first, "Central Command" proposes that these systemic responses are also dictated by the motor cortex; i.e., parallel activation of central circuits that control movement and cardiovascular / ventilatory responses (200, 433). The second theory proposes that responses are reflex in nature, originating within the contracting / exercising muscles (200). Focusing on the second theory, this thesis addresses the issue of exercise-evoked reflex vasoconstriction and $O_2$'s direct and/or indirect involvement in this response. The following discussion briefly introduces this topic.

The pioneering experiments of Alam and Smirk showed that the pressor responses were maintained following cessation of activity if *post*-exercise circulatory occlusion (PECO) were applied / maintained to trap the arterial and venous blood within the contracting muscles (5). Subsequent studies have confirmed this finding, and the response is now termed "Chemoreflex, Metaboreflex, or Exercise Pressor Reflex" (117, 128, 200, 246). For example, Mark et al. observed that an increase in HR, MABP, and peroneal nerve MSNA evoked in response to

isometric handgrip exercise returned to control values soon after cessation of exercise (246). However, application of PECO maintained the increase in MABP and MSNA. Later, the same group showed that this response was also evoked by rhythmic exercise (3, 421).

Experiments of Kaufman and colleagues are of vital importance regarding modern scientific understanding of neural pathways that are involved in reflex vasoconstriction; i.e., thinly myelinated group III afferents largely respond to mechanical stimuli while un-myelinated group IV afferents respond to metabolic factors. Following the discussion regarding the contribution of metabolic factors to exercise hyperaemia (See section 1.2.3), their involvement in the peripheral reflex responses is of particular interest. Metabolites such as lactate (347, 372), $K^+$ (261), and those involved in COX pathway (AA, $PGE_2$, and $TXA_2$) (207, 231, 262, 347) have all been shown to stimulate the group III and IV afferents. For example, Rotto and Kaufman showed that activity of group III and IV afferents in the triceps-surae of anesthetized cats significantly increased following intra-arterial injection of either lactate or AA (347); interesting observations considering the concentrations of these metabolites increase with exercise (10, 198, 199, 208, 216, 239, 435).

The presence of a relationship between exercise and hypoxic stimulation of the metaboreceptors is also of particular interest. For example, Hill et al. noticed that the contraction sensitive group III and IV afferents of cat triceps-surae are also stimulated by ~ 3 min of hypoxic breathing (3 – 5% $O_2$) at rest (174). Moreover, the responsiveness of group III and IV afferents to rhythmic and isometric contractions is compounded by blood flow occlusion (3, 4, 203). In agreement with these observations, Victor and Seals failed to observe any increase in peroneal MSNA with zero-load arm cycling, but under ischemia the same exercise evoked 120% increase in MSNA (421).

The relevance of these results lies in the observation that while responsiveness of group III and IV afferents is increased by ischemic exercise, maximal-effort physical activity is associated with profound decline in interstitial / intramuscular $PO_2$ (47, 337-339). Furthermore, as the response is not just related to the increase in concentrations of $H^+$ and lactate (4, 371) but has also been linked to COX pathway (207, 262, 347), blockade of this pathway attenuates the stimulation of these metaboreceptor afferents not only under rest but also during exercise (79, 267, 346, 347). These results clearly implicate COX products in the reflex vasoconstriction response. However Win and Marshall's observations regarding COX pathway's involvement in exercise hyperaemia of isometric handgrip contractions in an $O_2$-dependent manner raise questions regarding the $O_2$-dependent nature of the exercise-evoked reflex vasoconstriction response. Further, it is also not known whether the role of these hypothesized $O_2$-dependent metabolites changes with age. Chapter 5 and 6 address this topic in further detail.

## 1.5 General aims and hypotheses of the project

The general aim of the project was to investigate the relationship between $O_2$ and COX products that contribute towards exercise hyperaemia and exercise-evoked / reflex vasoconstriction of recreationally active healthy young and older individuals. Moreover, their relationship with adenosine / adenosine-receptors and the affect of different $O_2$ concentrations on endothelial function was also investigated.

Hypotheses of different experimental studies included in this thesis were as follows:

- *Chapter 3 (Methodology Chapter)* – Reduction in time taken to inflate the venous occlusion cuff for venous occlusion plethysmography would allow significantly higher FBF values to be recorded; reflecting the original rate of arterial inflow.

- *Chapter 4* – Similar to the isometric handgrip contractions, *post*-exercise hyperaemia of rhythmic handgrip contractions is also contributed to by $O_2$-dependent vasodilating prostaglandins (PGs). COX inhibition and 40% $O_2$ would similarly attenuate *post*-exercise hyperaemia response, along with the venous concentrations / efflux of these PGs. Lastly, *post*-exercise hyperaemia of older recreationally active individuals is also contributed to by $O_2$-dependent PGs.
- *Chapter 5* – $O_2$-dependent COX products produced in the exercising arms of young men contribute to the exercise-evoked vasoconstriction in the resting calf: this contribution to vasoconstriction is reflex in nature.
- *Chapter 6* – Isometric handgrip contractions at 60% MVC would not completely occlude muscle perfusion. Moreover, the increased muscle perfusion *during* these contractions would be similarly attenuated by 40% $O_2$ and COX inhibition in both age groups. Lastly, the $O_2$-dependent COX products contribute to exercise-evoked vasoconstriction in the resting arm / muscle in both age groups.
- *Chapter 7* – Adenosine's direct contribution to exercise / *post*-exercise hyperaemia is also $O_2$-dependent; further COX inhibition following adenosine-receptor antagonism would not add to the attenuation of the hyperaemic response.
- *Chapter 8* – 40% $O_2$ does not attenuate the endothelium-dependent dilation in either age group (i.e., young and old), but higher $O_2$ concentrations do; this attenuation is related to oxidative stress.

The specific aims and hypotheses of each study will be properly introduced and clearly stated in each relevant experimental chapter.

# Chapter 2

# General materials and methods

The majority of the studies described in this thesis were performed on human subjects; exceptions were the experiments described in Chapter 7 which were performed using a rat model. The methodology used in that study is described within Chapter 7.

## 2.1 Subjects

Participants of the studies were mainly young and older men; female participation was restricted to two studies (Chapter 3 & 8) in order to avoid the effects of female hormones on cardiovascular system. All subjects were non-smoking, recreationally active individuals who consumed less than 21 units of alcohol/week. Health and activity status of subjects was assessed with help of a questionnaire (See Appendix 2 and 3). Younger subjects were recruited from the University of Birmingham's student body while the older subjects were either recruited from the University's faculty, or via the Birmingham 1000 Elders group. All subjects were required to refrain from caffeinated drinks and heavy meals for 12 hours prior to the experiments. They were also required to refrain from any alcohol consumption, use of any non-steroidal anti-inflammatory drugs (NSAIDs), or participation in any strenuous exercise / activity for 24 hours prior to each experimental visit. Studies were approved by the University of Birmingham's Science, Technology, Engineering, and Mathematics Ethical Review Committee (application number ERN 12-1377) and complied with the Deceleration of Helsinki (15).

## 2.2 General experimental conditions

Experiments were performed at room temperature while the subjects were seated on a couch with backrest at ~ 65° to the horizontal. In general, legs were stretched out horizontally and both arms rested at approximately the level of the heart. The exception was the experiments discussed in Chapter 8 for which the subjects were seated in a chair with backrest at ~ 85° to the horizontal. In order to minimise any distractions, the room was organised so that the subjects

were unable to see the recording traces and equipment while the noise and visual distractions were kept at an absolute minimum. Subjects were allowed to watch a movie of their choice during long periods of rest.

In each study, subjects attended an initial familiarization visit followed by the relevant experimental visits. After completing the health / activity questionnaire and providing written and informed consent (See Appendix 4), subjects who were accepted were habituated to the equipment and the protocol of the study during the familiarization visit. All of the experiments were performed in a randomized single-blind cross-over manner.

## 2.3 Handgrip exercise

The familiarization visit was also used to record the maximum voluntary contraction (MVC) force. Each subject was asked to sit in the experimental position, grip the handgrip dynamometer with their dominant hand as powerfully as possible and hold the contraction for 5 s. The force measured at the end of the $5^{th}$ s was recorded as the MVC. This was done 3 times separated by at least 30 s in order to confirm reproducibility, and avoid snap contraction readings. The average of the 3 contractions was taken as 100% MVC.

As shown Figure 2.1, a handgrip dynamometer (Lafayette 70718, Loughborough, UK) with a voltage-output and an interfaced visual display-unit was used for all studies in which subjects were required to perform handgrip contractions. The dynamometer was calibrated before each experiment so that 1.0 volt on the display-unit represented subject's 100% MVC; allowing each subject to visualize the percentage of force produced and maintain the level requested. The voltage-output also allowed tension time integral (TTI) to be computed; this was done as the area under the tension curve from exercise onset to cessation. During experimental protocols, handgrip contractions were performed in either a rhythmic (1:1 s duty cycle) or an isometric

manner at 60% or 70% MVC intensities. Termination criteria included inability to maintain the force for 5 s and/or decrease in the generated isometric force by 5% of the MVC despite vigorous verbal encouragement; for example 55% when the target force required was 60% MVC. A Web-based metronome was used to aid subjects with rhythmic contractions. Figure 2.2 shows that the elbow of each subject's exercising arm rested on a polystyrene block and as the dynamometer was fastened to the bench, this allowed the subject to release their grip when asked to do so and completely relax the arm, thus facilitating blood flow measurements.

## 2.4 Delivery of medical air or 40% $O_2$

As shown in Figure 2.3, gas cylinders were used for the delivery of both normoxic medical air (Medical Air, BOC Medical, UK) and hyperoxic gas (Medical Oxygen, BOC Medical, UK). Gas was delivered to a facemask via tubing and a Venturi-valve (Intersurgical, Wokingham, UK). The mask was secured over the nose and mouth with help of elastic straps, which encircled the head. According to Bernoulli's principle of pressure differential, the velocity of a liquid or gas being forced from an area of high-pressure to an area of low-pressure through a narrow aperture accelerates the flow of the stream. This increase in velocity of flow simultaneously decreases surface pressure around it, allowing an influx of gas though the sides of the stream until the pressure is normalised. Thus, when a 40% Venturi-valve is supplied with 100% $O_2$ at 10 L/min, it entrains approximately 31 L/min of air with a final volume of 41 L/min. As the atmospheric air contains approximately 21% $O_2$ which equates to 6.5 L/min, the total $O_2$ delivery is (10 + 6.5) 16.5 L/min making the final $O_2$ concentration in the mixture approximately 40%.

In Chapter 8, 60 and 100% $O_2$ concentrations were also delivered to the subjects in a similar manner. For 60% $O_2$, the flow of 100% $O_2$ was 15 L/min which draws in a further 15 L/min of room air making the final volume of gas mixture to be 30 L/min. In this situation, the entrained

room air contains 3.15 L/min of $O_2$ making the final $O_2$ delivery (15 + 3.15) 18.15 L/min; the final $O_2$ concentration is 60.5%. For 100% $O_2$ delivery, the small openings which draw the room air in the Venturi-valve were sealed and a comparable flow rate of ~ 15 L/min was maintained. As shown in Figure 2.3, the concentration of gas that was delivered to the subjects was checked at regular intervals by using an $O_2$-sensor connected to a display-unit (ProOx 100, BioSpherix, USA). As a control for the hyperoxic gas, medical air was delivered in a similar fashion with similar flow rates via the facemask and Venturi-valve. Subject were blinded to the treatment conditions by ensuring that they did not see when gas flow was switched from one gas cylinder to another.

## 2.5 Measurements

### 2.5.1 Blood pressure

An automatically-calibrating Finapres monitor (Ohmeda 2300, Englewood, USA) was used to measure arterial blood pressure (ABP) from the non-dominant hand at the level of the heart. As shown in Figure 2.3, this was done by wrapping a finger-cuff around the middle phalanx of the middle finger. The monitor uses the principles of infrared photo-plethysmography in combination with an inflatable bladder. The photo-sensor detects the scatter and absorption of the infrared light while the bladder inflates and deflates in response to changes in volume and pressure, allowing a continuous measure of ABP. To ensure Finapres recordings closely reflected the actual blood pressure, initial readings were also taken with help of either an electronic (microlife, BP3BXO, Windnau, Switzerland) or a traditional mercury sphygmomanometer (Metpak MK3, Accoson, UK).

### 2.5.2 Limb blood flow

Depending on the protocol (See Methods Section of relevant Chapters; i.e., 3, 4, and 5), either FBF or calf blood flow (CBF) was measured using the technique of venous occlusion plethysmography (430). As shown in Figure 2.2, an appropriate-sized Indium-Gallium silastic strain-gauge was mounted on the widest part of the limb. A small (6.5 cm wide) sphygmomanometer cuff was wrapped around either the wrist or ankle, and a large (10.5 cm wide) sphygmomanometer cuff was wrapped around either the upper arm or the thigh. Blood flow measurements were made by inflating the small cuff to ≥ 250 mmHg which occluded blood flow to the hand or foot, and thereby allowed the focus of measurement to be on the skeletal muscle of the limb. This was done manually using a commercially available sphygmomanometer cuff. The larger cuff was then inflated to ~ 50 mmHg which restricted the venous drainage but allowed arterial flow. This was generally done by using a rapid cuff inflation system (E20, Rapid Cuff-Inflator, D.E. Hokanson Inc., USA); except in Chapter 3 where comparison was made between blood flow recordings made following manual inflation or automatic rapid inflation of the venous occlusion cuff. The smaller cuff was always inflated ~ 5 – 10 s before the larger cuff.

An electrically calibrating strain-gauge plethysmograph device (EC6 Plethysmograph, D.E. Hokanson Inc., USA) was used to measure limb blood flow (178). As shown in Figure 2.4, the device was calibrated by generating a step deflection of 1% in the voltage-output of the trace. This method of 2 point calibration (0 and 1%) simulates 1% change in the length of the mounted strain-gauge (i.e., limb circumference), and a similar change in limb volume (178, 411) (See Chapter 3). This was done 3 times and the average calibration value along with the initial gradient of the slope of plethysmograph trace was then used to calculate blood flow using the following algorithm;

$$\text{Limb Blood Flow (ml/dl/min)} = \frac{2 \times b \times 60}{c}$$

Where; b = “initial” gradient of the plethysmograph trace

c = value of 1% calibration deflection

The technique of venous occlusion plethysmography, the importance of the “initial” gradient of the plethysmograph trace, and the algorithm are further discussed in Chapter 3. Nonetheless for the sake of completeness, Figure 2.4 shows a typical plethysmograph recording along with a corresponding ABP trace used for the measurement of blood flow during rest and immediately after exercise. If the plethysmograph trace moved off scale during exercise (See Figure 2.4), the device was re-balanced so as to move the plethysmograph trace to the middle of the chart. This did not affect the calibration of the mounted strain-gauge and allowed for blood flow recordings to be made following handgrip contractions.

### 2.5.3 Near-infrared spectroscopy

Near-infrared spectroscopy (NIRS) was used to measure an index of change in deoxygenated and total haemoglobin in the flexor digitorum-superficialis of the exercising and non-exercising forearm in response to isometric handgrip contractions. This is discussed in Chapter 6.

### 2.5.4 Red cell flux

Laser-Doppler (Laser-Doppler Perfusion and Temperature Monitor, DRT4, Moor Instruments Ltd, UK) was used to record cutaneous red cell flux (RCF) from the ventral surface of the forearm in response to ACh-iontophoresis. This is discussed in Chapter 8.

## 2.6 Drug administration

Pharmacological agents were given orally in a drink (250 ml) before the experiment; details are provided in the relevant Chapters. In most studies, it contained 600 mg of aspirin (A; Aspirin Dispersible Tablets, Boots Pharmaceuticals, UK), or orange-flavoured squash alone acted as placebo treatment. In Chapter 8, 2000 mg of Vitamin-C (Effervescent Vitamin-C, Boots Pharmaceuticals, UK) was used instead of aspirin as a treatment. The dose of aspirin used here has previously been shown to provide near-maximal COX inhibition for 0.5 to 1.5 hours (161, 437).

## 2.7 Data acquisition

Data was recorded on a desktop computer (Dell Inc., USA) using a bridge amplifier unit (Power-Lab, AD Instruments, USA) and Lab-Chart data acquisition software (Version 7.3.3, AD Instruments, USA) at the sampling frequency of 400 Hz.

Heat rate (HR) was derived and monitored online with the help of Finapres and Lab-Chart software from the interval between successive systolic blood pressure peaks; MABP was derived off-line using the blood pressure trace. FBF and CBF were also calculated off-line. FVC was calculated as (FBF/MABP) while calf vascular resistance (CVR) was calculated as (MABP/CBF) on Microsoft Excel: data for this purpose was extracted from the same cardiac-cycle. If this was not possible due to automatic re-calibration of the Finapres monitor, MABP (and HR) was taken from the most adjacent cardiac-cycle. Their values are expressed in either conductance units (CU) or resistance units (RU), respectively. TTI data were computed offline using the tension recording. Figure 2.5 shows examples of the raw data recordings made during rhythmic and isometric handgrip contractions in a venous occlusion plethysmography study. For the purpose

of analysis, measurements of variables were taken at different time points (See Methods Section of relevant Chapter).

## 2.8 Statistical analysis

Appropriate Analysis of Variance (ANOVA) was used to identify time, treatment, age, condition, and their interaction effects. Once a significant main effect was detected, the data was further analyzed using an appropriate post-hoc test (Tukey's HSD) to detect the exact point of difference. Where appropriate other comparisons were made by using Student's t-test, Pearson's correlation coefficient (r), and coefficient of determination ($r^2$). Statistical significance was assumed when $P < 0.05$. Results are expressed as Mean±SEM.

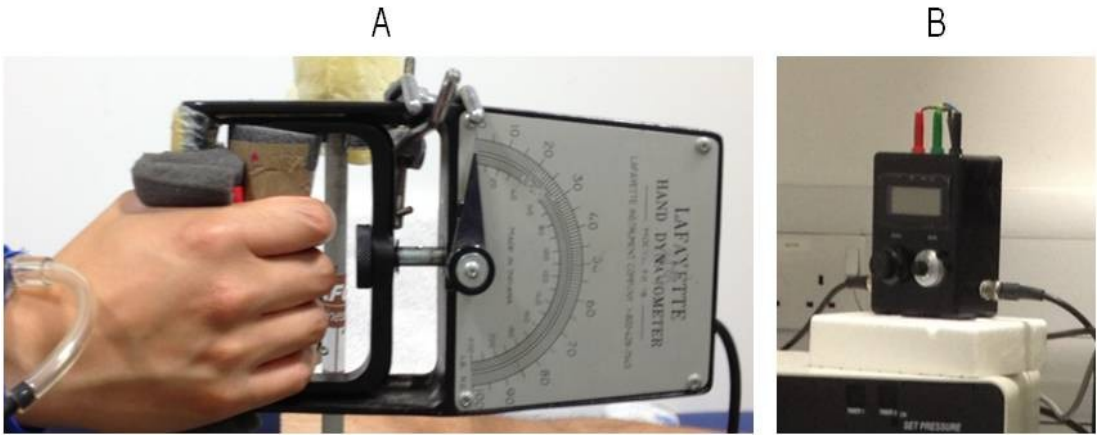



Figure 2.1 **Illustration of equipment used for handgrip.** (A) shows the handgrip dynamometer and (B) shows the visual display-unit that was used to perform rhythmic and isometric handgrip contractions at required MVC intensities.

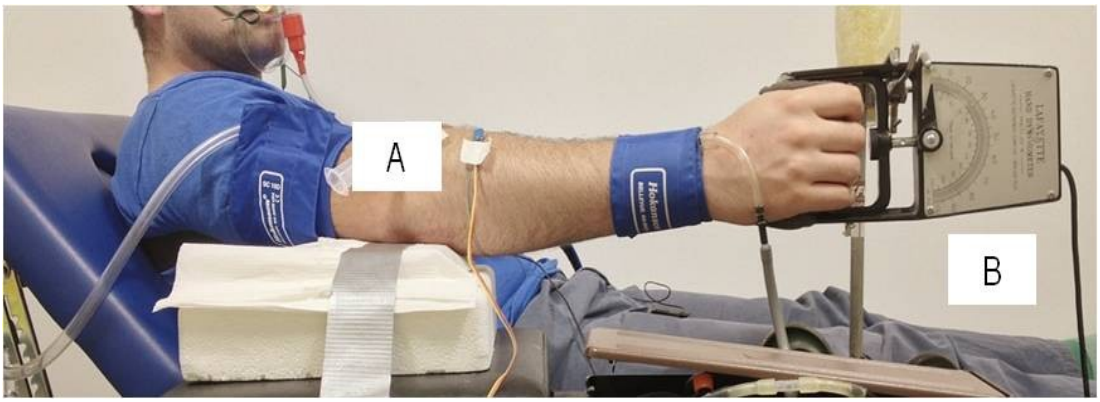



Figure 2.2 **Arrangement used for venous occlusion plethysmography and handgrip exercise.** Strain-gauge (A) was placed around the widest part of the forearm and secured with paper tape. Handgrip dynamometer (B) was secured with a clamp and elbow of each subject allowed to rest on a polystyrene block. This aided in recording blood flow at the level of the heart.

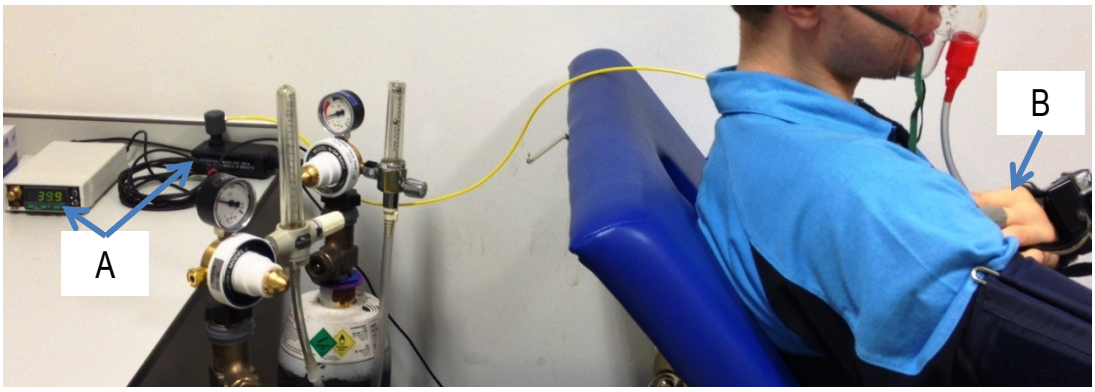



Figure 2.3 **Arrangement of gas cylinders relative to the subject.** Medical Air / $O_2$ cylinders were kept behind the subject to keep them blinded while the gas was delivered to the facemask via a Venturi-valve. $O_2$-sensor with its display-unit (A) was connected to the facemask to monitor $O_2$ concentration of the delivered gas. The contra-lateral hand rested at the level of the heart while ABP recordings were made from the middle finger (B).

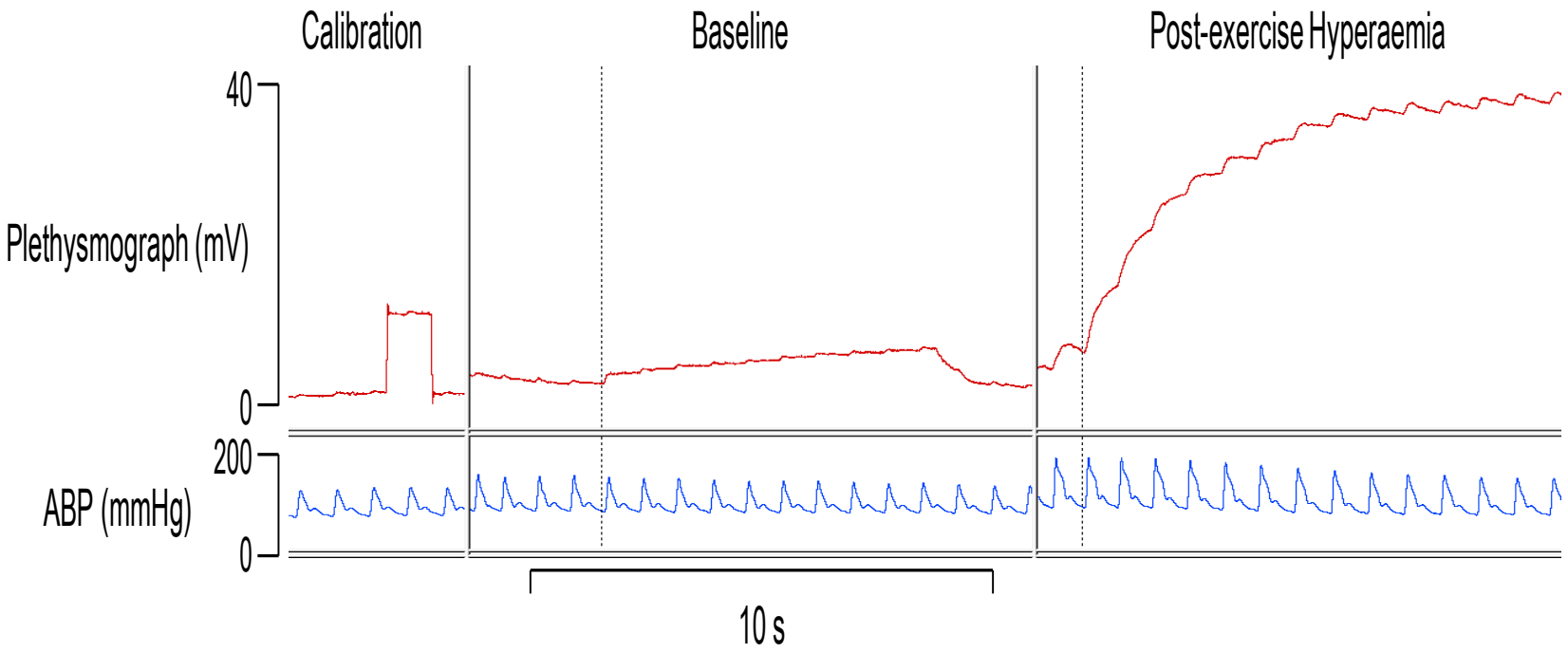



Figure 2.4 **Raw plethysmograph and ABP traces.** These examples show application of 0 – 1% calibration deflection, recording made following inflation of venous occlusion cuff under baseline condition, and a recording under *post*-exercise condition. Dotted lines represent the time of venous occlusion cuff inflation to ~ 50 mmHg and help visualise the close relationship between the pulses recorded on the plethysmograph and those on the ABP trace.

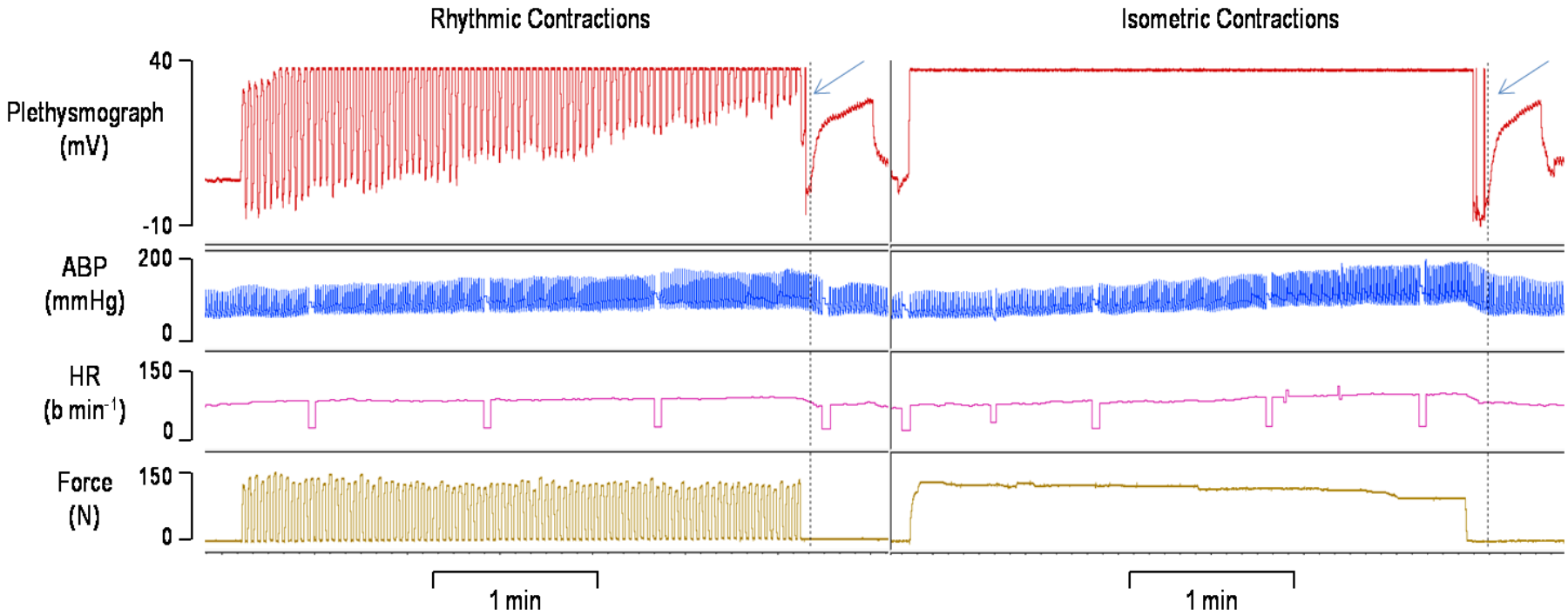



Figure 2.5 **Raw traces showing plethysmograph output, ABP, HR, and Force recordings immediately before, during, and after rhythmic (left) and isometric handgrip contractions (right).** The small gaps in ABP and HR trace show regular auto-calibration of Finapres monitor. Exercise often drove the plethysmograph trace off scale. Thus, immediately after cessation of contraction, Wheatstone-bridge was rebalanced before venous occlusion was applied: blue arrows show rebalancing while dotted lines show time of the venous cuff inflation immediately after exercise.

# Chapter 3

# Methodological considerations: Comparison of measurements of forearm blood flow using strain-gauge venous occlusion plethysmography with manual or rapid inflation of the venous occlusion cuff

## 3.1 Introduction

Our group have previously employed Whitney Plethysmograph with manual inflation of the venous occlusion cuff to study FBF in response to different stimuli (122, 123, 437). However in this thesis, the Hokanson strain-gauge venous occlusion plethysmograph with automatic rapid cuff inflation system was used to record blood flow in 3 studies (including this one). The initial pilot experiments conducted to replicate our group's established protocols consistently revealed FBF values following exercise that were approximately twice those previously observed (122, 123, 437). As the technique employed by Whitney and Hokanson strain-gauge plethysmographs is similar (explained below), this initial study was undertaken to investigate the influence of the speed of venous occlusion on the observed / calculated limb blood flow values.

In order to appreciate venous occlusion plethysmography, a brief history of the technique and the basic principles utilized in its development need to be understood. Venous occlusion plethysmography was first used by Schafer and Moore (357) to measure blood flow in the spleen; and later employed by Hewlett and Van Zwaluwenburg (170) to measure limb blood flow. The basic idea of plethysmography is that when the venous occlusion cuff (also known as collecting cuff) is inflated below diastolic but above venous pressure; blood comes in (i.e. into the limb) but cannot escape (431). The limb increases in size due to the continuing arterial inflow that gradually fills the venous vessels so increasing limb volume: measurement of the rate of change in volume reflects the arterial inflow (191, 431). This in theory implies that the rate of change of volume depends on the difference between arterial and venous pressures; i.e., any decrease in arterial pressure or an increase in venous pressure would negatively affect perfusion pressure, and subsequently, limb perfusion. This means that with venous occlusion plethysmography, inflation of the collecting cuff would over time increase the venous pressure within the limb with each successive heart beat and subsequently influence rate of arterial inflow.

In the original volume plethysmographs, the limb was sealed in a rigid box containing water and the rate of blood flow was measured on a volume recorder by inflating the venous occlusion cuff and measuring the displacement of water surrounding the limb (191, 430). This method had two main limitations to it. Firstly, the subject had to remain completely still as any movement on their part would separately influence the displacement of water around the limb (430). However, the biggest limitation of the technique was trying to maintain the perfect watertight seal around the limb (191, 430).

Whitney used mathematical considerations to address these problems (430). In an ideal cylinder, the percentage change in a specific area of any given section will always be twice the percentage change in the circumference ($\Delta$ V/V = 2 $\Delta$ C/C or $\Delta$ V = 2 $\Delta$ C). Assuming that the length of a cylinder (limb) remains unaltered, the volume change following venous occlusion would be accommodated in the cross-sectional area. Theoretically, the length would only remain unaltered if no restrictions are to be applied to the cross-sectional area. However, as this would remain nearly impossible if $\Delta$ C was to be measured, a calibrated girth measuring device could overcome this limitation. He employed these two assumptions with Ohm's Law (voltage = current x resistance) to provide the basis for the use of one such device (i.e., silastic strain-gauge), which could record change in limb circumference; and used it to measure the change in volume. Thus in Whitney's Plethysmograph, thin mercury-in-rubber silastic tubing attached to a strain-gauge with a small amount of current passing through it was used to encircle the widest part of the limb. Occlusion of venous outflow increased the circumference of the limb which lengthened the tubing of the strain-gauge and caused an increase in the electrical resistance (191, 430). The circuit was connected to a galvanometer which showed voltage deflections corresponding to the volume change in the limb: "initial" gradient (i.e., the rate of change) of these deflections has since been employed to calculate blood flow (147, 430). Traditionally, this involved measuring

the gradient over at least 3 – 4 heart beats (411). The device itself was calibrated by stretching the strain-gauge with help of an adjustment screw by a known length and recording the change in voltage (191, 430). The blood flow recordings made with venous occlusion plethysmography have typically been expressed as changes relative to dl of tissue volume per unit time; but can be expressed as ml/min using the volume displacement method (325, 430, 431). This expression of flow as a percentage unit "normalizes" the readings to allow for easier comparison of flow values recorded from different limbs (explained in discussion) (178). The algorithm employed in measuring limb blood flow with Whitney plethysmograph is:

$$\text{Blood Flow (ml/dl/min)} = \frac{2 \times b \times 60\ (\text{s/min}) \times 100}{c\ (\text{volts/turn}) \times \text{no. of turns for 1 cm change in strain-gauge} \times \text{initial ilmb circumference (cm)}}$$

(325)

Where; b = "initial" gradient of the plethysmograph trace (Δ voltage / unit time) (147)

c = calibration factor

In the 1970s Hokanson et al. noted that if the length of a strain-gauge is exactly the same as the circumference of the limb, the linearly proportional relationship between resistance and volume can simply be expressed as: $\Delta R/R = \Delta V/V$ or $\Delta R = \Delta V$ (178, 411). Using this observation, they developed a system that allowed 1 arm of the Wheat-stone bridge to be altered by 1% to provide a simpler electrical calibration of the *mounted* strain-gauge (178). In the Hokanson plethysmograph a step deflection of the plethysmograph trace provides a 2-point calibration (c) signal and the difference between the two values not only signifies 1% change in volume, but also a similar change in the length of the mounted strain-gauge (i.e. limb circumference) (411). This removed the need to separately account for the initial limb circumference and simplified the algorithm to:

$$\text{Limb Blood flow (ml/dl/min)} = \frac{2 \times b \times 60\ (\text{s/min}) \times 100}{c\ (\text{difference between 0 and 1\% voltage-deflection with 1\% change in limb circumference})}$$

Alternatively, this could simply be expressed as:

$$\text{Limb Blood Flow (ml/dl/min)} = \frac{2 \times b \times 60\ (\text{s/min})}{c}$$

Where; b = "initial" gradient of the plethysmograph trace

c = value of calibration deflection (voltage difference between 0 and 1% deflection)

It might appear that the initial gradient of the plethysmograph trace always rises linearly; and this has sometimes been suggested in the literature (147, 431). For example, Greenfield et al. mentioned that the rate of arterial inflow is rarely affected by mild degrees of rise in venous pressure (147). Further, while discussing the examples of plethysmograph traces, they mentioned that if the rate of inflow appeared to decline from one heart beat to the next, the data could be interpreted with less confidence, but the solution to the problem was "*to reduce the jump to a minimum*" (147). However, they did acknowledge that this phenomenon occurred when the rate of arterial inflow was high (147), raising the possibility of perfusion pressure being affected by changes in arterio-venous pressure. Experiments conducted by Tschakovsky et al. using a rapid cuff inflation system (similar to the one used in this study) provided evidence that the inflation of venous occlusion cuff at 50 mmHg decreased the rise in the slope of plethysmograph trace (i.e. arterial inflow) over subsequent beats, and that this decrease in blood flow was not due to any changes in arterial diameter (411). This important observation not only provides experimental evidence for the curvilinear nature of the plethysmograph trace but underlines the need to measure blood flow as quickly as possible after cuff inflation to obtain the actual blood flow (411).

However consideration of these findings raises additional issues. As flow measurements can only be made once the venous occlusion cuff has reached the desired pressure (for example, 50 mmHg), the rate of inflation of the venous occlusion cuff itself might affect the arterio-venous pressure; and therefore the gradient of the plethysmograph trace. Recommendations of Tschakovsky et al. following their study to only use the 1st heart beat's gradient for calculation of blood flow addresses the issue of underestimation of arterial inflow by measuring gradient over multiple heart beats. However, it fails to address the influence of time taken to inflate the venous occlusion cuff. Traditionally venous occlusion cuffs were inflated manually with help of sphygmomanometers. Indeed, as mentioned previously, our group has also used manual inflation of venous occlusion of cuff to record FBF (122, 123, 437). The observations made by Tschakovsky et al. and in our own pilot experiments underlined the need to investigate the influence on FBF values of an apparently negligible time delay (~ 1 – 2 s) in inflation of venous occlusion cuff when done manually vs. by automatic rapid cuff inflation. To our knowledge, no study has compared the FBF values calculated from the "initial" rising slopes by the two different venous occlusion methods.

### 3.1.1 Aims and hypothesis

The aim of the present study was to compare FBF values recorded when the venous occlusion cuff was inflated either manually or by the use of a rapid cuff inflation system. This was investigated under baseline and *post*-exercise conditions within the same individuals. Our hypothesis was that reduction in an apparently negligible time delay (~ 1 – 2 s) in inflation of venous occlusion cuff by inflating automatically, rather than manually, would allow significantly higher FBF values to be recorded that would more closely reflect the original initial rate of inflow.

## 3.2 Methods

Nine subjects (20 – 30 years) participated in the study. HR, MABP, and TTI were measured as described in Chapter 2.

### 3.2.1 Protocol

Each subject visited the laboratory on one occasion. Once a subject had been accepted and had consented to the study, anthropometric measurements of age, weight, height, body mass index (BMI), and forearm circumference (FAC) of the widest part were taken. This was followed by recording each subject's MVC (See Section 2.3). Subjects were then allowed to rest for 30 min before 3 baseline FBF measurements were taken, 15 s apart. Following these, subjects were asked to produce and maintain an isometric handgrip contraction at 70% MVC for 1 min. As shown in Figure 3.1, FBF was measured at 0, 15, 30 s, and 1 min after exercise, and then at 1 min intervals for further 9 min. Subjects were then allowed to rest for a further 30 min before the entire protocol was repeated. The only difference between both exercise bouts was the use of either manual inflation of the venous occlusion cuff using a normal sphygmomanometer (Metpak MK3, Accoson, UK), or rapid inflation with an automatic rapid cuff inflation system (E20, Rapid Cuff-Inflator, D.E. Hokanson Inc., USA). Manual inflation of the upper arm cuff occurred in < 2 s while automatic rapid inflation took ≤ 0.3 s. The order of venous occlusion was randomized. However due to the nature of experiments, subjects could not be blinded to the treatment condition.

### 3.2.2 Blood flow measurements

Blood flow was measured as described in Chapter 2 (See Section 2.5.2) using a Hokanson plethysmograph with an appropriate size Indium-Gallium strain-gauge for each subject. The average value of calibration signal was used in the aforementioned Hokanson plethysmograph

algorithm. Venous occlusion cuffs were inflated for approximately 8 s to 50 mmHg. Figure 3.2 shows the gradient of plethysmograph trace used for calculation of baseline FBF, while Figure 3.3 shows an example of the plethysmograph slopes used for calculation of FBF immediately after exercise when the cuff was inflated either manually or by rapid inflation system. Notice that the gradient was calculated from the "*initial*" rising slope of the plethysmograph deflection following venous occlusion. Using average gradient calculation (best-fitting line) option on the Lab-Chart, the gradient is drawn from the initial complete beat (or 2 – 3 beats) of manual cuff inflation and only the 1st complete pulsatile beat of rapid cuff inflation. The use of more than one beat was employed in manual inflation only when no difference was observed in the gradient between them. This was done based on the traditional recommendations of Greenfield et al. (147). Use of only the 1st pulsatile beat for rapid cuff inflation was employed because of the observations made by Tschakovsky et al. to the subsequent pulsatile beats in their studies (411).

In addition, 3 different gradients from the 3 successive beats after inflation of venous occlusion cuff with rapid cuff inflation were taken for comparison with FBF values calculated from "initial" rising slopes of manual cuff inflation. An example of these gradients is shown in Figure 3.4.

### 3.2.3 Data analysis

Cardiovascular / haemodynamic data were analyzed using 2-way repeated-measures ANOVA for the detection of time, treatment, and time*treatment effects. 1-way repeated-measures ANOVA was also used to detect time effects within each treatment. Tukey's HSD was used as a post-hoc test after a significant main effect was detected. Degree of correlation between FBF values from 2 different cuff inflation methods was tested using Pearson's correlation coefficient (r) and coefficient of determination ($r^2$) along with a regression-line fitted through 0 on axes. Dynamometer force data was analyzed using Student's paired t-test.

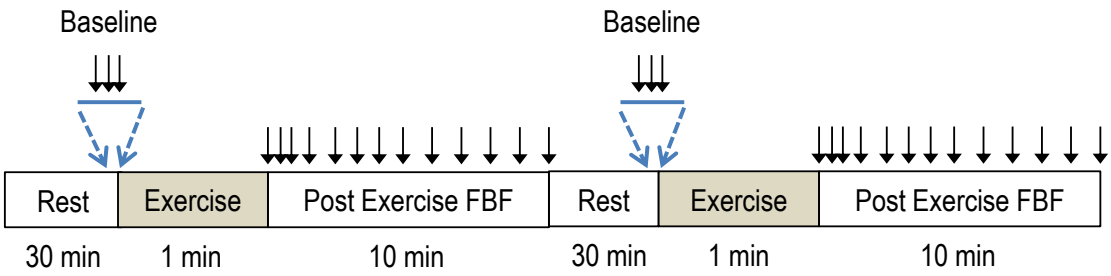



Figure 3.1 **Schematic diagram of the protocol.** ↓ represents the times at which FBF measurements were made using either manual inflation or automatic rapid cuff inflation of the venous occlusion cuff.

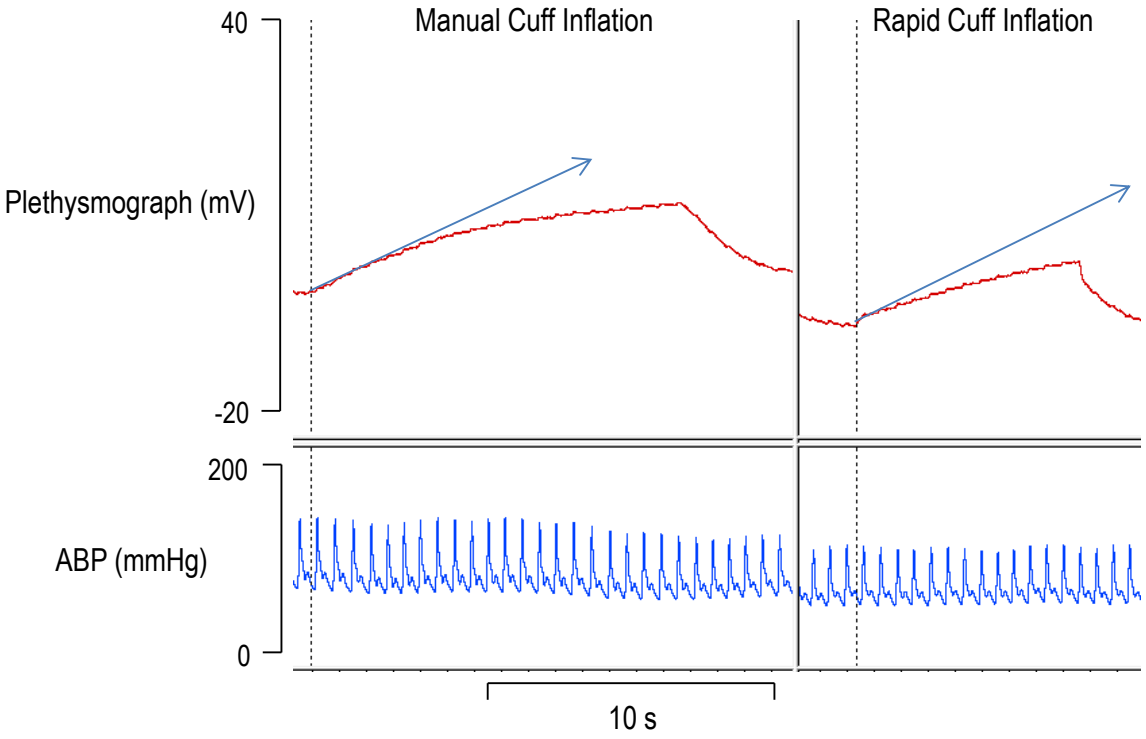



Figure 3.2 **Baseline plethysmograph traces of manual and automatic rapid cuff inflation with corresponding ABP trace.** Dashed lines represent the point of venous cuff inflation while the blue → lines represent the gradient of the plethysmograph trace used for FBF measurements. Pulsatile ABP trace aids in visualization of the pulsatile increments on the plethysmograph trace.

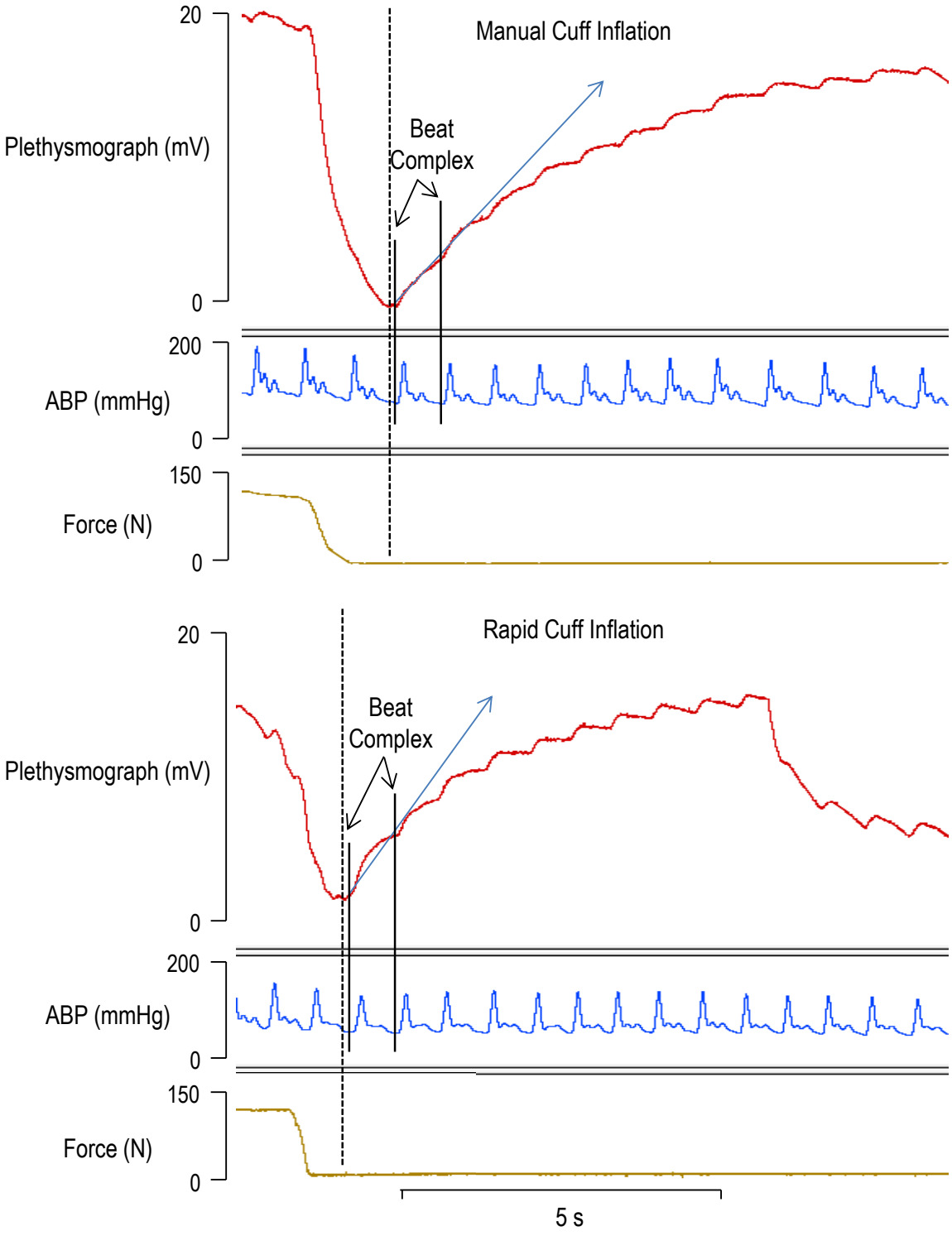



Figure 3.3 **Plethysmograph and ABP traces during manual cuff inflation (upper panel) and automatic rapid cuff inflation (lower panel) protocols, immediately after corresponding handgrip contractions.** Dashed lines represent the point of venous occlusion cuff inflation while the blue → lines represent the "initial" rising gradient calculated from the initial complete beats (i.e., beat complex).

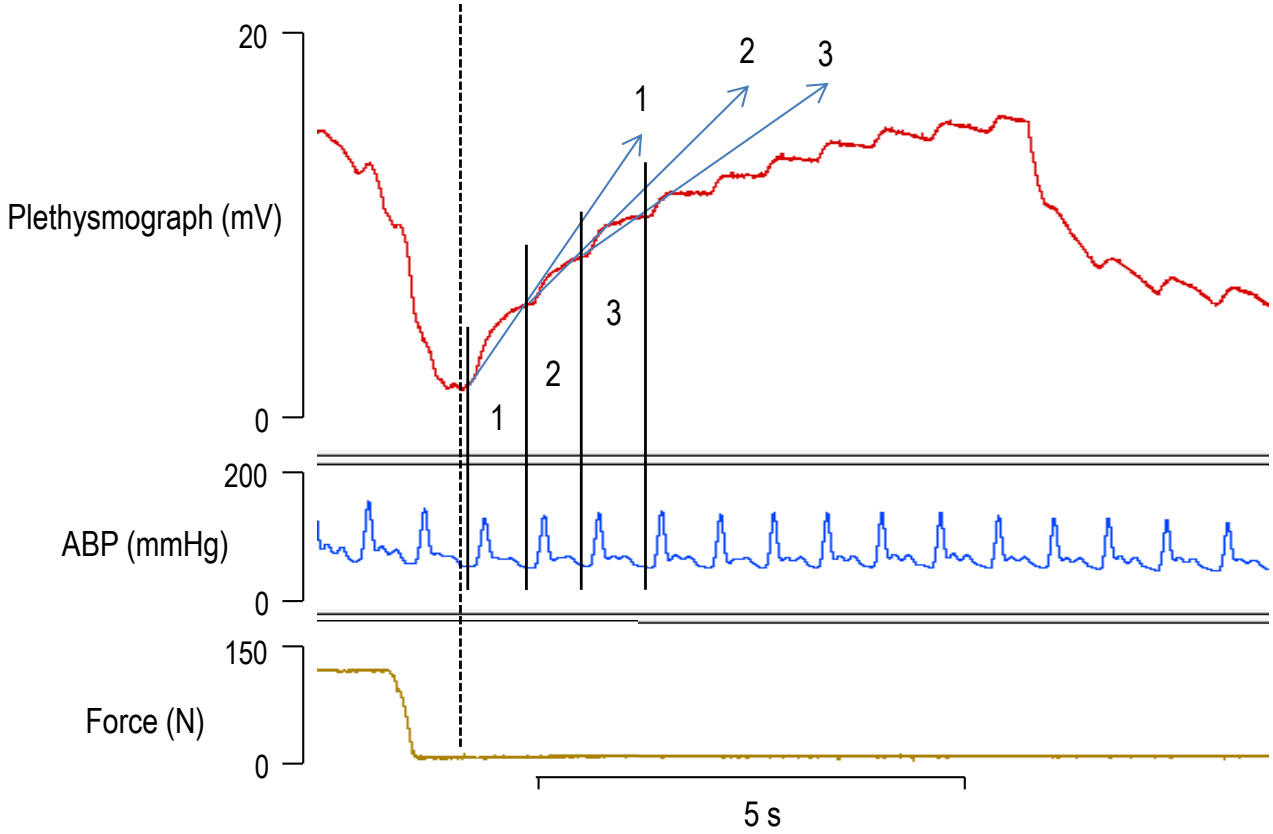



Figure 3.4 **Plethysmograph trace with corresponding pulsatile ABP for FBF measurements made with automatic rapid cuff inflation immediately after exercise.** Blues → lines represent the 3 gradients drawn from the 3 successive pulsatile deflections shown here as individual beat complexes after venous occlusion at 50 mmHg. The 1st gradient was used to compare FBF values from manual cuff inflation and automatic rapid cuff inflation.

## 3.3 Results

Anthropometric data of subjects is presented in Table 3.1: 3 female and 6 male subjects participated in the study. All subjects were healthy individuals with no diagnosed medical conditions.

TTI generated by subjects during the two isometric handgrip contractions was not different between manual and rapid cuff inflation trials (P = 0.82; Figure 3.5). HR and MABP were also not different between the treatment conditions before, during, and after handgrip contractions (Figure 3.6). However, as expected, HR and MABP increased significantly from baseline values during exercise (P < 0.01), and after cessation of handgrip contractions both returned back to resting values.

### 3.3.1 FBF responses

Baseline FBF values recorded from the "initial" gradients of plethysmograph trace were not different between manual and automatic rapid cuff inflation (Table 3.2). However, as shown in Figure 3.7, comparison of FBF values recorded from cuff inflation during rest and *post*-exercise revealed significant treatment (P < 0.01) and time (P < 0.001) effects. With manual cuff inflation FBF values increased from ~ 9.6±1.3 ml/dl/min at rest to 33.8±4.2 ml/dl/min immediately after handgrip contraction (P < 0.001), and remained significantly elevated for 4 min *post*-contraction. By contrast, FBF values recorded with automatic rapid cuff inflation increased from baseline of ~ 10.5±1.4 ml/dl/min to as high as 54.6±5.1 ml/dl/min immediately after handgrip contraction (P < 0.001). These values remained significantly elevated from baseline FBF values for 7 min *post*-contraction; confirming our hypothesis that rapid cuff inflation would show FBF values which were much higher than those recorded by manual cuff inflation.

However, when FBF was calculated from the 2nd and 3rd pulsatile beat of the plethysmograph trace after rapid inflation of the venous occlusion cuff as shown in Figure 3.8, the FBF recorded from the gradient of the 1st pulsatile beat was significantly higher than those recorded from the 2nd ($P < 0.05$) and 3rd pulsatile beats ($P < 0.01$). FBF recorded from the 2nd pulsatile deflection remained elevated for 6 min *post*-contraction from its respective baseline, while that recorded from 3rd pulsatile deflection remained elevated for only 4 min. Interestingly, FBF values recorded from 3rd complete pulsatile beat after rapid cuff inflation were similar to those recorded from the "initial" gradient of the manual cuff inflation ($P = 0.66$).

Comparison of mean FBF values recorded from the 1st, 2nd, and 3rd individual pulsatile beats following rapid cuff inflation with mean FBF recorded from manual cuff inflation revealed high correlation with r values of 0.98, 0.98, and 0.96; and $r^2$ values of 0.91, 0.95, and 0.92, respectively (Figure 3.9). Similarly, when individual FBF values from 1st, 2nd, and 3rd pulsatile beats following rapid inflation were compared with individual FBF values of manual cuff inflation, a reasonably high correlation of 0.79, 0.82, and 0.79; and $r^2$ values of 0.61, 0.68, and 0.61 respectively were detected (Figure 3.10).

| | Age (yr) | Weight (Kg) | Height (m) | BMI ($Kg/m^2$) | MVC (N) | FAC (cm) |
|---|---|---|---|---|---|---|
| | 27♂ | 72 | 1.73 | 24.06 | 196 | 27 |
| | 30♂ | 80 | 1.80 | 24.69 | 196 | 28 |
| | 20♀ | 51 | 1.60 | 19.92 | 137 | 20 |
| | 26♀ | 57 | 1.60 | 22.27 | 108 | 20 |
| | 24♀ | 86 | 1.70 | 28.31 | 157 | 25 |
| | 30♂ | 99 | 1.87 | 28.31 | 177 | 29 |
| | 28♂ | 70 | 1.65 | 25.71 | 186 | 24 |
| | 28♂ | 82 | 1.74 | 27.08 | 157 | 25 |
| | 26♂ | 70 | 1.68 | 24.80 | 137 | 25 |
| Mean | 26.56 | 73.78 | 1.71 | 25.08 | 161.32 | 24.78 |
| SEM | 1.04 | 4.69 | 0.03 | 0.97 | 10.09 | 1.05 |

Table 3.1 **Anthropometric data of subjects (n=9).**

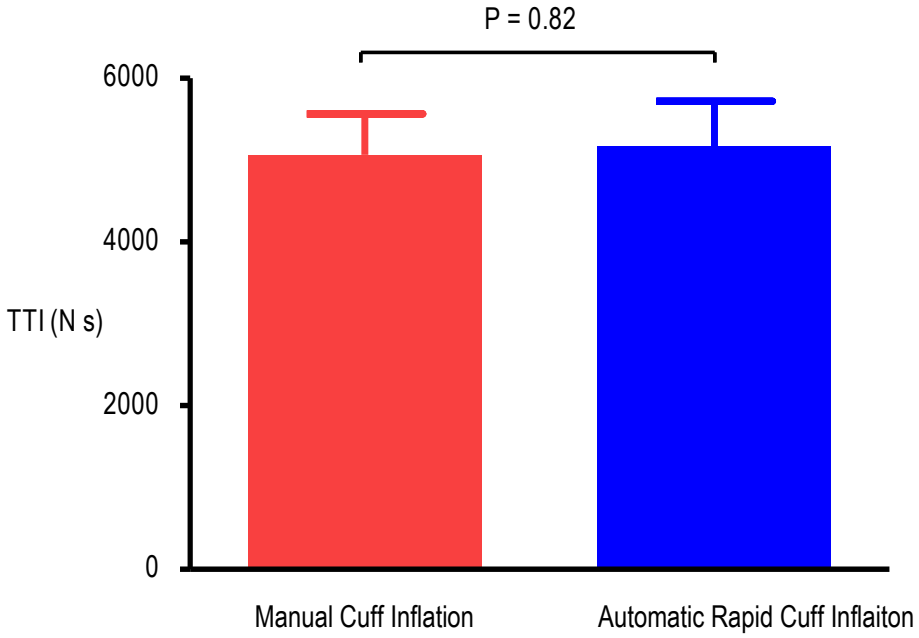



Figure 3.5 **TTI recorded from the isometric handgrip contraction at 70% MVC for 1 min.** No significant difference was found between manual cuff inflation (red bar) and automatic rapid cuff inflation (blue bar) treatment conditions.

| | Baseline FBF Responses | | | | |
|---|---|---|---|---|---|
| | Manual Inflation | Rapid Cuff Inflation (1st Pulse) | Rapid Cuff Inflation (2nd Pulse) | Rapid Cuff Inflation (3rd Pulse) | P Value |
| | 9.44±0.91 | 11.09±1.80 | 8.41±1.94 | 8.27±2.02 | *0.24* |
| | 9.67±1.19 | 10.16±1.42 | 8.44±1.38 | 7.84±1.39 | *0.11* |
| | 9.56±1.29 | 10.52±1.14 | 8.02±1.20 | 7.65±1.13 | *0.09* |
| P Value | *0.97* | *0.71* | *0.87* | *0.77* | |

Table 3.2 **Baseline FBF responses measured with manual cuff inflation and automatic rapid cuff inflation.** No significant difference was found between treatments.

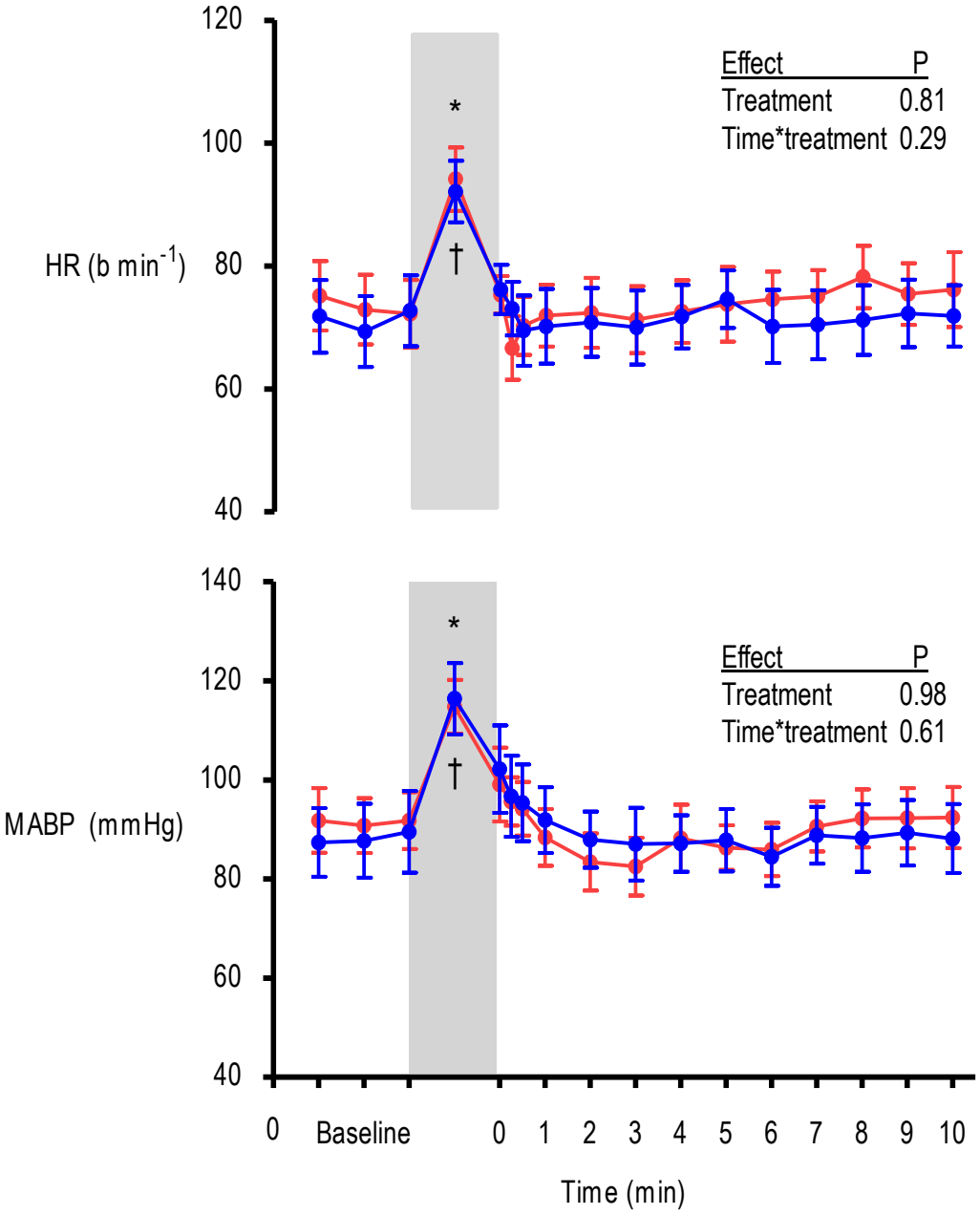



Figure 3.6 **HR and MABP responses of isometric handgrip contraction at 70% MVC.** Handgrip contraction is shown by grey bar. As expected, no significant treatment or time*treatment effect was detected between manual cuff inflation (red) or automatic rapid cuff inflation values (blue). */† show values significantly different from their respective baseline ($P < 0.001$).

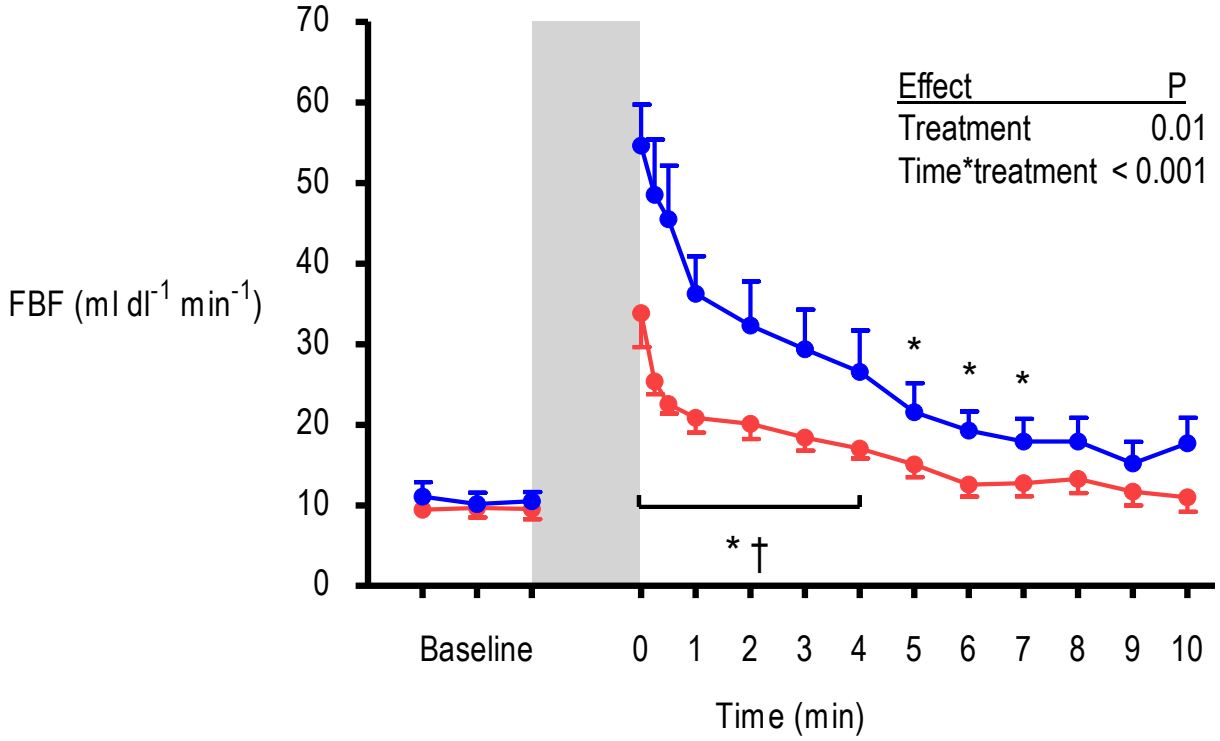



Figure 3.7 **FBF responses evoked before and following isometric handgrip contraction at 70% MVC for 1 min.** Handgrip contraction is shown by grey bar. FBF values recorded with manual cuff inflation (red) were significantly different from those measured with automatic rapid cuff inflation (blue). */† show values significantly different from their own respective baselines ($P < 0.001$). This includes all time points within the bracket.

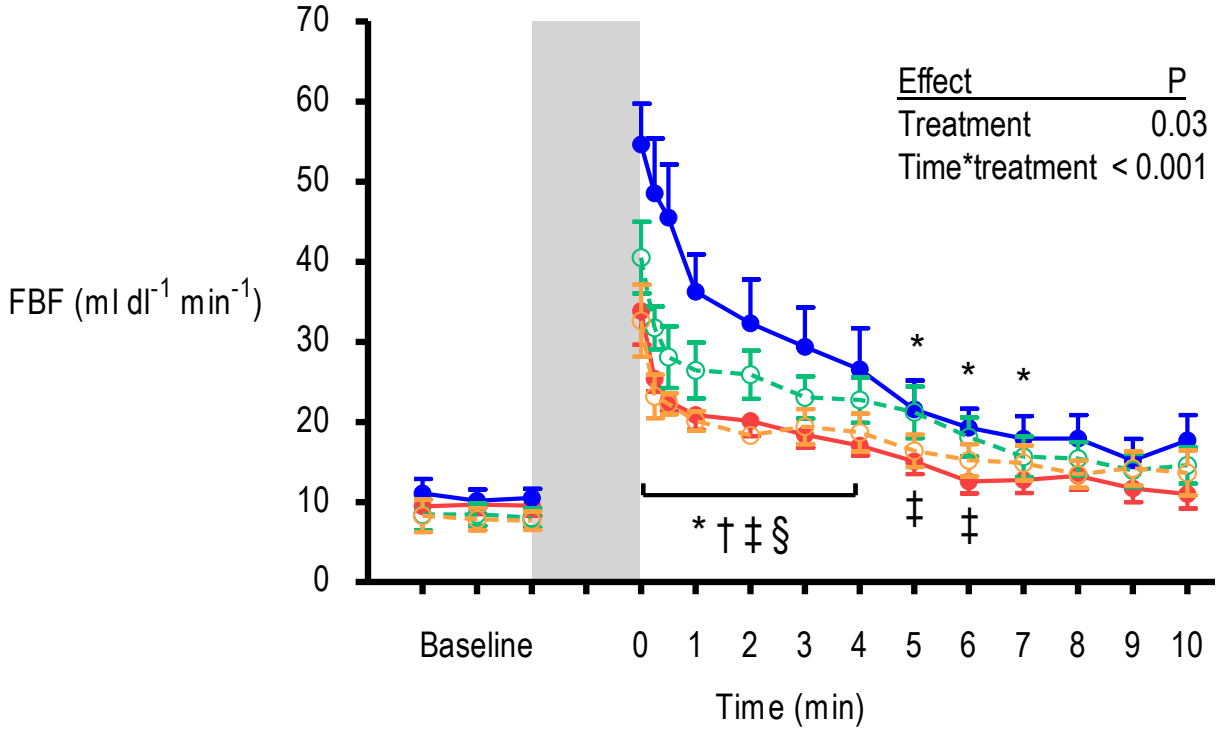



Figure 3.8 **Comparison of FBF values recorded before and after isometric handgrip contraction with automatic rapid cuff inflation and manual cuff inflation.** Handgrip contraction is shown by grey bar. Values calculated from the "initial" gradient after manual cuff inflation (red) and automatic rapid cuff inflation (blue) are shown alongside FBF values recorded from the 2nd (green) and 3rd (orange) pulsatile beats after rapid cuff inflation. Significant treatment effect was detected in FBF values measured from the 1st pulsatile beat gradient of automatic rapid cuff inflation, and those from 2nd, and 3rd pulsatile beats. */†/‡/§ represent FBF values significantly different from respective baselines (1st beat of rapid cuff inflation, manual cuff inflation, 2nd beat, and 3rd beat of rapid cuff inflation; $P < 0.001$ vs. respective baselines). This includes all time points within the bracket.

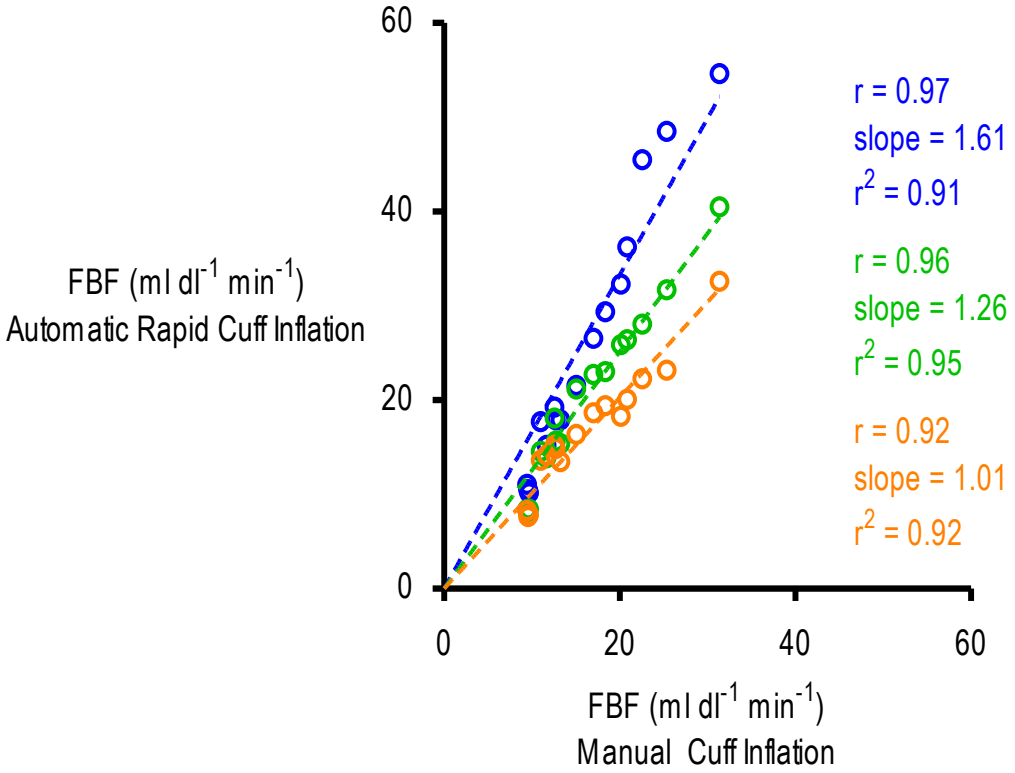



Figure 3.9 **Graph comparing mean FBF values from 1st, 2nd, and 3rd beat of rapid cuff inflation with mean FBF values from manual cuff inflation.** FBF values from 1st beat (blue), 2nd beat (green), and 3rd beat (orange) showed a high correlation (r) values of ~ 0.9 and $r^2$ values of ~ 0.9 when compared with FBF values recorded from manual inflation of the venous occlusion cuff. Regression line was forced to fit through 0 on both axes, revealing a slope of 1 for FBF values from 3rd beat (orange) vs. those from manual cuff inflation.

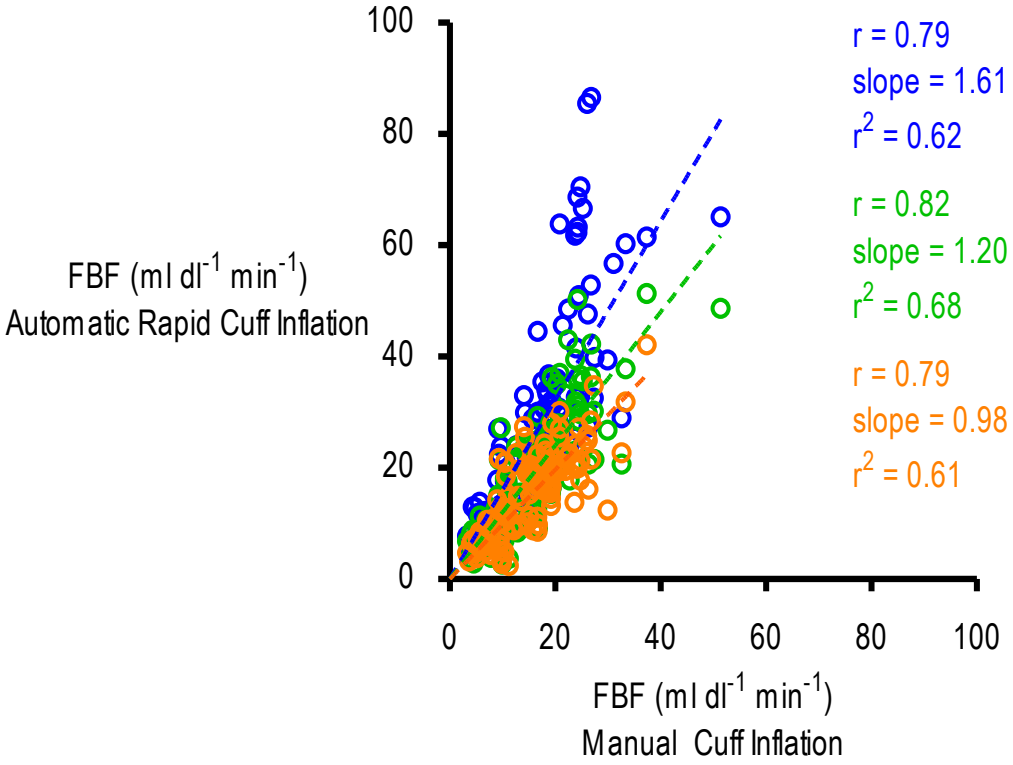



Figure 3.10 **Graph comparing individual FBF values from 1st, 2nd, and 3rd beat of rapid cuff inflation with individual FBF values from manual cuff inflation.** FBF values from 1st beat (blue), 2nd beat (green), and 3rd beat (orange) showed a high correlation (r) values of ~ 0.8 and fairly high $r^2$ values of 0.6, 0.7, and 0.6, respectively when compared with FBF values recorded from manual inflation of the venous occlusion cuff. Regression line was forced to fit through 0 on both axes, and revealed a slope of 0.98 for FBF values from 3rd beat (orange) vs. those from manual cuff inflation.

## 3.4 Discussion

To our knowledge this is the first study to directly compare FBF values calculated by venous occlusion plethysmography when venous occlusion is done manually and by automatic rapid inflation under resting and *post*-exercise hyperaemia conditions. The principle finding of this study was that the FBF values recorded with the automatic rapid cuff inflation were ~ 2 fold higher compared with traditional manual cuff inflation immediately following isometric handgrip contractions. Secondly, consideration of the baseline and 10 min *post*-exercise recordings provides the important observation that the discrepancy in FBF values between the methods is not present during resting conditions, but only appears when FBF is substantially raised. Therefore, the results of this study offer evidence that the rise in initial gradient following rapid cuff inflation provides a "better" estimate of limb blood flow; especially when it is substantially elevated from baseline conditions. Furthermore, it also confirms the observations of Tschakovsky et al. (411) that FBF calculated from pulsatile beats following the 1$^{st}$ complete beat underestimates flow with each consecutive beat. The present study also shows that by the 3$^{rd}$ pulsatile beat, FBF is similar to that observed when the traditional manual inflation of the venous occlusion cuff is employed.

It is important to mention here that despite the high correlation in FBF measurements made with two techniques, the actual magnitude of the exercise hyperaemia response, and its gradual attenuation were not similar in individual subjects. This is indicated by the significantly high $r^2$ values when *mean* FBF values from 1$^{st}$, 2$^{nd}$, and 3$^{rd}$ pulsatile beat following rapid cuff inflation are compared with *mean* FBF values from manual cuff inflation (Figure 3.9). Whereas comparison of *individual* FBF values reveals moderate $r^2$ values (Figure 3.10). Figure 3.8 shows that as time passes, the magnitude of difference between FBF values measured with the two inflation techniques decreases following handgrip contraction. Moreover, as shown in Figure

3.10, the change in the magnitude of the difference in hyperaemia values is not consistent and is therefore the reason for moderate $r^2$ values. This is understandable as the arterial inflow, the subsequent decline in perfusion pressure, and hence the measured blood flow values following venous occlusion would be dependent on each individual's hyperaemia response and their unique arterio-venous architecture. Nonetheless, the mean data do show a high correlation (r) and degree of determination ($r^2$) between FBF values measured from the two cuff inflation techniques.

Work done by Tschakovsky et al. (411) provided the important observation that with each pulsatile beat following automatic rapid venous occlusion, the pulsatile voltage beats of the plethysmograph trace gradually "lose the rise in their gradient". Therefore, calculation of FBF as an average over several beats as done traditionally underestimates flow. This means that flow calculations from 2$^{nd}$ or 3$^{rd}$ beat of plethysmograph trace following venous occlusion, or from the average gradient of the first few beats, do not reflect that "actual" hyperaemic response, but instead, underestimate it.

In the light of the present study, it is apparent that the very short delay (1 – 2 s) induced by inflating the venous occlusion cuff by using traditional manual inflation method also leads to underestimation of peak hyperaemic response, and subsequent FBF during the ~ 7 min of *post*-exercise hyperaemia. These observations therefore question the absolute values reported in previous studies performed when limb blood flow was measured under hyperaemic condition by either taking an average of the gradient of the first few beats or by employing manual inflation of the venous occlusion cuff.

One explanation for the discrepancy in FBF values could be that the approximate 1 – 2 s delay in manual cuff inflation to ~ 50 mmHg allows the hyperaemic response to "decrease" before any

measurements can be made. Certainly, when FBF was measured following automatic rapid cuff inflation, the drop in FBF was nearly 12% from 0 to 15 s *post*-contraction (See Figure 3.7). However, this would not explain the discrepancy in FBF values recorded from manual and automatic rapid inflation of ~ 40%, with a time delay of only 1 – 2 s. The alternative explanation for the discrepancy between FBF values recorded with the two methods is related to the evidence provided by Tschakovsky et al. (411) regarding their observation of underestimating FBF with each successive pulsatile beat. They showed that the decrease in FBF was due to the reduction in pressure gradient caused by increased venous filling and subsequent back pressure, and not due to a decrease in arterial diameter. Certainly, the time of ~ 1 – 2 s in the inflation of venous occlusion cuff to 50 mmHg with manual inflation does not necessarily mean that venous occlusion only occurs at that pressure (148). It is more likely that some hindrance in venous drainage occurs during the process of cuff inflation, which allows blood to "accumulate" in the veins of the forearm before cuff pressure actually reaches 50 mmHg. This must cause reduction in the arterio-venous pressure gradient, and as flow can only be measured once the desired venous occlusion cuff pressure has been achieved, the result is underestimation of FBF. Indeed, the calculations of FBF from the 3 successive pulsatile beats after rapid inflation of venous occlusion cuff also seems to suggest that reduction in pressure gradient is the cause of underestimation of flow: by the 3$^{rd}$ beat (~ 1 – 2 s delay from the point of rapid cuff inflation) FBF was similar to that observed with traditional manual cuff inflation (Figure 3.8). This is also evident if a regression line is fitted between FBF values recorded from the 3$^{rd}$ beat of rapid cuff inflation and those from manual cuff inflation; the slope between them is ~ 1 and is independent of the use of *mean* or *individual* FBF data points (Figure 3.9 and Figure 3.10). Nonetheless, as the act of venous occlusion cuff inflation has previously been associated with attenuated arterial diameter (171), the results and conclusion of this study remain slightly ambiguous. Nonetheless,

keeping in mind the limitation of the technique of Doppler-ultrasound (discussed below), its use to monitor brachial artery diameter along with the two cuff inflation techniques may have further clarified the observed responses: this remains a small limitation of this study.

Although there is no absolute or correct way of measuring limb blood flow, venous occlusion plethysmography has been compared with the popular flow-transducer and Doppler-ultrasound techniques in the past to reveal a consistently high correlation / linear relationship between them (238, 411). For example, Longhurst et al. compared discontinuous FBF measurements made with strain-gauge plethysmography to continuous recordings obtained from the brachial artery using an electromagnetic flow-transducer. They found a high correlation (0.8 – 0.9) between them (238). Comparison of FBF values measured from Hokanson strain-gauge plethysmograph with rapid inflation system and Doppler-ultrasound provided by Tschakovsky et al. over simultaneous beats also showed a tight linear relationship (~ 0.9) between them (411). These two studies provide validity for the robustness of the technique of strain-gauge venous occlusion plethysmography. However, these parallel techniques will only provide an accurate quantitative measure of changes in volume flow if the angle of insonation of the Doppler-probe is properly normalized, and the vessel diameter is continuously monitored. Tschakovsky et al. showed that it cannot be assumed that changes in velocity reflect changes in flow because vessel diameter can also change (411). Indeed, their observations led them to recommend that calibration of ultrasound flow-meters against venous occlusion plethysmography at the beginning and end of each experimental stimulus, along with continuous recording of vessel diameter change should be employed in order to truly reflect qualitative and quantitative changes to the limb volume.

Further, observations made by Casey et al. (57) while investigating exercise hyperaemia in response to rhythmic handgrip contractions (1 s contraction: 2 s relaxation) during normoxia or hypoxia provide good examples of the importance of expressing limb blood flow relative to tissue

volume. In one of their study, FBF was recorded from the brachial artery using Doppler-ultrasound, together with end-diastole diameter measurements. With rhythmic handgrip exercise performed at 10% MVC under systemic hypoxia, FBF was recorded to be ~ 289 and ~ 172 ml/min in young men and women respectively (57): significantly greater in men than women. However, when arm volume was assessed by volume displacement, the same data analyzed as changes in blood flow relative to the original tissue volume showed that FBF was ~ 26 and ~ 23 ml/dl/min in men and women respectively; with no gender difference between them (57).

Thus, the simple technique of venous occlusion, which automatically allows FBF to be calculated relative to limb volume, holds considerable advantage over its main rival. The problem of cuff artefact hindering gradient calculation from the initial rise in plethysmograph trace can easily be avoided by use of computerized data collection software (for example, Lab-Chart). These software products are able to collect a detailed record of the experiments and can easily be used to exclude the inflation artefact from calculations. Nevertheless, the main drawback of venous occlusion plethysmography remains its inability to continuously record blood flow changes (191).

As stated above, it is of profound importance that especially under hyperaemia conditions, venous occlusion cuff is inflated as quickly as possible *and* only the first complete pulsatile beat of the plethysmograph trace be used for blood flow calculation. Any delay in cuff occlusion to the required pressure level will result in underestimation of hyperaemia response. This would also be the case if the venous occlusion cuff were inflated quickly (using rapid cuff inflation systems), but the gradient is calculated from the average of a few pulsatile beats. An example of this second problem can be seen in the PhD thesis of Davies where Hokanson plethysmograph was used in combination with rapid cuff inflation system to record FBF responses (83). The estimated values for FBF in that thesis are actually similar to those observed with manual venous occlusion cuff inflation (122, 123, 437). Moreover, as the limb arterio-venous architecture of his subjects did not

change between treatments, calculation of averaged blood flow from a few pulsatile beats following rapid venous occlusion may well have been the reason for masked treatment effects between conditions: i.e., larger hyperaemia would negatively affect perfusion pressure more quickly than an attenuated response.

In conclusion, the use of manual inflation of the venous occlusion cuff results in significant underestimation of FBF as compared with the values obtained with rapid cuff inflation. Therefore, our hypothesis that reduction in time taken to inflate the venous occlusion cuff will provide a better estimate of FBF was accepted. However, even when the automatic system is used, to be sure that the calculated FBF value is accurate, only the initial / first pulsatile beat of the plethysmograph trace should be used for calculation of the gradient of deflection; especially under hyperaemic conditions.

# Chapter 4

# 40% $O_2$ and COX blockade similarly attenuate forearm *post*-exercise hyperaemia and venous efflux of PGs following handgrip contractions in young and older men

## 4.1 Introduction

As discussed in Chapter 1 (See Section 1.2.3.4), vasodilator COX products are generally considered to contribute towards exercise hyperaemia. Indeed, a large number of studies indicate that PG production increases during exercise (29, 198, 199, 302, 390, 435, 447, 448). For example, Symons et al. observed that $PGE_2$ and 6-keto-$PGF_{1\alpha}$ concentrations doubled in cat triceps-surae following their 30 s isometric contractions (390). In humans, Nowak and Wennmalm observed an exercise-related increase in the venous concentrations of PGE-like activity following isometric and rhythmic handgrip exercise (302). Further, Wilson and Kapoor observed an increase in the concentrations of both $PGE_2$ and $PGI_2$ in response to wrist-flexion exercise (435). This was completely abolished by COX inhibition, which also attenuated the hyperaemia by ~ 20%. In addition, $PGE_2$ concentrations increase in vastus-lateralis and vastus-medialis interstitial fluid following leg exercise (29, 199).

Subsequently, a number of studies have confirmed that PGs contribute towards exercise hyperaemia regulation in that COX blockade attenuates the hyperaemic response (73, 74, 100, 209, 359, 437). For example, Cowley et al. showed that the *post*-exercise hyperaemia of 30 min treadmill exercise halved in response to COX inhibition with either indomethacin or aspirin (74). Separately, Duffy et al. observed attenuation in peak-exercise hyperaemia following rhythmic wrist-flexion during intra-arterial aspisol infusion (100). Similarly, ~ 35% attenuation in *post*-exercise hyperaemia of isometric handgrip contractions (60% MVC; 2 min) was observed by our group (437). Regarding the influence of COX inhibition on the vasodilatation that occurs during exercise, Schrage et al. showed that intra-brachial ketorolac infusion attenuated exercise hyperaemia recorded during rhythmic handgrip contractions by ~ 12% (358).

On the other hand, it should be noted (See Section 1.2.3.4 and 1.2.3.7) that some studies which have involved the use of COX inhibitors have concluded that PGs play a minor role in exercise hyperaemia; or that their contribution can only be demonstrated when NO synthase is also inhibited because the contributions of PGs and NO are interdependent (287, 329). Importantly, none of these studies have assayed PG release during contractions.

Interestingly, the release of PGs during exercise was shown to be related to the work intensity / $VO_2$ (29, 198, 447, 448). Moreover, isolated arterial vessels show augmented release of PGs under hypoxic conditions (45, 46, 266). Together this is suggestive of an intimate relationship between PG production and localized tissue hypoxia. Consistent with this idea, Win and Marshall reported that the *post*-exercise hyperaemia that followed 60% MVC isometric handgrip contraction was equally attenuated by COX inhibition and 40% $O_2$ supplementation (437). As the combination of COX inhibition and 40% $O_2$ did not yield any added attenuation in *post*-exercise hyperaemia, their results suggested $O_2$-dependent PG production during isometric exercise. However, they did not directly test this idea by assaying PG release. Further, although studies have in the past indicated a contribution of vasodilator PGs to the exercise hyperaemia of rhythmic contractions, in that COX inhibition attenuated the response (74, 100, 435), it is not known whether PG production under these conditions is $O_2$-dependent. The present study therefore addressed these very questions, including whether the release / action of $PGE_2$ and/or $PGI_2$ might be $O_2$-dependent.

A question then arises regarding the primary site of the hypoxic stimulus that may cause increased PG production. Busse et al. measured intra-luminal $PGE_2$ and $PGI_2$ release from isolated but intact, canine coronary and femoral arteries, along with the rat tail artery segments (45). Under hypoxic perfusion, an increase was mainly observed in the concentration of $PGI_2$ which was abolished by either endothelium removal or pre-incubation of vessels with

indomethacin. Further, Messina et al. measured diameter of isolated cremaster muscle arterioles under hypoxic, normoxic, and hyperoxic conditions (263). Arteriolar dilatation evoked when bath $O_2$ was reduced to ~ 0 was completely abolished by either endothelium's removal or COX inhibition. Interestingly when bath $O_2$ was raised to ~ 95%, arteriolar diameter decreased to become comparable to that measured when endothelium was removed or when COX was inhibited with indomethacin. Taken together, these studies suggest that $PGI_2$ production during hypoxia is the result of the vascular endothelium sensing / responding to the changes in localized $PO_2$.

Fordy and Marshall's work suggests an additional possibility (123). They asked their subjects to perform two bouts of exhaustive isometric exercise, separated by 7 min of recovery. Subjects were given 40% $O_2$ either during the exercise *or* during the 7 min of recovery period. As *post*-exercise hyperaemia was only attenuated if subjects were breathing supplementary $O_2$ during exercise, the source of hypoxic stimulus for PG production during exercise must lie within the working muscles. Moreover, the fact that the increase in venous efflux of lactate was also reduced by breathing 40%, suggested that additional $O_2$ must have reached the contracting muscle fibres and affected their metabolism. This observation is consistent with studies that have assessed intra-muscular $PO_2$ concentrations and found that they decrease significantly during exercise (47, 337-339). As the primary PG shown to be released by the skeletal muscles belongs to the E series (400), the concentrations of the stable metabolites of both $PGE_2$ and $PGI_2$ were measured in the present study in hope of distinguishing the source / target of exercise-related hypoxic stimulus for PG production.

Lastly, controversy exists regarding the influence of aging on the contribution of vasodilator PGs to exercise hyperaemia. Recent work by Schrage et al. suggested that their contribution is completely lost with aging (358). They measured FBF of 15 older subjects while they performed

rhythmic handgrip contractions (10% MVC; 20/min), and found no change in their steady-state exercise hyperaemia with intra-brachial infusion of the COX inhibitor, ketorolac. This raises an interesting question as to whether the results obtained by Schrage et al. were due to the use of light-exercise intensity, or to a global age-related decline in the generation of PGs, or in their effective contribution to exercise hyperaemia. Moreover, based on the recent data from Nyberg and colleagues (285, 305), a reduction in the contribution of PGs to exercise hyperaemia in the elderly might be a reflection of their relatively sedentary life styles, rather than age *per-se*. The present study tried to address these questions.

### 4.1.1 Aims and hypotheses

As stated above, the potential $O_2$-dependent contribution of vasodilator PGs to hyperaemia associated with rhythmic exercise has not been investigated. Moreover, the idea that the vasodilator PGs involved in hyperaemia of isometric exercise are $O_2$-dependent is based on indirect evidence: i.e., no one has measured changes in the release of PGs. This study was designed to address these questions. Furthermore, although the relative contributions of vasoactive ions to exercise hyperaemia were discussed in Chapter 1 (See Section 1.2.3.3); it remains unclear whether COX inhibition or supplementary $O_2$ influence their production / function. Therefore, this study was also designed to allow pH, lactate, and $K^+$ concentrations to be measured intermittently in the venous efflux. Lastly, the present study allowed the influence of age on the production / action of all these vasoactive molecules and exercise hyperaemia to be assessed.

We hypothesized, that the *post*-exercise hyperaemia associated with rhythmic handgrip contractions would be similarly $O_2$-dependent to that of isometric handgrip exercise. We also hypothesized that the concentrations of vasodilator PGs would be similarly attenuated by COX

blockade and by breathing 40% $O_2$. Furthermore, based on the recent work from Copenhagen (285, 305), we hypothesized that PGs do play a significant role in exercise hyperaemia of recreationally active older individuals, and that their production will remain $O_2$-dependent. To test these hypotheses, we investigated the effect of COX blockade, breathing 40% $O_2$, and their combination on exercise hyperaemia following rhythmic and isometric handgrip contractions (60% MVC) performed by healthy, active, young and older men.

## 4.2 Methods

Twelve young (19 – 25 years) and eleven older (60 – 76 years) men were recruited; all were healthy, recreationally active, non-smoking adults (2 older subjects quit smoking over 40 years ago). Medication history included occasional use of anti-histamines for allergies by 1 young subject, occasional use of NSAIDs for knee pain by 1 older subject, use of sodium-valproate as a child by 1 older subject, and discontinued use of statins and low-dose aspirin (at least 3 months) by further 2 older men. Supplemental medication use included whey-protein, including creatine and glutamine by 3 young men, multivitamins by 3 young and 1 older male, fish-oil supplementation by 2 young and 1 older male, and occasional use of ascorbic-acid and glucosamine by 1 older male. All subjects were required to adhere to dietary, medicinal, and exercise restrictions previously described in Chapter 2 (See Section 2.1). Subjects were also asked to stop taking multivitamins / fish-oil for at least 7 days prior to the experiments. Administration of air or 40% $O_2$, and placebo or aspirin along with measurements of MVC, TTI, HR, MABP, FBF, and FVC were done as described in Chapter 2.

### 4.2.1 Protocol

Subjects visited the laboratory on five occasions. The initial visit was used to complete the health and activity questionnaire, provide familiarization with the experimental protocol, and to record each subject's MVC (See Section 2.3). The following four experimental visits were carried out in a randomized single-blind cross-over manner; placebo (P), aspirin (A), 40% $O_2$, and a visit with combination of aspirin and 40% $O_2$ (A+$O_2$).

On experimental days the handgrip dynamometer was set up for subject's MVC. The subject was then allowed to rest for a few min before an intra-venous cannula was inserted into the antecubital vein of the exercising (dominant) arm and made secure. The subject then consumed

the treatment drink (P or A; 600 mg). All recording equipment (FBF and MABP) was then connected to the subject as described in Chapter 2. After ~ 25 min of rest, the facemask was put on the subject to deliver medical air or 40% $O_2$; it was made secure with elastic-straps around the head. The subject was asked to breathe normally and after 5 min, a resting blood sample was taken. Immediately following this, three FBF measurements were made with venous occlusion plethysmography (See Section 2.5.2) to confirm their reproducibility; each ~ 15 s apart. As mentioned in Chapter 2, ABP and HR were recorded from the resting contra-lateral hand.

30 s to 1 min following the last baseline FBF measurement, the subject performed rhythmic handgrip contractions (1 s contraction: 1 s relaxation) for 3 min at 60% MVC. As shown in Figure 4.1 (protocol schematic), following the cessation of exercise, FBF was measured at 0, 30 s, and 1 min, and then at 1 min intervals for 7 min; MABP was extracted from the same cardiac-cycles (See Section 2.7). *Post*-exercise venous blood samples were taken at 0 s for PG analysis and at 0, 3, 5, and 7 min for blood gas and metabolite analysis. Gas flow was then ceased and the facemask was removed; the subject rested for a further 25 min.

Following this, either medical air or 40% $O_2$ was again delivered; resting blood samples and FBF data were taken as previously described after 5 min. The subject then performed an isometric handgrip contraction at 60% MVC for 3 min; FBF measurements and venous blood samples were taken as described above. At the end of the experiment, all recording equipment and the intra-venous cannula were disconnected. The subject rested for 5 – 10 min before leaving the laboratory.

### 4.2.2 Blood sample collection

An intra-venous cannula (22 – 24 G, BD Venflon, BD) was inserted in an easily accessible vein of the subject's exercising forearm for the purpose of blood sampling (Figure 4.2). The cannula

was connected to a multiple-sample luer-adapter (BD Vacutainer Multiple-Sample Luer-Adapter, BD) via a plastic tube connector (10 cm BD Connecta, BD). Blood was collected into either 4 ml vacutainers (Sodium-Heparin BD Vacutainer, BD) for PG analysis or 1 ml syringes (BD Plastipak, BD) for blood gas and metabolite analysis at specific time points (See Figure 4.1). The samples collected into a syringe were immediately transferred into heparinised capillary tubes (Capillary Sample-Kit, Instrumentation Laboratory) and capped. The intra-venous cannula with its connector tube was periodically flushed with 3 ml bolus of 0.9% sterile-saline (BD PosiFlush SP Syringe, BD) to stop blood clotting. At each time point of blood sample collection, 0.8 ml of blood was initially drawn out and discarded in order to get rid of the dead-space volume from the connector tube. All blood samples were kept on ice during the period of experimentation. In order to prevent *ex-vivo* formation of PGs, 1 µl/ml of 10 µM Indomethacin (Sigma-Aldrich, UK) stock was added to the vacutainers immediately after the experiment; indomethacin stock was prepared using ethanol. All vacutainers were then centrifuged (Mistral 3000i, MSE Ltd) at 2500 rpm at 4°C for 20 min; supernatant plasma was collected, snap frozen using liquid nitrogen, and then stored in a - 80°C freezer for later analysis. Capillary samples were immediately analyzed after each experiment using a blood gas analyzer (GEM Premier 4000, Instrumentation Laboratory) for venous $PO_2$, $PCO_2$, $H^+$, lactate, and $K^+$. In total, ~ 36 ml of blood was collected during each experiment.

### 4.2.3 Enzyme linked immuno-sorbent assay

#### 4.2.3.1 Prostacyclin

Enzyme linked immuno-sorbent assay (ELISA) of 6-keto-$PGF_{1\alpha}$ was carried out on the venous blood samples of 6 randomly selected subjects from each age group using a commercially available kit (Item-515211, Cayman Chemical Company). This assay is based on the competition between 6-keto-$PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$ tracer (6-keto-$PGF_{1\alpha}$ acetylcholinesterase conjugate)

for a restricted number of 6-keto-$PGF_{1\alpha}$-specific rabbit antiserum binding sites. The 6-keto-$PGF_{1\alpha}$ antiserum binds to the mouse monoclonal anti-rabbit immunoglobulin-G (IgG) previously attached to the plate wells. The concentration of bound $PGF_{1\alpha}$ is inversely proportional to the concentrations of bound tracer. Unbound reagents were washed away; Ellman's reagent was added to develop the plate. The yellow substance produced as a result of the enzymatic reaction was measured by Spectrophotometery. 120 min after development of plate in the dark, it was read at 405 nm using a plate reader (VeraMax micro-plate reader, Molecular Devices LLC). Each sample was analyzed in duplicate and a mean absorbance was calculated: values converted into concentrations of 6-keto-$PGF_{1\alpha}$ using the standard curve (Figure 4.3).

#### 4.2.3.2 Prostaglandin E metabolite

ELISA of the stable metabolite of $PGA_2$ and $PGE_2$, Prostaglandin E metabolite (PGEM) was carried out on the same 6 subjects from each age group using a commercially available kit (Item-514531, Cayman Chemical Company). Samples were left overnight in the dark at room temperature for derivation of 13,14-dihydro-15-keto-$PGA_2$ and 13,14-dihydro-15-keto-$PGE_2$ to stable PGEM for quantification. The competitive assay is between PGEM and PGEM-acetylcholinesterase conjugate (PGEM Tracer) for limited PGEM-specific, rabbit antiserum binding sites. The antiserum binds to the mouse monoclonal anti-rabbit IgG which is already attached the wells. Ellman's reagent was added to develop the plate after washing away unbound reagent and the substance produced was measured spectrophotometrically. The plate was read at 405 nm after 90 min of development in a dark room. Each sample was analyzed in duplicate and mean absorbance was calculated. Concentrations of PGEM were detected from the absorbance values using the standard curve (Figure 4.4).

### 4.2.4 Statistical analysis

Factorial mixed-model ANOVA was used to identify time, treatment, exercise type, age, and their interaction effects. The data of each treatment condition were also individually analyzed using 1-way repeated measures ANOVA to detect within time effects. Percentage changes in *post*-exercise FVC, 6-keto-$PGF_{1\alpha}$, and PGEM efflux were compared between treatments and age groups using 2-way ANOVA. Once a statistically significant effect was detected, Tukey's HSD was used to detect the exact point of statistical difference within and/or between conditions. Pearson's correlation coefficient (r), regression analysis for best-fitting line, and the coefficient of determination ($r^2$) were calculated for comparison between baseline and *post*-exercise FBF and PG (6-keto-$PGF_{1\alpha}$ and PGEM) concentrations.

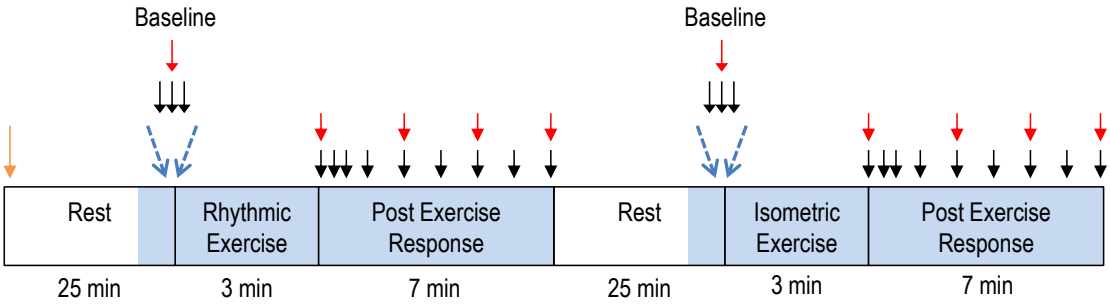



Figure 4.1 **Schematic diagram of the protocol.** Initial ↓ (orange) represents the consumption of treatment drink. ↓ (black) represents times at which FBF was measured using venous occlusion plethysmography and ↓ (red) represents time points of blood sample collection. Blue area represents medical air or 40% $O_2$ inhalation. Air or $O_2$ flow was always turned on 5 min before any FBF measurements or blood samples were taken. Protocol was repeated 4 times in a single-blind randomized manner with placebo, 40% $O_2$, aspirin, and A + $O_2$ as treatment conditions.

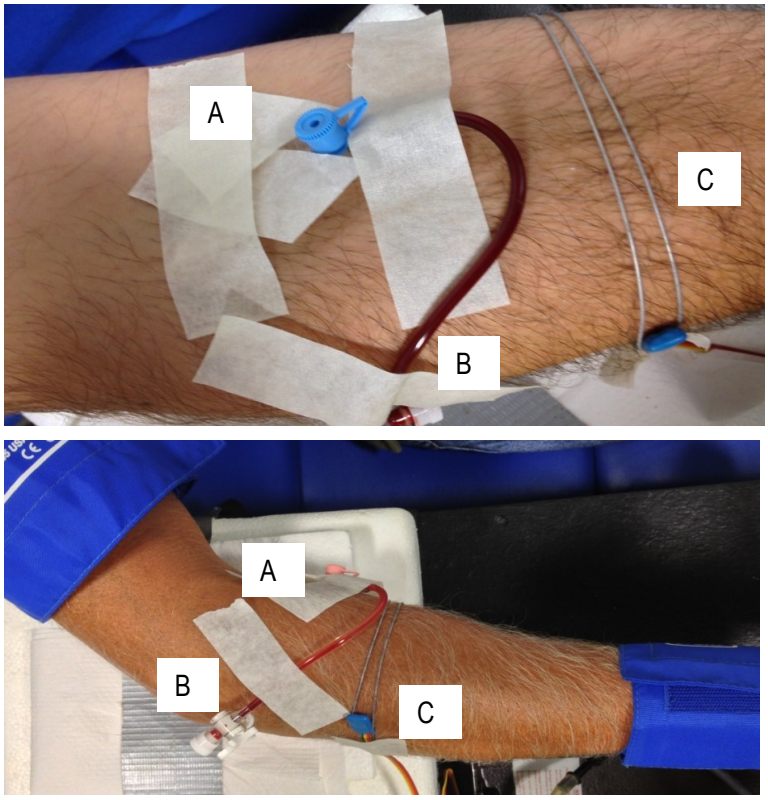



Figure 4.2 **Arrangement of the cannula in the antecubital vein (upper and lower panel).** Blood samples were collected using the intra-venous cannula (A); connected to the luer-adapter via plastic tube connector (B). 0.9% sterile-saline was used to keep the cannula patent and dead space fluid in the plastic tube connector (~ 0.8 ml) was always discarded before any samples were taken. Strain-gauge (C) can be seen wrapped around the widest part of the forearm.

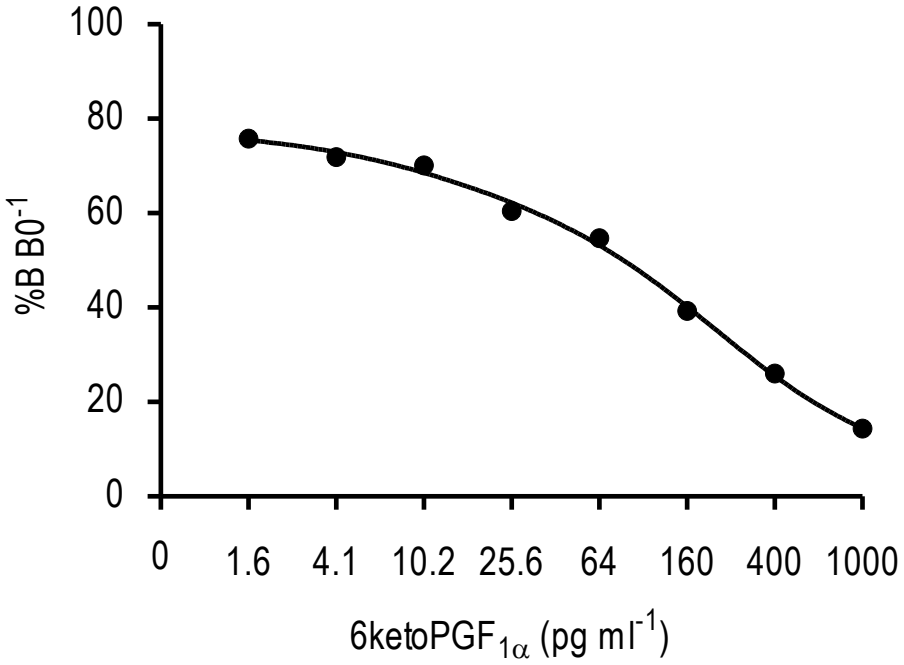



Figure 4.3 **Standard curve calculated from the standards prepared for 6-ketoPGF$_{1\alpha}$ ELISA.**

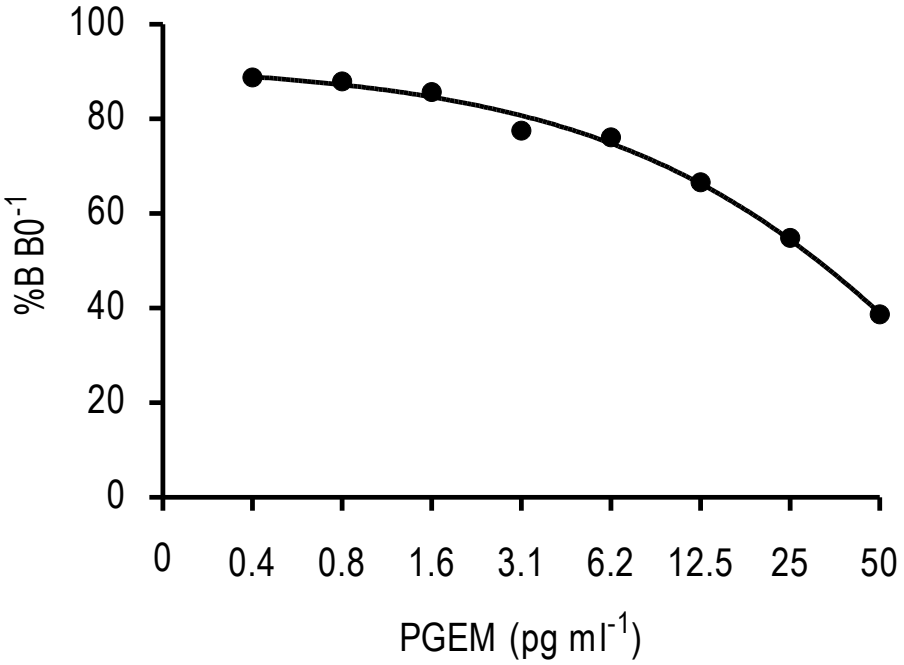



Figure 4.4 **Standard curve calculated from the standards prepared for PGEM ELISA.**

## 4.3 Results

Anthropometric data of the young and older subjects is shown in Table 4.1.

### 4.3.1 Young men

TTI was not different between treatment conditions during either exercise bout. However, TTI recorded during rhythmic contractions was lower than that generated during isometric handgrip contractions (Table 4.2). Most young subjects stopped isometric handgrip contractions around 2 min leading to statistically significant time difference between the two types of exercise (Table 4.2).

#### 4.3.1.1 Hemodynamic responses

The measured baseline hemodynamic responses were not affected by treatment conditions during rhythmic (Table 4.3) or isometric (Table 4.4) handgrip exercise bouts.

HR and MABP were also not different between treatment conditions during rhythmic and isometric handgrip contractions (Figure 4.5 and Figure 4.6, respectively). However as expected, both HR and MABP increased significantly during handgrip exercise, returning back to normal following cessation of exercise. Under placebo conditions, following both rhythmic and isometric contractions, FBF and FVC were increased, gradually returning to baseline over 7 – 8 min (Figure 4.7 and Figure 4.8). The magnitude of increase in *post*-exercise FVC was higher during isometric handgrip exercise ($P < 0.05$ vs. rhythmic). 40% $O_2$, aspirin, and A+$O_2$ caused similar attenuation of both FBF and FVC following both rhythmic and isometric contractions (Figure 4.7 and Figure 4.8, respectively). Supplemental data are shown in Appendix 5.

#### 4.3.1.2 Venous efflux of $K^+$, pH, lactate and blood gases

**$K^+$**: Under all treatment conditions, venous $K^+$ concentrations were increased immediately following both rhythmic and isometric handgrip exercise (Table 4.5 and Table 4.6, respectively). However by 3 min *post*-exercise, venous $K^+$ concentrations had returned back to baseline. 40% $O_2$, aspirin, or A+$O_2$ had no significant effect on the $K^+$ concentrations in the venous efflux for either rhythmic or isometric contractions.

**pH and lactate**: As expected venous pH decreased and venous lactate efflux increased immediately following rhythmic and isometric handgrip contractions (Table 4.5 and Table 4.6, respectively). A treatment effect was not detected during either handgrip contraction. Similarly, there was no difference between treatments following rhythmic or isometric contractions for lactate efflux (Table 4.5 and Table 4.6, respectively).

**$PO_2$ and $PCO_2$**: Overall $PO_2$ measured in venous blood samples following both rhythmic and isometric handgrip contractions were significantly higher during 40% $O_2$ breathing conditions (40% $O_2$ and A+$O_2$) than air breathing ($P < 0.05$; Table 4.5 and Table 4.6). Immediately following rhythmic contraction, only $PO_2$ values measured during air breathing (P and A) were lower than their respective baselines ($P < 0.05$ vs. baseline; Table 4.5) whereas following isometric handgrip contractions, venous $PO_2$ values were significantly lower for all treatment conditions except 40% $O_2$ ($P < 0.05$ vs. baseline; Table 4.6). By 3 min and onwards, venous $PO_2$ values were higher than their respective baselines in all treatment conditions. Overall, $PCO_2$ values measured in venous efflux following rhythmic and isometric handgrip contractions were not different between treatments (Table 4.5 and Table 4.6). Immediately following rhythmic and isometric handgrip, venous $PCO_2$ values were increased significantly from their respective baselines ($P < 0.01$ vs. baseline), but by 3 min *post*-contraction, had returned to baseline (Table 4.5 and Table 4.6).

#### 4.3.1.3. Venous PGs

**$PGI_2$ (6-keto-$PGF_{1\alpha}$)**: $PGI_2$ concentrations measured in the venous efflux before and immediately after rhythmic and isometric handgrip contractions and *normalized* $PGI_2$ (the product of $PGI_2$ efflux and the appropriate FBF value) are shown in Figure 4.9 (upper and lower panels, respectively). During placebo, *normalized* $PGI_2$ values increased significantly from baseline values of 18.03±5.98 pg/dl/min to 349.78±62.69 pg/dl/min following rhythmic handgrip contractions and from 20.13±8.55 pg/dl/min to 384.81±138.64 pg/dl/min following isometric contractions (Figure 4.9). As expected, COX inhibition with aspirin reduced baseline $PGI_2$ concentrations ($P < 0.05$ vs. P; Figure 4.9) but failed to influence baseline FBF or FVC (Table 4.3 and Table 4.4). No treatment effect was detected for baseline *normalized* $PGI_2$ during either handgrip trials. Further under interventional treatment conditions of 40% $O_2$, aspirin, and their combination (A+$O_2$), the *post*-exercise increase in $PGI_2$ efflux was significantly attenuated following both rhythmic and isometric contractions (Figure 4.9). Correlation of each individual's baseline and *post*-exercise FBF with venous $PGI_2$ concentrations is shown in Figure 4.10. The line of best-fit shows an increase in the *post*-exercise FBF with $PGI_2$ concentrations under placebo conditions during both rhythmic and isometric handgrip. This was not seen with the interventional treatments: i.e., the attenuated *post*-contraction hyperaemia during these interventional treatments was not accompanied by any significant increase in venous $PGI_2$ concentrations (Figure 4.10).

**PGEM**: The PGEM concentrations in venous efflux before and immediately after rhythmic and isometric handgrip exercise and the *normalized* PGEM data are shown in Figure 4.11 (upper and lower panels respectively). Baseline PGEM efflux and the *normalized* PGEM data were not significantly decreased by the treatments for either the rhythmic or isometric handgrip trials (Figure 4.11). During placebo, *normalized* PGEM increased significantly following rhythmic

handgrip contractions from the baseline of 20.47±6.55 pg/dl/min to 559.46±133.38 pg/dl/min and from 33.12±12.47 to 733.77±163.27 pg/dl/min following isometric contractions (Figure 4.11). Similar to $PGI_2$ efflux, under conditions of 40% $O_2$, aspirin, and A+$O_2$, these *post*-exercise PGEM values were similarly decreased following both rhythmic and isometric handgrip contractions (Figure 4.11). The correlation of each individual's baseline and *post*-exercise FBF and venous PGEM concentrations is shown in Figure 4.12. The line of best-fit shows an increase in the *post*-exercise FBF with PGEM concentrations under placebo conditions during rhythmic and isometric handgrip. Again, this was not seen with the interventional treatments (Figure 4.12).

### 4.3.2 Older men

The results obtained in older men were similar in almost every respect with those obtained in young men. Thus, in order to avoid repetition, the findings are described in brief with emphasis on the differences between young and older men; details are shown in Tables and Figures.

TTI in older men was not influenced by treatments during either rhythmic or isometric handgrip contractions (Table 4.2), but as in young men the TTI generated during rhythmic contractions was significantly lower than that generated by isometric contractions (Table 4.2). In contrast to young men, older men performed both rhythmic and isometric handgrip contractions for the full 3 min under all treatment conditions (Table 4.2). Despite the lower maximal handgrip contraction force of older men and thereby, the lower 60% MVC (See Table 4.1), TTIs recorded during rhythmic and isometric contractions were not significantly different between age groups ($P > 0.05$ vs. young; Table 4.2).

#### 4.3.2.1 Hemodynamic responses

As in young men, baselines were not affected by treatment conditions (Table 4.7 and Table 4.8). As expected, HR and MABP increased during both rhythmic and isometric contractions and by 1

min *post*-exercise had returned to the baselines; as in young men these changes were not affected by treatment condition (Figure 4.13 and Figure 4.14).

Under placebo condition, FBF and FVC increased significantly from their respective baselines following both rhythmic and isometric handgrip contractions (Figure 4.15 and Figure 4.16). Similar to the results obtained in young subjects, 40% $O_2$, aspirin, and A+$O_2$ equally attenuated the *post*-exercise hyperaemia of both rhythmic and isometric handgrip contractions. However the magnitude of this attenuation was not similar between young and older subjects; i.e., the attenuation of the increase in *post*-exercise FVC was weaker in the older subjects. See Figure 4.17 which compares the percentage attenuation of the 7 min period of *post*-exercise hyperaemia induced by 40% $O_2$, aspirin, and A+$O_2$ relative to the placebo responses. Supplemental data are shown in Appendix 5.

#### 4.3.2.2 Venous efflux of $K^+$, pH, lactate and blood gasses

**$K^+$**: As in young men, venous $K^+$ concentrations increased significantly after rhythmic and isometric handgrip trails under all treatments but by 3 min *post*-exercise, the values were similar to respective baselines (Table 4.9 and Table 4.10): treatment effect was not detected. The increase in venous $K^+$ efflux was not different between young and older men.

**pH and lactate**: Again, as expected venous pH decreased while venous lactate efflux increased following rhythmic and isometric handgrip contractions of the older men (Table 4.9 and Table 4.10); these changes were not affected by treatment condition. Following both rhythmic and isometric handgrip contractions, the decline venous pH was slightly more in older, than young subjects when breathing air (placebo and aspirin; Table 4.9 and Table 4.10). Further, venous efflux of lactate was significantly lower in the older men following both handgrip contractions.

**$PO_2$ and $PCO_2$**: As in young men, venous $PO_2$ decreased and $PCO_2$ increased immediately following rhythmic and isometric contractions, (Table 4.9 and Table 4.10). In contrast to young, venous $PO_2$ in older men was not affected by treatments at baseline or following contractions; values were not different between young and older subjects (Table 4.9 and Table 4.10). The increase in venous $PCO_2$ evoked by either handgrip exercise was not different between treatments and age groups (Table 4.9 and Table 4.10).

#### 4.3.2.3 Venous PGs

**$PGI_2$ (6-keto-$PGF_{1\alpha}$)**: $PGI_2$ concentrations and *normalized* $PGI_2$ efflux before and immediately after rhythmic and isometric handgrip contractions are shown in Figure 4.18 (upper and lower panels, respectively). In contrast to young subjects, baseline $PGI_2$ values were not affected by treatment conditions (See Figure 4.18). However, as in young subjects, *normalized* $PGI_2$ values increased significantly following rhythmic contractions from 14.34±6.79 pg/dl/min to 284.36±43.43 pg/dl/min under placebo; and from 27.57±13.20 pg/dl/min to 404.51±155.84 pg/dl/min following isometric contractions (Figure 4.18) Comparable to the observations made in young subjects, the treatment conditions 40% $O_2$, aspirin, and A+$O_2$ similarly attenuated these increases following both contractions (Figure 4.18). Correlation of each individual's FBF and venous $PGI_2$ concentrations is shown in Figure 4.19. The line of best-fit shows an increase in the *post*-exercise FBF with $PGI_2$ concentration under placebo conditions, during both rhythmic and isometric handgrips. As in the young men, this was not seen with the interventional treatments: the attenuated hyperaemia was not accompanied by a significant increase in $PGI_2$ concentration for either type of handgrip (Figure 4.19). Moreover, the values of degree of correlation (r) are in agreement with the idea that contribution of PGs to *post*-contraction hyperaemia responses decreases with age. The older group's *r* values for 6-keto-$PGF_{1\alpha}$ and FBF were ~ 0.5 compared

to ~ 0.8 of the young men. The percentage attenuation in $PGI_2$ efflux was similar between treatments and not different between young and older subjects (Figure 4.20).

**PGEM**: PGEM concentrations in venous efflux and the *normalized* PGEM data are shown in Figure 4.21). As described for $PGI_2$, baseline PGEM concentrations were not affected by treatments. During placebo, *normalized* PGEM increased significantly from 16.64±6.62 pg/dl/min to 781.66±192.47 pg/dl/min following rhythmic handgrip contractions and from 47.12±15.62 pg/dl/min to 1095.18±309.74 pg/dl/min (Figure 4.21). Similar to $PGI_2$ efflux, the treatment conditions of 40% $O_2$, aspirin, and A+$O_2$ similarly attenuated the increases in PGEM (Figure 4.21). Just as for $PGI_2$ efflux (Figure 4.20), the percentage attenuation in PGEM efflux was similar between interventional treatments and the extent of this attenuation was not different between young and older men (Figure 4.23). Correlation of each individual's FBF and venous PGEM concentrations for both handgrip contractions is shown in Figure 4.22. The line of best-fit shows an increase in the *post*-exercise FBF along with PGEM concentration under placebo during both rhythmic and isometric handgrip: again, this was not seen with the interventional treatments (Figure 4.22).

| | Young Subjects | | | | | | Older Subjects | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Age (yr) | Weight (Kg) | Height (m) | BMI (Kg/m$^2$) | MVC (N) | FAC (cm) | Age (yr) | Weight (Kg) | Height (m) | BMI (Kg/m$^2$) | MVC (N) | FAC (cm) |
| | 21 | 80 | 1.73 | 26.73 | 216 | 27 | 60 | 86 | 1.80 | 26.54 | 216 | 25 |
| | 21 | 88 | 1.88 | 24.90 | 294 | 28 | 65 | 65 | 1.78 | 20.52 | 177 | 22 |
| | 20 | 90 | 1.89 | 25.20 | 451 | 29 | 67 | 70 | 1.80 | 21.60 | 226 | 26 |
| | 20 | 65 | 1.72 | 21.97 | 186 | 25 | 66 | 88 | 1.76 | 28.41 | 196 | 25 |
| | 19 | 77 | 1.84 | 22.74 | 196 | 25 | 65 | 75 | 1.65 | 27.55 | 137 | 26 |
| | 22 | 81 | 1.84 | 23.92 | 255 | 29 | 61 | 78 | 1.73 | 26.06 | 137 | 25 |
| | 19 | 66 | 1.75 | 21.55 | 177 | 26 | 63 | 100 | 1.94 | 26.57 | 147 | 29 |
| | 21 | 74 | 1.73 | 24.73 | 157 | 26 | 63 | 76 | 1.75 | 24.82 | 196 | 24 |
| | 19 | 68 | 1.75 | 22.20 | 163 | 23 | 75 | 70 | 1.81 | 21.37 | 196 | 25 |
| | 25 | 66 | 1.70 | 22.84 | 235 | 25 | 76 | 66 | 1.67 | 23.67 | 128 | 24 |
| | 24 | 56 | 1.63 | 21.08 | 147 | 23 | 69 | 76 | 1.70 | 26.30 | 137 | 24 |
| | 22 | 88 | 1.88 | 24.90 | 275 | 29 | | | | | | |
| Mean | 21.08 | 74.92 | 1.78 | 23.56 | 229.31 | 26.25 | 66.39 | 77.27 | 1.78 | 24.85 | 172.12 | 25.00 |
| SEM | 0.56 | 3.14 | 0.02 | 0.50 | 24.39 | 0.63 | 1.57 | 3.16 | 0.02 | 0.81 | 10.78 | 0.52 |

Table 4.1 **Anthropometric data of young (n=12) and older (n=11) men**.

| | | | P | 40% $O_2$ | A | A + $O_2$ | P Value |
|---|---|---|---|---|---|---|---|
| Young | TTI (N.s) | Rhythmic | 10596.40±1416.83 | 10777.21±1404.50 | 10647.45±1186.36 | 10844.35±1364.23 | *0.99* |
| | | Isometric | 16948.32±3125.62 | 16396.91±2496.92 | 17247.65±2071.54 | 16235.30±2587.68 | *0.99* |
| | P Value (between Exercise type) | | *< 0.001* | | | | |
| | Time (s) | Rhythmic | 179.52±0.36 | 180.04±0.38 | 180.08±0.56 | 179.31±0.36 | *0.49* |
| | | Isometric | 139.71±8.61 | 138.28±7.94 | 156.28±8.86 | 138.29±9.34 | *0.39* |
| | P Value (between exercise type) | | *< 0.001* | | | | |
| | | | | | | | |
| Older | TTI (N.s) | Rhythmic | 8332.82±736.70 | 7794.71±704.28 | 8121.06±788.83 | 8203.02±686.12 | *0.96* |
| | | Isometric | 15594.85±1394.80 | 15379.84±1439.94 | 16156.77±1270.94 | 15924.16±1261.73 | *0.98* |
| | P Value (between Exercise type) | | *< 0.001* | | | | |
| | Time (s) | Rhythmic | 180.32±0.31 | 179.98±0.17 | 180.60±0.28 | 180.47±0.36 | *0.47* |
| | | Isometric ‡ | 180.68±0.26 | 180.63±0.19 | 180.84±0.16 | 178.03±2.70 | *0.41* |
| | P Value (between exercise type) | | *0.68* | | | | |
| ‡ represents between age*exercise type effect ( P Value) = *< 0.001* | | | | | | | |

Table 4.2 **TTI and time recorded for rhythmic and isometric handgrip contractions performed by young and older men**. No treatment effect was detected between Placebo, 40% $O_2$, aspirin, and A+$O_2$. However, significant difference was detected in TTI between rhythmic and isometric handgrip exercise bouts. Moreover, significant difference was also detected between young and old isometric exercise time.

| Young Rhythmic Handgrip | | Baseline 1 | Baseline 2 | Baseline 3 | Mean ± SEM | P Value |
|---|---|---|---|---|---|---|
| HR (b.min$^{-1}$) | P | 65 ± 3 | 64 ± 2 | 64 ± 2 | 64 ± 1 | *0.83* |
| | 40% $O_2$ | 63 ± 3 | 62 ± 3 | 62 ± 3 | 62 ± 1 | *0.72* |
| | A | 68 ± 3 | 67 ± 4 | 67 ± 3 | 67 ± 2 | *0.71* |
| | A + $O_2$ | 64 ± 3 | 61 ± 3 | 61 ± 3 | 62 ± 2 | *0.27* |
| P Value | | | | | *0.52* | |
| MABP (mmHg) | P | 79 ± 5 | 78 ± 6 | 78 ± 5 | 79 ± 3 | *0.71* |
| | 40% $O_2$ | 76 ± 6 | 76 ± 6 | 76 ± 5 | 76 ± 3 | *0.75* |
| | A | 77 ± 5 | 78 ± 5 | 78 ± 5 | 77 ± 3 | *0.22* |
| | A + $O_2$ | 73 ± 3 | 74 ± 4 | 73 ± 4 | 73 ± 2 | *0.25* |
| P Value | | | | | *0.81* | |
| FBF (ml.dl$^{-1}$.min$^{-1}$) | P | 4.86 ± 0.94 | 4.57 ± 0.72 | 3.93 ± 0.54 | 4.45 ± 0.43 | *0.46* |
| | 40% $O_2$ | 4.74 ± 0.77 | 4.00 ± 0.59 | 4.27 ± 0.62 | 4.34 ± 0.38 | *0.43* |
| | A | 5.42 ± 0.80 | 4.77 ± 0.95 | 4.14 ± 0.57 | 4.78 ± 0.45 | *0.13* |
| | A + $O_2$ | 5.38 ± 0.86 | 4.19 ± 0.72 | 4.25 ± 0.56 | 4.59 ± 0.41 | *0.09* |
| P Value | | | | | *0.93* | |
| FVC (CU) | P | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.05 ± 0.01 | 0.06 ± 0.01 | *0.62* |
| | 40% $O_2$ | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.06 ± 0.01 | *0.51* |
| | A | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.05 ± 0.01 | 0.06 ± 0.01 | *0.35* |
| | A + $O_2$ | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.06 ± 0.01 | *0.31* |
| P Value | | | | | *0.99* | |

Table 4.3 **Baseline HR, MABP, FBF, and FVC responses of young men during rhythmic handgrip exercise**. Measurements were made just before the handgrip exercise bouts. No time or treatment effect was detected.

| Young Isometric Handgrip | | Baseline 1 | Baseline 2 | Baseline 3 | Mean ± SEM | P Value |
|---|---|---|---|---|---|---|
| HR ($b.min^{-1}$) | P | 67 ± 3 | 67 ± 3 | 69± 3 | 68 ± 2 | *0.78* |
| | 40% $O_2$ | 64 ± 4 | 63 ± 3 | 63 ± 4 | 64 ± 2 | *0.87* |
| | A | 71 ± 3 | 68 ± 3 | 67 ± 3 | 69 ± 2 | *0.14* |
| | A + $O_2$ | 64 ± 2 | 63 ± 2 | 61 ±2 | 62 ± 1 | *0.18* |
| P Value | | | | | *0.32* | |
| MABP (mmHg) | P | 81 ± 5 | 80 ± 5 | 79 ± 5 | 80 ± 3 | *0.46* |
| | 40% $O_2$ | 81 ± 6 | 79 ± 5 | 79 ± 5 | 80 ± 3 | *0.71* |
| | A | 78 ± 3 | 79 ± 4 | 79 ± 4 | 79 ± 2 | *0.33* |
| | A + $O_2$ | 77 ± 5 | 75 ± 5 | 76 ± 5 | 75 ± 3 | *0.92* |
| P Value | | | | | *0.85* | |
| FBF ($ml.dl^{-1}.min^{-1}$) | P | 6.02 ± 0.99 | 6.13 ± 1.16 | 5.60 ± 0.73 | 5.92 ± 0.55 | *0.54* |
| | 40% $O_2$ | 6.58 ± 0.83 | 5.51 ± 0.67 | 5.85 ± 0.69 | 5.98 ± 0.42 | *0.31* |
| | A | 6.86 ± 0.76 | 5.91 ± 0.75 | 5.90 ± 0.93 | 6.22 ± 0.46 | *0.22* |
| | A + $O_2$ | 7.00 ± 1.37 | 8.11 ± 1.79 | 6.59 ± 1.06 | 7.23 ± 0.81 | *0.44* |
| P Value | | | | | *0.43* | |
| FVC (CU) | P | 0.08 ± 0.01 | 0.08 ± 0.01 | 0.07 ± 0.01 | 0.08 ± 0.01 | *0.70* |
| | 40% $O_2$ | 0.09 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.08 ± 0.01 | *0.21* |
| | A | 0.10 ± 0.01 | 0.08 ± 0.01 | 0.08 ± 0.01 | 0.09 ± 0.01 | *0.16* |
| | A + $O_2$ | 0.10 ± 0.02 | 0.09 ± 0.01 | 0.09 ± 0.02 | 0.09 ± 0.01 | *0.73* |
| P Value | | | | | *0.68* | |

Table 4.4 **Baseline HR, MABP, FBF, and FVC responses of young men during isometric handgrip exercise**. Measurements were made just before the handgrip exercise bouts. No time or treatment effect was detected.

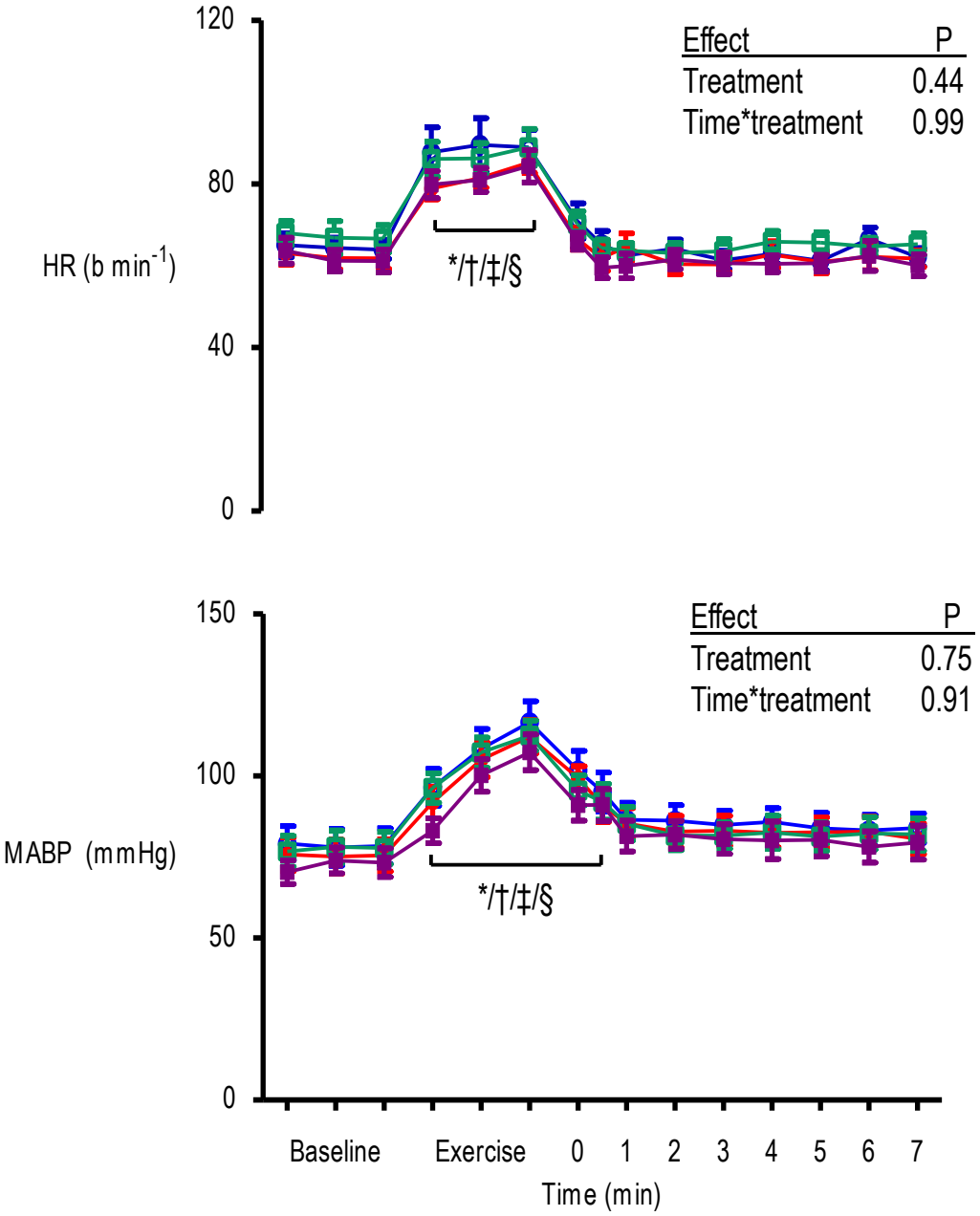



Figure 4.5 **HR and MABP responses of rhythmic handgrip contractions performed by young men.** Placebo is represented in blue, and 40% $O_2$ in red, aspirin (A) is shown in green, and their combination (A+$O_2$) in purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.001$).

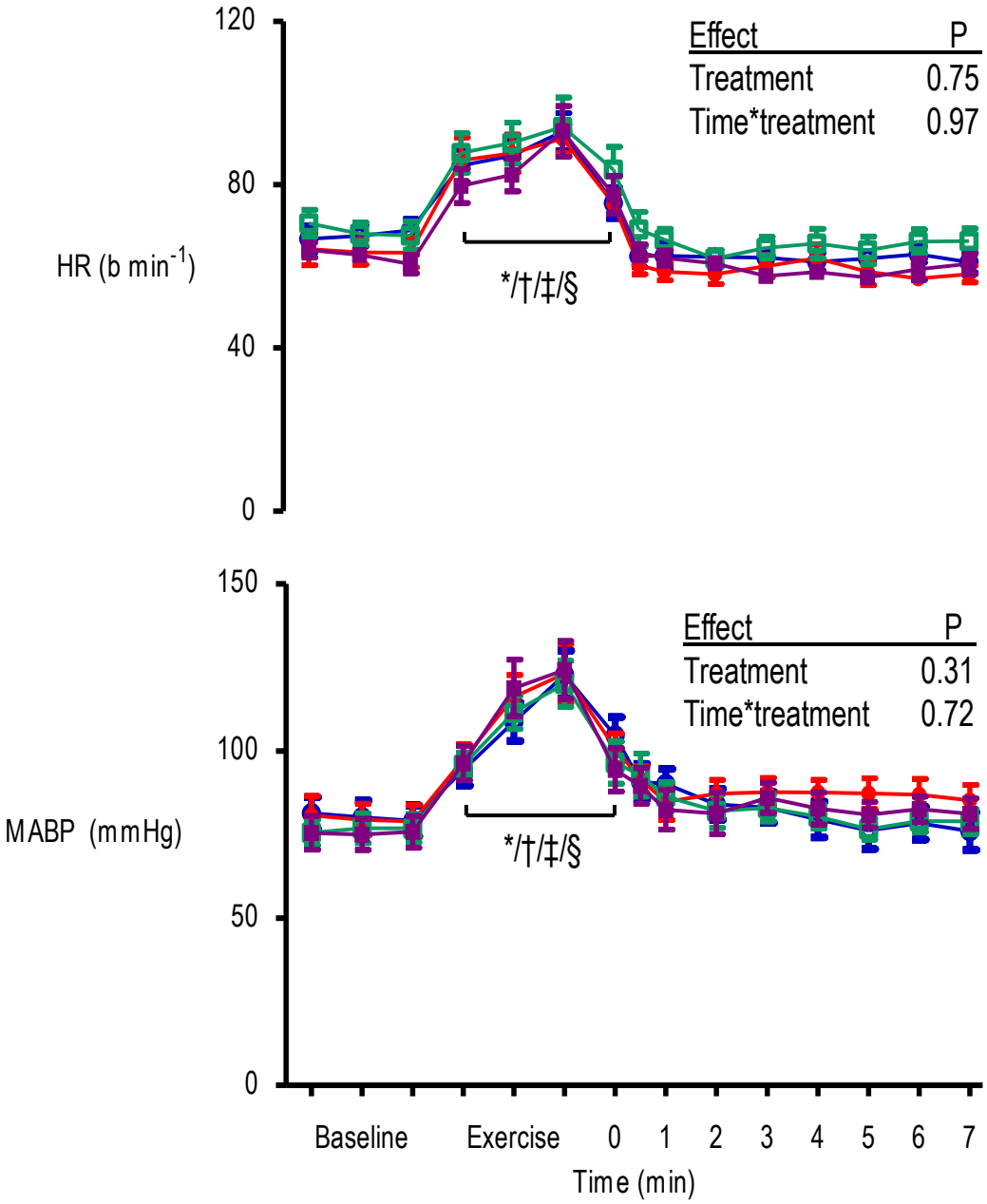



Figure 4.6 **HR and MABP responses of isometric handgrip contractions performed by young men.** Placebo is represented in blue, and 40% $O_2$ in red, aspirin (A) is shown in green, and their combination (A+$O_2$) in purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.001$).

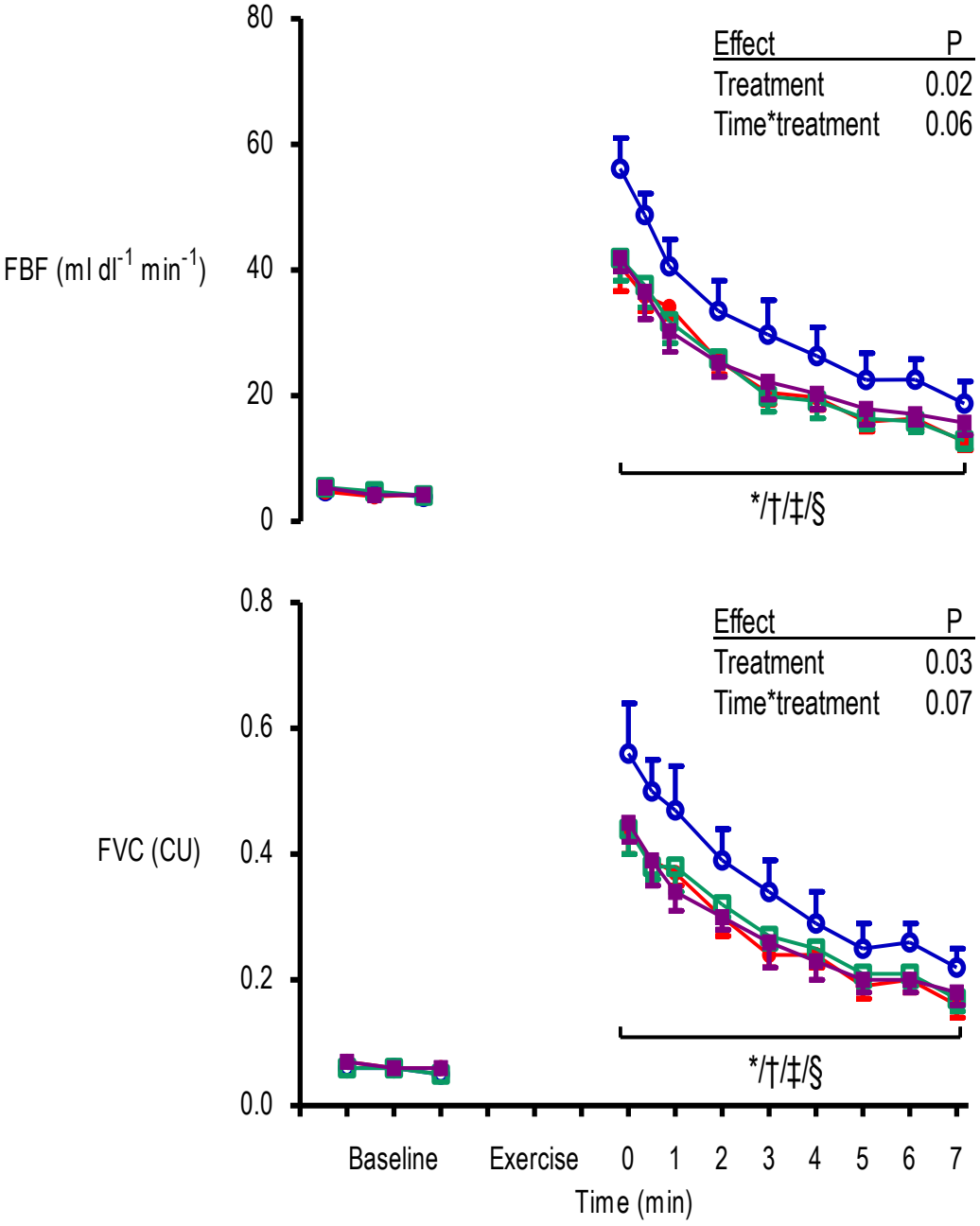



Figure 4.7 **FBF and FVC responses of rhythmic handgrip contractions performed by young men.** Placebo is represented in blue, and 40% $O_2$ in red, aspirin (A) is shown in green, and their combination (A+$O_2$) in purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.001$).

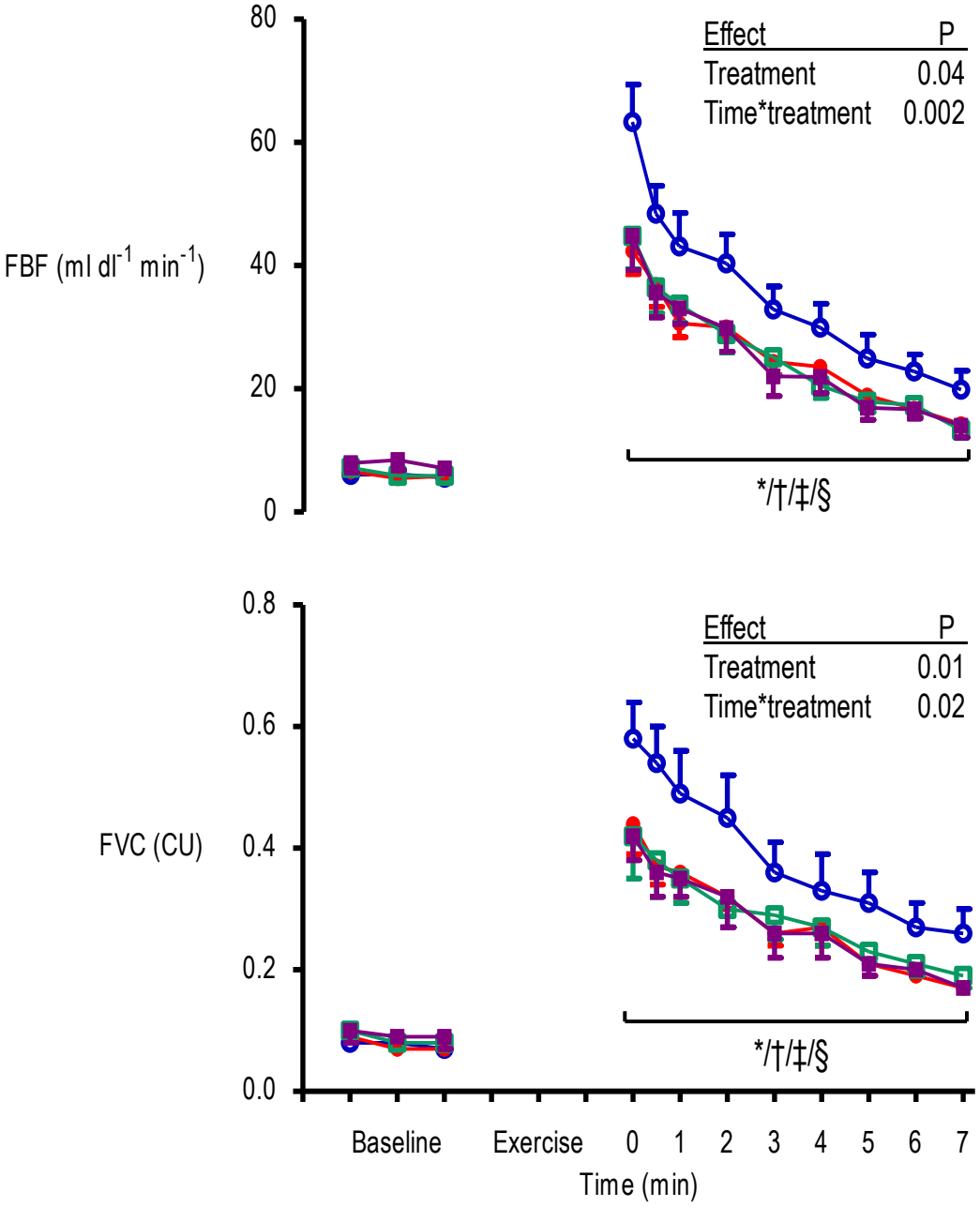



Figure 4.8 **FBF and FVC responses of isometric handgrip contractions performed by young men.** Placebo is represented in blue, and 40% $O_2$ in red, aspirin (A) is shown in green, and their combination (A+$O_2$) in purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.001$).

| Young - Rhythmic Exercise | | Baseline | Post Exercise Responses | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | Venous Samples | | 0 s | 3 min | 5 min | 7 min | * represents Time effect P Value |
| $PO_2$ (mmHg) | Placebo | 44 ± 5 | 34 ± 3 * | 52 ± 3 | 51 ± 3 | 51 ± 3 | *0.02* |
| | 40% $O_2$ † | 44 ± 4 | 39 ± 3 | 58 ± 4 * | 60 ± 4 * | 58 ± 4 * | < *0.001* |
| | Aspirin | 42 ± 3 | 32 ± 2 * | 51 ± 3 | 51 ± 2 | 51 ± 3 | < *0.001* |
| | A + $O_2$ † | 41 ± 3 | 35 ± 3 | 57 ± 2 * | 58 ± 2 * | 57 ± 2* | < *0.001* |
| | † represents Treatment effect P Value = *0.04*<br>Time*Treatment P Value = *0.12* | | | | | | |
| $PCO_2$ (mmHg) | Placebo | 53 ± 2 | 99 ± 6 * | 63 ± 2 | 54 ± 2 | 52 ± 2 | < *0.001* |
| | 40% $O_2$ | 52 ± 3 | 93 ± 5 * | 60 ± 1 | 51 ± 1 | 52 ± 1 | < *0.001* |
| | Aspirin | 50 ± 2 | 88 ± 6 * | 58 ±1 | 55 ± 1 | 50 ± 1 | < *0.001* |
| | A + $O_2$ | 51 ± 3 | 94 ± 10 * | 60 ± 2 | 54 ± 1 | 53 ± 2 | < *0.001* |
| | Treatment P Value = *0.61*<br>Time*Treatment P Value = *0.96* | | | | | | |
| $K^+$ ($mmol.L^{-1}$) | Placebo | 4.74±0.08 | 5.28±0.15 * | 4.60±0.11 | 4.96±0.28 | 4.98±0.26 | *0.02* |
| | 40% $O_2$ | 4.72±0.11 | 5.07±0.30 * | 4.45±0.10 | 4.41±0.07 | 4.70±0.17 | *0.03* |
| | Aspirin | 4.61±0.15 | 5.32±0.18 * | 4.42±0.09 | 4.45±0.10 | 4.56±0.09 | < *0.001* |
| | A + $O_2$ | 4.66±0.13 | 5.41±0.10 * | 4.51±0.12 | 4.62±0.11 | 4.66±0.14 | < *0.001* |
| | Treatment P Value = *0.16*<br>Time*Treatment P Value = *0.20* | | | | | | |
| pH | Placebo | 7.36±0.01 | 7.17±0.01 * | 7.25±0.01 * | 7.29±0.01 * | 7.31±0.01 * | < *0.001* |
| | 40% $O_2$ | 7.36±0.01 | 7.19±0.02 * | 7.26±0.01 * | 7.32±0.01 | 7.32±0.01 | < *0.001* |
| | Aspirin | 7.38±0.01 | 7.20±0.02 * | 7.28±0.01 * | 7.30±0.02 * | 7.33±0.01 * | < *0.001* |
| | A + $O_2$ | 7.37±0.02 | 7.18±0.02 * | 7.26±0.01 * | 7.29±0.01 * | 7.32±0.01 | < *0.001* |
| | Treatment P Value = *0.53*<br>Time*Treatment P Value = *0.78* | | | | | | |
| Lactate ($mmol.L^{-1}$) | Placebo | 1.3 ± 0.1 | 5.2 ± 0.6 * | 5.0 ± 0.4 * | 4.5 ± 0.4 * | 4.0 ± 0.5 * | < *0.001* |
| | 40% $O_2$ | 1.3 ± 0.2 | 5.0 ± 0.6 * | 5.1 ± 0.5 * | 4.5 ± 0.5 * | 3.8 ± 0.4 * | < *0.001* |
| | Aspirin | 1.4 ± 0.2 | 4.7 ± 0.4 * | 4.5 ± 0.4 * | 4.1 ± 0.5 * | 3.7 ± 0.4 * | < *0.001* |
| | A + $O_2$ | 1.3 ± 0.1 | 5.1 ± 0.4 * | 5.2 ± 0.3 * | 4.8 ± 0.4 * | 4.2 ± 0.4 * | < *0.001* |
| | Treatment P Value = *0.86*<br>Time*Treatment P Value = *0.97* | | | | | | |

Table 4.5 **$PO_2$, $PCO_2$, $K^+$, pH, and lactate concentrations in the venous efflux recorded before and after rhythmic handgrip contractions performed by young men.** * represent values significantly different from respective baselines while † represents values different from placebo.

| Young - Isometric Exercise | | Baseline | Post Exercise Responses | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | Venous Samples | | 0 s | 3 min | 5 min | 7 min | * represents Time effect P Value |
| $PO_2$ (mmHg) | Placebo | 47 ± 5 | 38 ± 5 * | 58 ± 3 * | 61 ± 5 * | 59 ± 3 * | < *0.001* |
| | 40% $O_2$ † | 49 ± 3 | 46 ± 4 | 71 ± 4 * | 69 ± 4 * | 72 ± 12 * | *0.002* |
| | Aspirin | 45 ± 5 | 38 ± 2 * | 55 ± 2 * | 57 ± 2 * | 60 ± 4 * | < *0.001* |
| | A + $O_2$ † | 52 ± 5 | 39 ± 2 * | 65 ± 3 * | 63 ± 3 * | 75 ± 12 * | *0.01* |
| | † represents Treatment effect P Value = < *0.05*<br>Time*Treatment P Value = *0 .83* | | | | | | |
| $PCO_2$ (mmHg) | Placebo | 48 ± 2 | 96 ± 7 * | 56 ± 3 | 50 ± 2 | 48 ± 2 | < *0.001* |
| | 40% $O_2$ | 48 ± 1 | 87 ± 8 * | 56 ± 2 | 52 ± 2 | 49 ± 1 | < *0.001* |
| | Aspirin | 49 ± 1 | 90 ± 7 * | 55 ± 2 | 50 ± 2 | 48 ±1 | < *0.001* |
| | A + $O_2$ | 46 ± 3 | 88 ± 10 * | 52 ± 2 | 51 ± 2 | 47 ±1 | < *0.001* |
| | Treatment P Value = *0.77*<br>Time*Treatment P Value = *0.99* | | | | | | |
| $K^+$ ($mmol.L^{-1}$) | Placebo | 4.74±0.10 | 6.32±0.36 * | 5.16±0.44 | 4.93±0.27 | 4.95±0.21 | < *0.001* |
| | 40% $O_2$ | 4.87±0.14 | 5.80±0.26 * | 5.15±0.39 | 4.80±0.29 | 5.24±0.39 | *0.04* |
| | Aspirin | 4.76±0.09 | 5.67±0.19 * | 4.93±0.23 | 4.73±0.10 | 4.76±0.16 | < *0.001* |
| | A + $O_2$ | 4.63±0.21 | 6.00±0.27 * | 4.74±0.12 | 5.19±0.45 | 5.04±0.24 | *0.04* |
| | Treatment P Value = *0.79*<br>Time*Treatment P Value = *0.80* | | | | | | |
| pH | Placebo | 7.39±0.01 | 7.19±0.02 * | 7.30±0.02 * | 7.33±0.01 | 7.34±0.02 | < *0.001* |
| | 40% $O_2$ | 7.38±0.01 | 7.19±0.03 * | 7.28±0.01 * | 7.29±0.02 * | 7.33±0.01 | < *0.001* |
| | Aspirin | 7.37±0.01 | 7.19±0.03 * | 7.29±0.02 * | 7.33±0.02 | 7.35±0.02 | < *0.001* |
| | A + $O_2$ | 7.39±0.02 | 7.21±0.03 * | 7.29±0.02 * | 7.31±0.02 * | 7.34±0.02 | < *0.001* |
| | Treatment P Value = *0.33*<br>Time*Treatment P Value = *0.36* | | | | | | |
| Lactate ($mmol.L^{-1}$) | Placebo | 1.4 ±0.1 | 5.7 ± 0.7 * | 4.8 ± 0.3 * | 4.2 ± 0.4 * | 3.6 ± 0.4 * | < *0.001* |
| | 40% $O_2$ | 1.6 ± 0.2 | 5.8 ± 0.6 * | 5.6 ± 0.6 * | 5.0 ± 0.5 * | 4.3 ± 0.5 * | < *0.001* |
| | Aspirin | 1.4 ± 0.1 | 5.7 ± 0.6 * | 4.7 ± 0.6 * | 4.3 ± 0.6 * | 3.6 ± 0.4 * | < *0.001* |
| | A + $O_2$ | 1.6 ± 0.2 | 5.4 ± 0.5 * | 5.1 ± 0.5 * | 4.8 ± 0.6 * | 3.8 ± 0.3 * | < *0.001* |
| | Treatment P Value = *0.69*<br>Time*Treatment P Value = *0.98* | | | | | | |

Table 4.6 **$PO_2$, $PCO_2$, $K^+$, pH, and lactate concentrations in the venous efflux recorded before and after isometric handgrip contractions performed by young men.** * represent values significantly different from respective baselines while † represents values different from placebo.

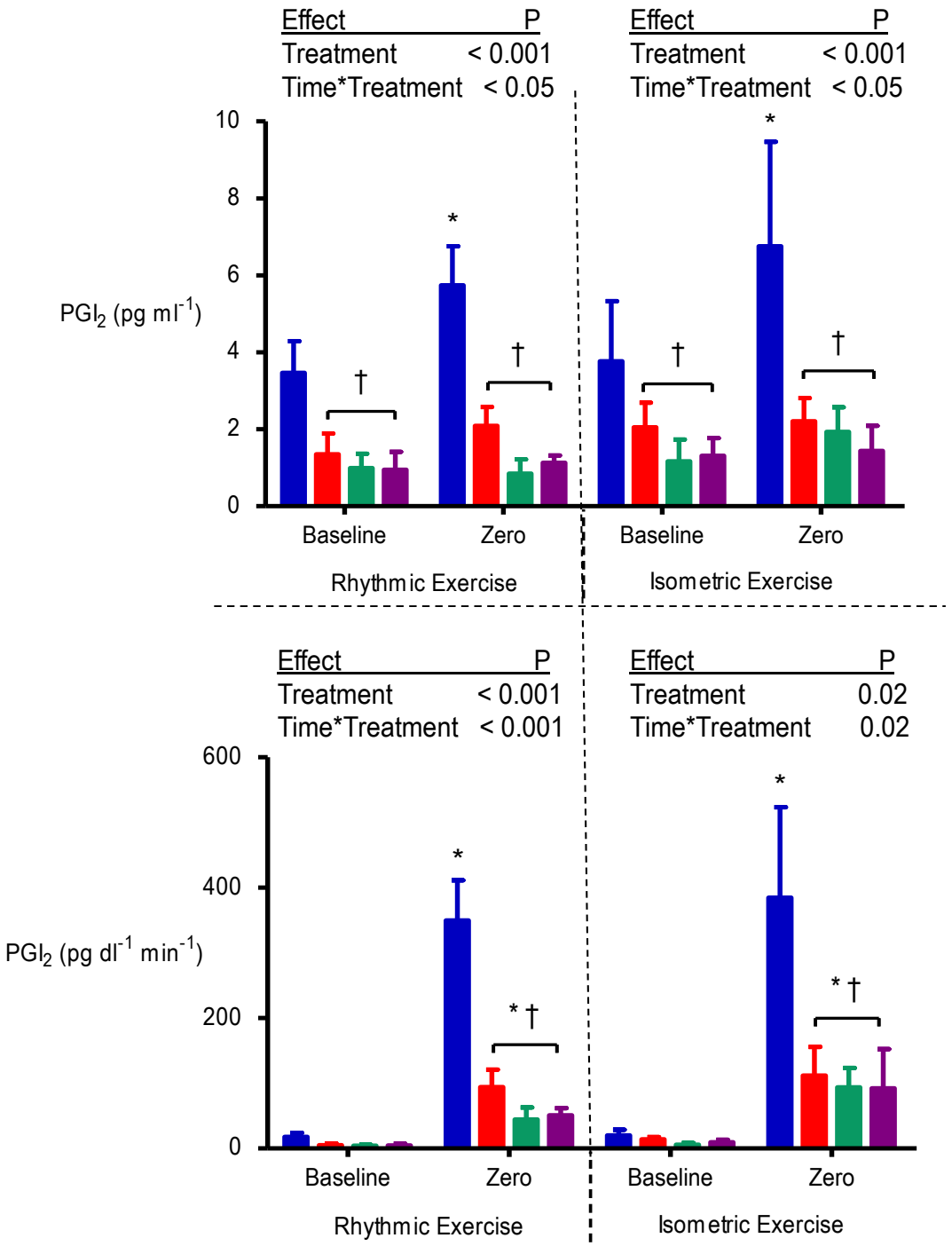



Figure 4.9 **$PGI_2$ (upper panel) measured in the venous plasma samples and normalized $PGI_2$ (lower panel) values recorded before and immediately after rhythmic and isometric handgrip contractions performed by young men.** P is represented in blue, 40% $O_2$ is red, A is green, and their combination (A+$O_2$) is shown in purple. * represent values significantly different from their respective baselines; † represents values significantly different from P condition ($P < 0.05$). This includes all values within the brackets.

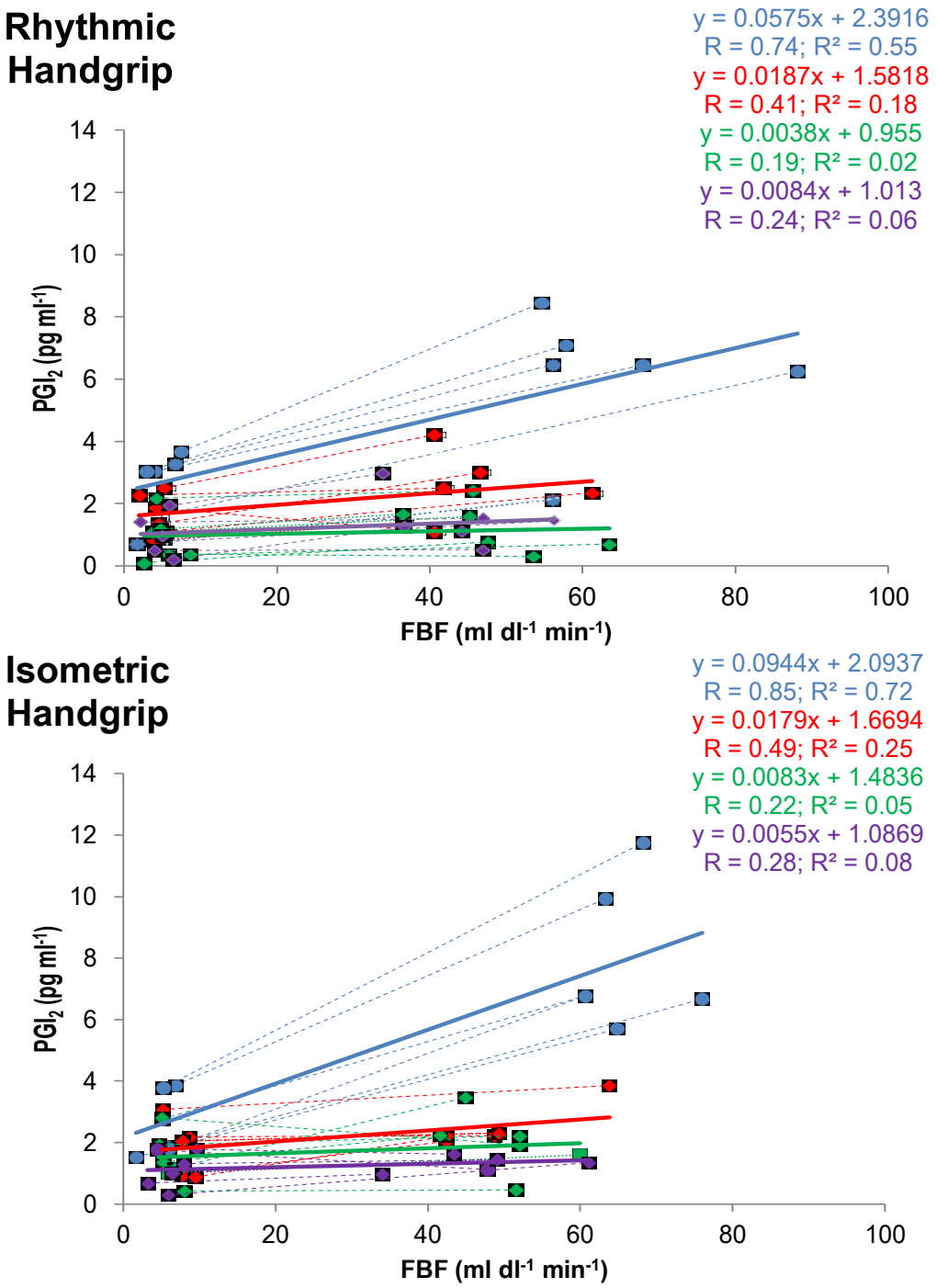



Figure 4.10 **Correlation between baseline and *post*-exercise FBF and $PGI_2$ (6-keto-$PGF_{1\alpha}$ ) concentrations for rhythmic (upper panel) and isometric (lower panel) exercise performed by young men.** P is represented in blue, 40% $O_2$ is red, A is green, and their combination (A+$O_2$) is shown in purple. Each individual's baseline and post-exercise response is shown by the dashed line while the solid line represents the line of best fit (regression analysis and $r^2$ values are provided for each treatment).

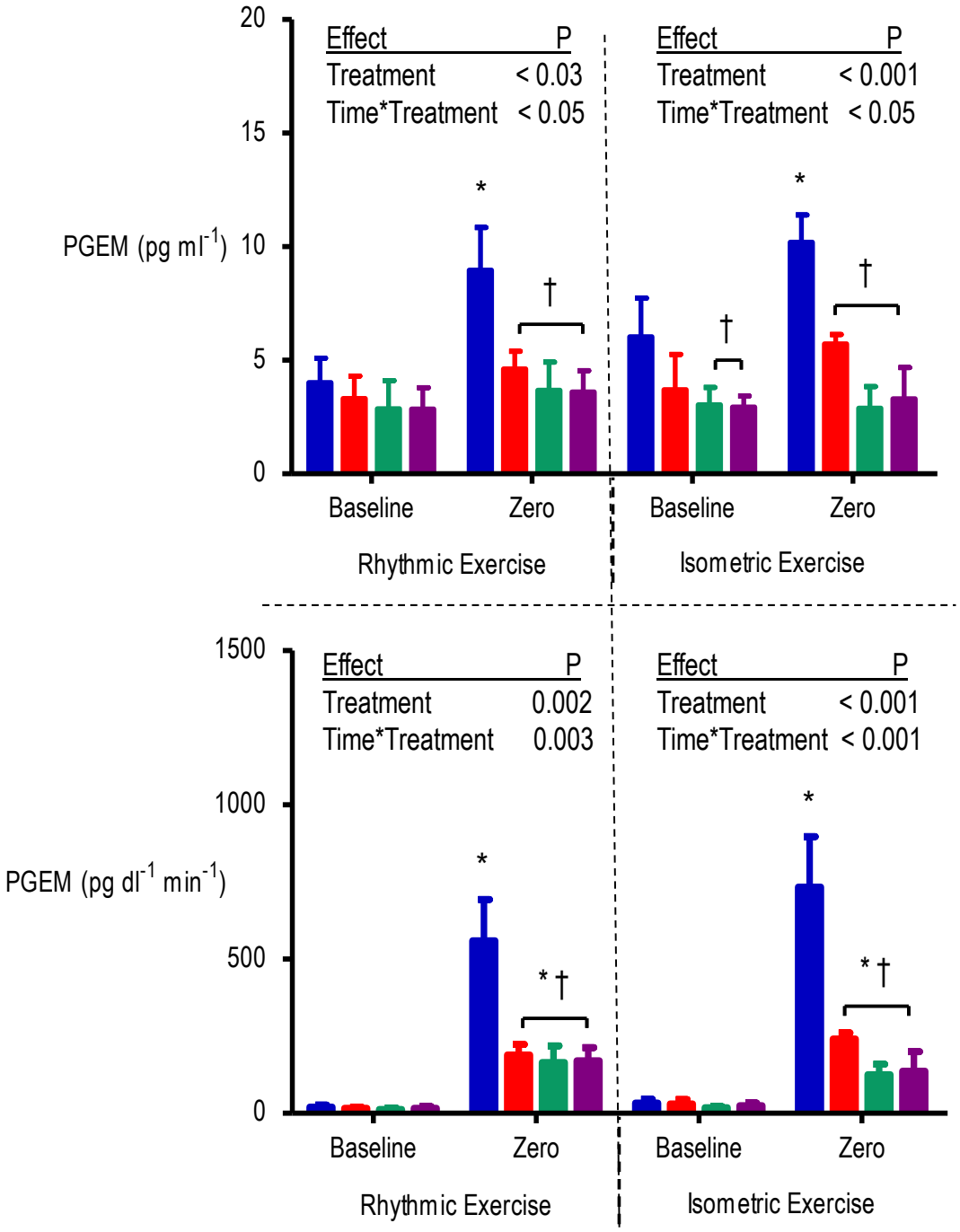



Figure 4.11 **PGEM (upper panel) measured in the venous plasma and the normalized PGEM (lower panel) values recorded before and immediately after rhythmic and isometric handgrip contractions performed by young men.** P is represented in blue, 40% $O_2$ is red, A is green, and their combination (A+$O_2$) is shown in purple. * represent values significantly different from their respective baselines; † represents values significantly different from P treatment condition ($P < 0.05$). This includes all values within the brackets.

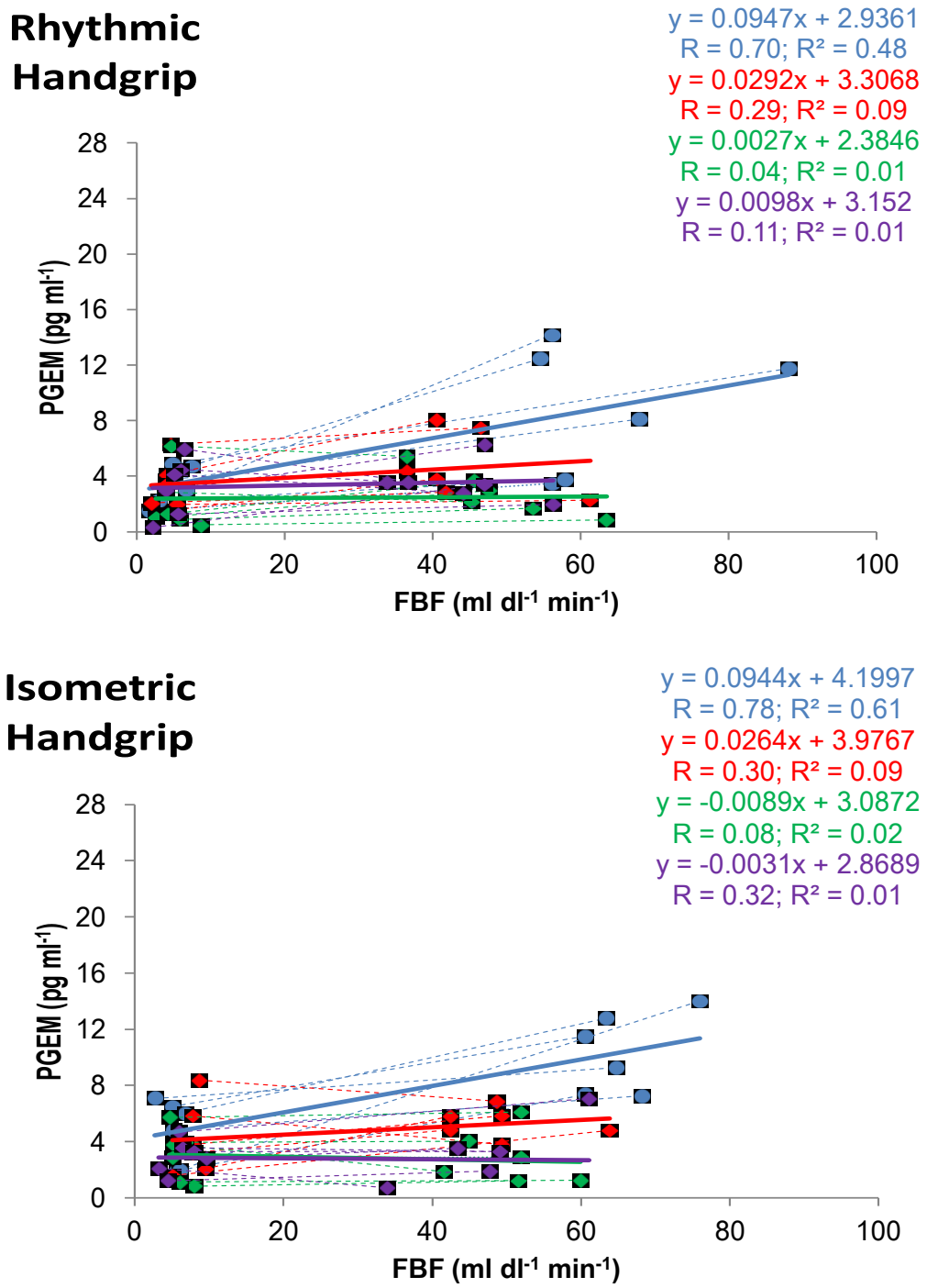



Figure 4.12 **Correlation between baseline and *post*-exercise FBF and PGEM concentrations for rhythmic (upper panel) and isometric (lower panel) exercise performed by young men.** P is represented in blue, 40% $O_2$ is red, A is green, and their combination (A+$O_2$) is shown in purple. Each individual's baseline and post-exercise response is shown by the dashed line while the solid line represents the line of best fit (regression analysis and $r^2$ values are provided for each treatment).

| Older Rhythmic Handgrip | | Baseline 1 | Baseline 2 | Baseline 3 | Mean ± SEM | P Value |
|---|---|---|---|---|---|---|
| HR (b $min^{-1}$) | P | 66 ± 3 | 65 ± 3 | 64 ± 3 | 65 ± 2 | *0.28* |
| | 40% $O_2$ | 62 ± 3 | 62 ± 3 | 64 ± 3 | 63 ± 2 | *0.40* |
| | A | 63 ± 3 | 61 ± 3 | 62 ± 3 | 62 ± 2 | *0.24* |
| | A + $O_2$ | 62 ± 3 | 61 ± 3 | 62 ± 3 | 62 ± 2 | *0.58* |
| P Value | | | | | *0.85* | |
| MABP (mmHg) | P | 84 ± 3 | 83 ± 3 | 84 ± 3 | 84 ± 2 | *0.59* |
| | 40% $O_2$ | 87 ± 5 | 85 ± 4 | 86 ± 5 | 86 ± 3 | *0.17* |
| | A | 89 ± 4 | 89 ± 4 | 87 ± 4 | 88 ± 2 | *0.29* |
| | A + $O_2$ | 85 ± 4 | 84 ± 4 | 85 ± 4 | 85 ± 2 | *0.29* |
| P Value | | | | | *0.88* | |
| FBF (ml.$dl^{-1}$.$min^{-1}$) | P | 7.14±1.02 | 6.11±0.74 | 6.53±2.00 | 6.59±0.77 | *0.72* |
| | 40% $O_2$ | 7.12±1.00 | 4.85±0.66 | 6.15±1.00 | 6.04±0.53 | *0.06* |
| | A | 5.77±1.10 | 4.49±0.63 | 5.11±0.83 | 5.12±0.50 | *0.18* |
| | A + $O_2$ | 5.35±0.56 | 5.33±0.49 | 4.37±0.50 | 5.02±0.30 | *0.12* |
| P Value | | | | | *0.49* | |
| FVC (CU) | P | 0.09±0.01 | 0.07±0.01 | 0.08±0.03 | 0.08±0.01 | *0.79* |
| | 40% $O_2$ | 0.08±0.01 | 0.06±0.01 | 0.07±0.01 | 0.07±0.01 | *0.12* |
| | A | 0.07±0.01 | 0.05±0.01 | 0.06±0.01 | 0.06±0.01 | *0.16* |
| | A + $O_2$ | 0.06±0.01 | 0.06±0.01 | 0.05±0.01 | 0.06±0.00 | *0.09* |
| P Value | | | | | *0.53* | |

Table 4.7 **Baseline HR, MABP, FBF, and FVC responses of older men during rhythmic handgrip exercise**. Measurements were made just before the handgrip exercise bouts. No time or treatment effect was detected.

| Older Isometric Handgrip | | Baseline 1 | Baseline 2 | Baseline 3 | Mean ± SEM | P Value |
|---|---|---|---|---|---|---|
| HR (b $min^{-1}$) | P | 62 ± 2 | 61 ± 2 | 62 ± 3 | 62 ± 1 | *0.26* |
| | 40% $O_2$ | 60 ± 3 | 60 ± 3 | 59 ± 3 | 60 ± 2 | *0.09* |
| | A | 61 ± 2 | 61 ± 3 | 61 ± 3 | 61 ± 1 | *0.88* |
| | A + $O_2$ | 61 ± 3 | 61 ± 3 | 60 ± 3 | 61 ± 2 | *0.25* |
| P Value | | | | | *0.97* | |
| MABP (mmHg) | P | 81 ± 3 | 81 ± 3 | 83 ± 3 | 81 ± 2 | *0.08* |
| | 40% $O_2$ | 85 ± 4 | 87 ± 4 | 85 ± 4 | 85 ± 2 | *0.23* |
| | A | 84 ± 4 | 86 ± 3 | 86 ± 5 | 85 ± 2 | *0.44* |
| | A + $O_2$ | 83 ± 3 | 82 ± 4 | 83 ± 3 | 83 ± 2 | *0.25* |
| P Value | | | | | *0.76* | |
| FBF ($ml.dl^{-1}.min^{-1}$) | P | 7.18±1.25 | 6.76±1.07 | 6.07±1.22 | 6.67±0.67 | *0.37* |
| | 40% $O_2$ | 6.55±0.74 | 5.92±0.62 | 5.81±0.60 | 6.09±0.37 | *0.28* |
| | A | 7.26±0.57 | 6.92±0.54 | 6.57±0.63 | 6.91±0.33 | *0.52* |
| | A + $O_2$ | 7.20±0.94 | 6.80±0.67 | 6.56±0.82 | 6.85±0.46 | *0.49* |
| P Value | | | | | 0.71 | |
| FVC (CU) | P | 0.09±0.02 | 0.08±0.02 | 0.07±0.02 | 0.08±0.01 | *0.10* |
| | 40% $O_2$ | 0.08±0.01 | 0.07±0.01 | 0.07±0.01 | 0.07±0.01 | *0.23* |
| | A | 0.09±0.01 | 0.08±0.01 | 0.08±0.01 | 0.08±0.01 | *0.53* |
| | A + $O_2$ | 0.09±0.02 | 0.08±0.01 | 0.08±0.01 | 0.08±0.01 | *0.46* |
| P Value | | | | | *0.67* | |

Table 4.8 **Baseline HR, MABP, FBF, and FVC responses of older men during isometric handgrip exercise**. Measurements were made just before the handgrip exercise bouts. No time or treatment effect was detected.

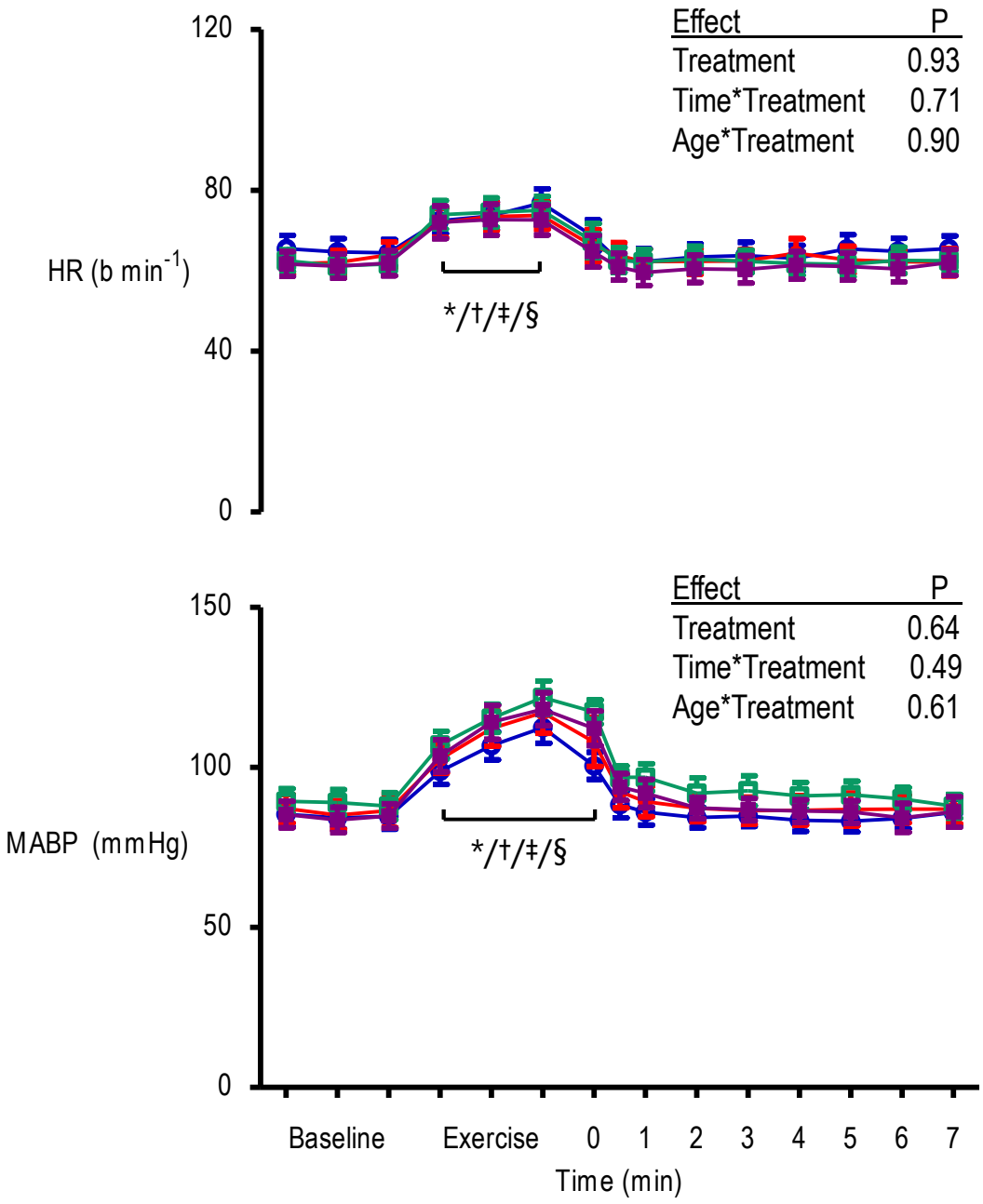



Figure 4.13 **HR and MABP responses of rhythmic handgrip contractions performed by older men.** Placebo is represented in blue, and 40% $O_2$ in red, aspirin is shown in green, and their combination (A+$O_2$) in purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.001$).

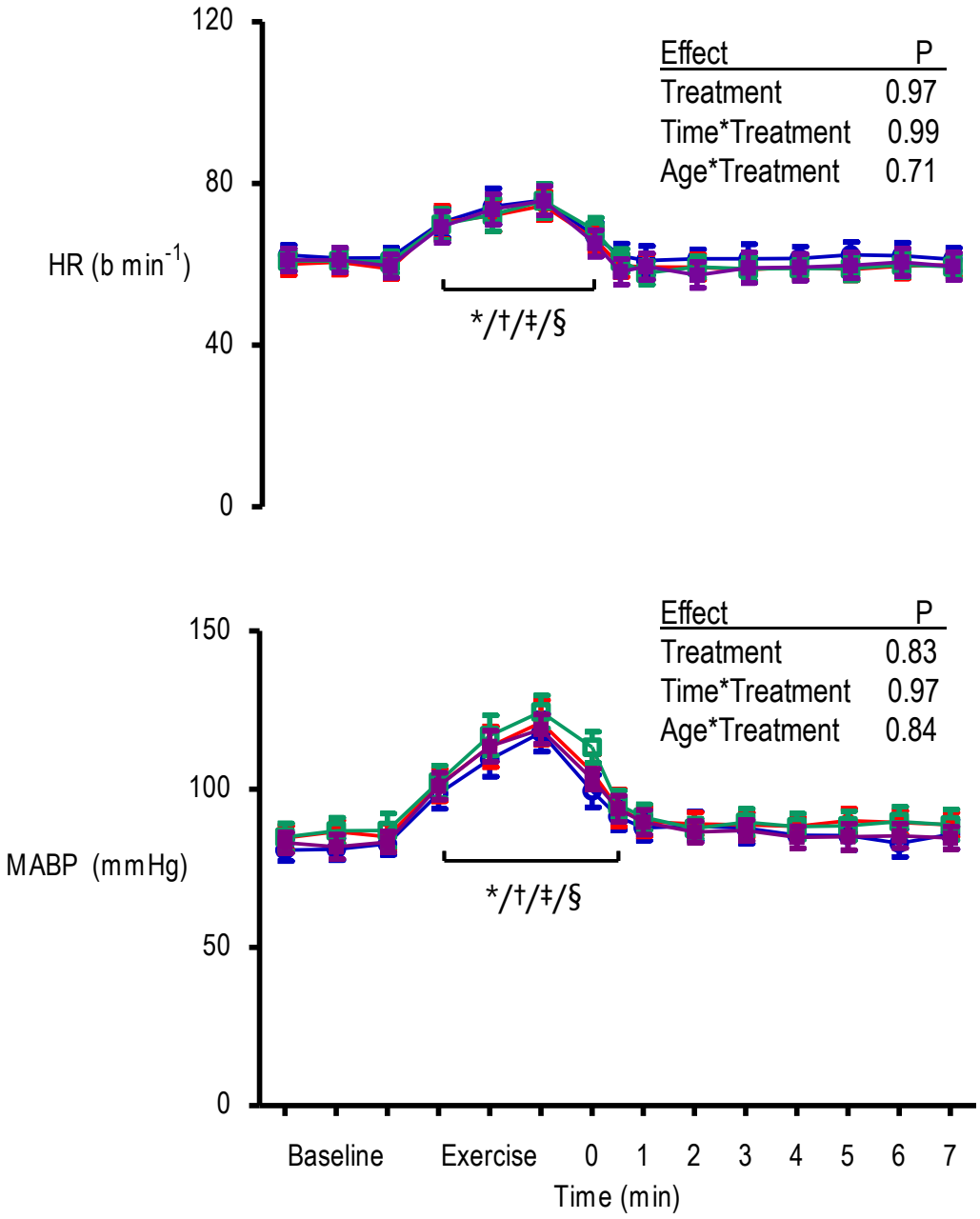



Figure 4.14 **HR and MABP responses of isometric handgrip contractions performed by older men.** Placebo is represented in blue, and 40% $O_2$ in red, aspirin is shown in green, and their combination (A+$O_2$) in purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.001$).

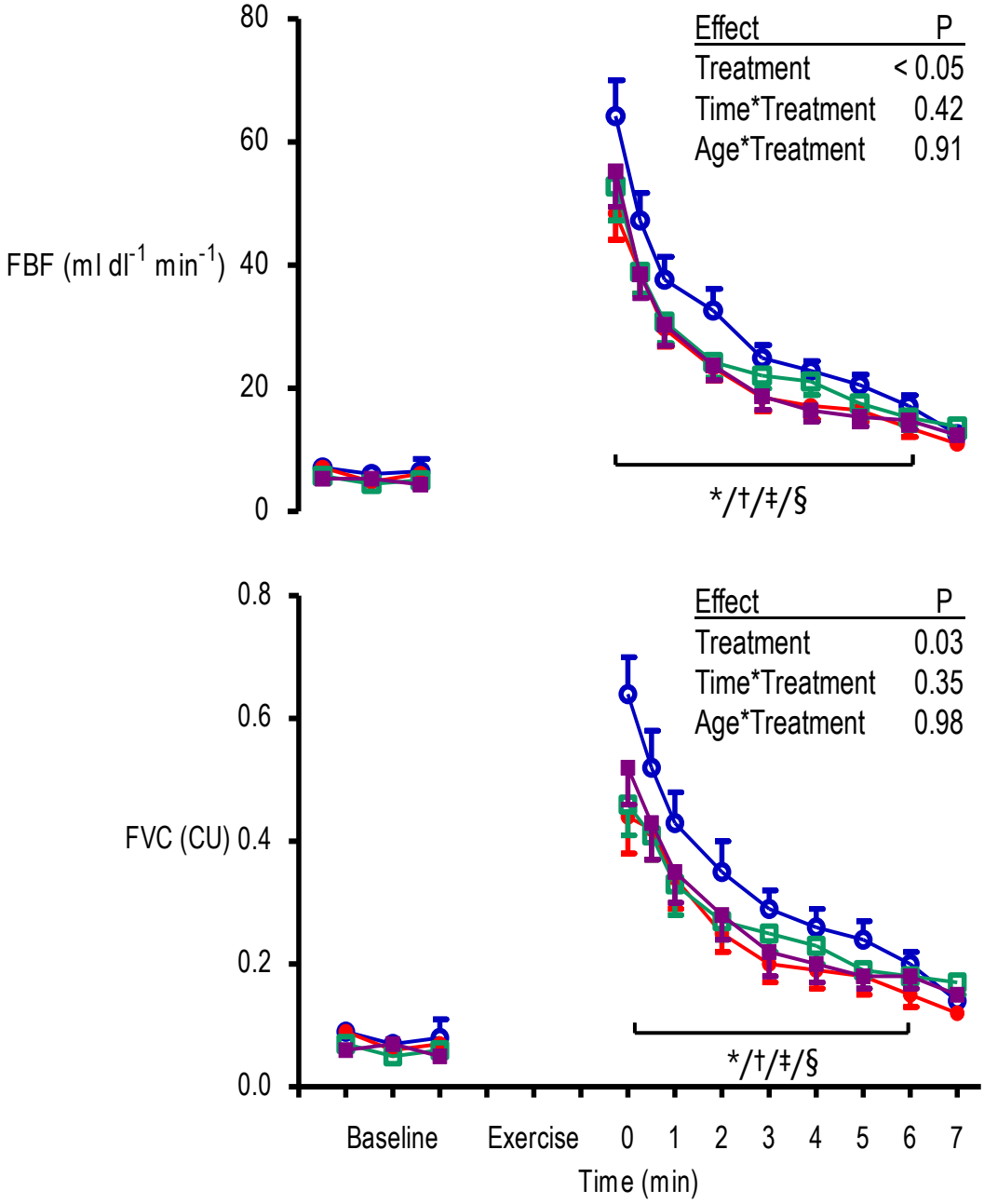



Figure 4.15 **FBF and FVC responses of rhythmic handgrip contractions performed by older men.** Placebo is represented in blue, and 40% $O_2$ in red, aspirin is shown in green, and their combination (A+$O_2$) in purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.001$).

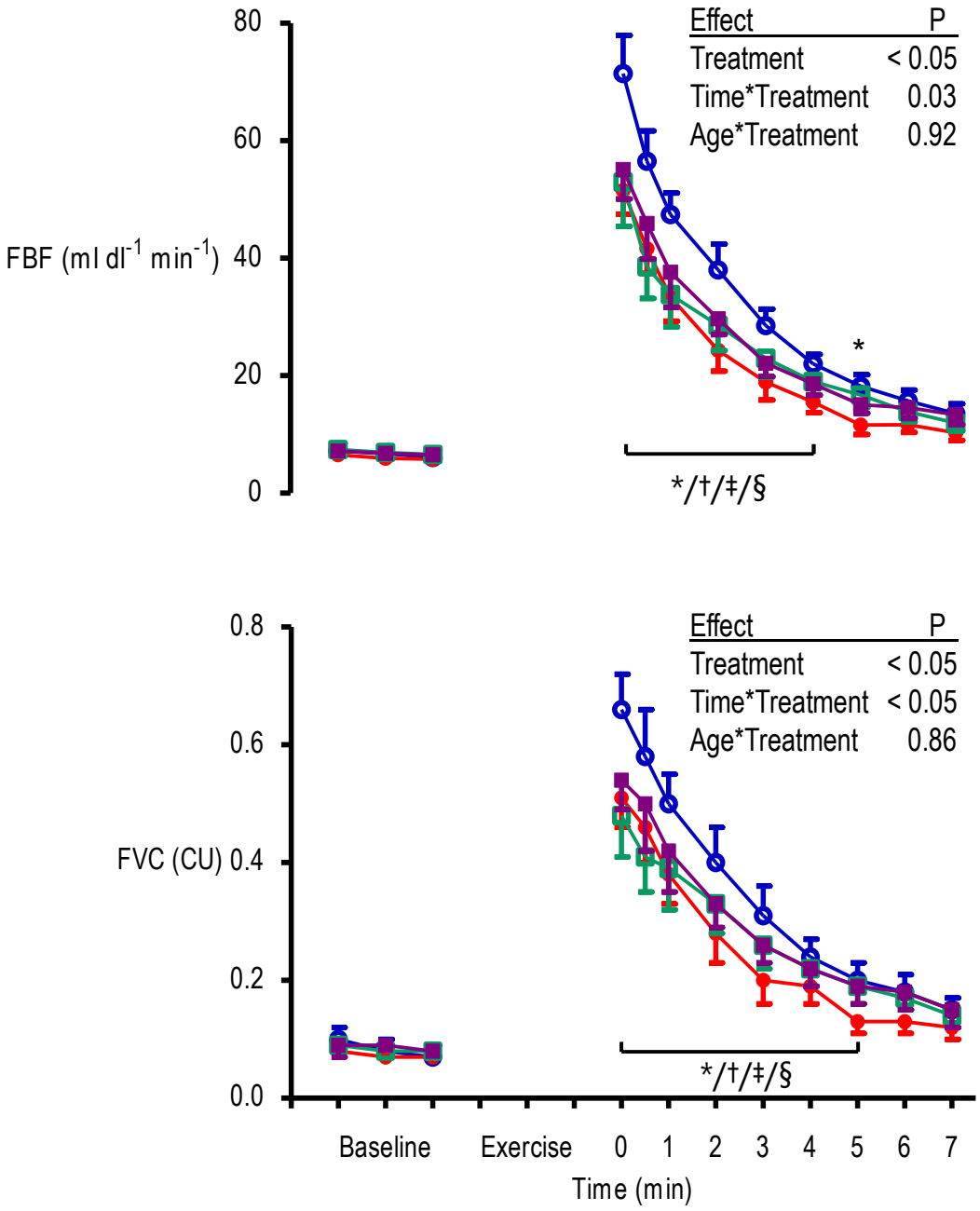



Figure 4.16 **FBF and FVC responses of isometric handgrip contractions performed by older men.** Placebo is represented in blue, and 40% $O_2$ in red, aspirin is shown in green, and their combination (A+$O_2$) in purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.001$).

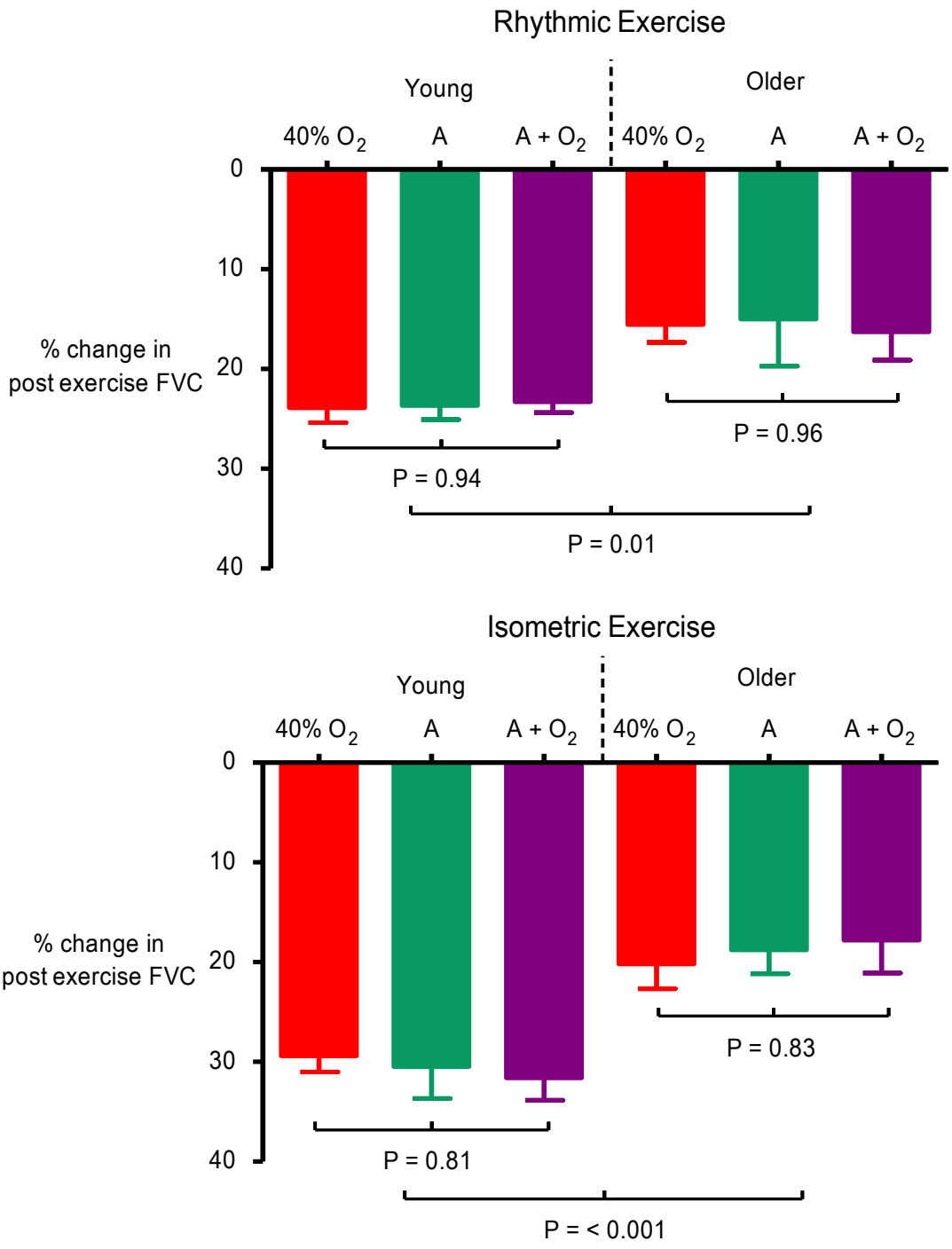



Figure 4.17 **Comparison of percentage reduction in FVC following rhythmic (upper panel) and isometric (lower panel) handgrip contractions performed by young and older men.** 40% $O_2$ is red, aspirin is green, and their combination (A+$O_2$) is shown in purple.

| Older - Rhythmic Exercise | | Baseline | Post Exercise Responses | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | Venous Samples | | 0 s | 3 min | 5 min | 7 min | * represents Time effect P Value |
| $PO_2$ (mmHg) | Placebo | 43 ± 5 | 30 ± 3 * | 51 ± 2 | 48 ± 4 | 46 ± 4 | < *0.001* |
| | 40% $O_2$ | 53 ± 6 | 31 ± 2 * | 53 ± 2 | 54 ± 3 | 55 ± 4 | < *0.001* |
| | Aspirin | 52 ± 3 | 30 ± 1 * | 51 ± 2 | 52 ± 2 | 53 ± 3 | < *0.001* |
| | A + $O_2$ | 53 ± 4 | 28 ± 2 * | 50 ± 3 | 51 ± 4 | 49 ± 4 | < *0.001* |
| | Treatment P Value = *0.91*<br>Time*Treatment P Value = *0.71* Age*Treatment P Value = *0.39* | | | | | | |
| $PCO_2$ (mmHg) | Placebo | 47 ± 2 | 82 ± 5 * | 55 ± 1 * | 50 ± 1 | 48 ± 2 | < *0.001* |
| | 40% $O_2$ | 46 ± 1 | 91 ± 6 * | 56 ± 1 * | 51 ± 1 | 50 ± 1 | < *0.001* |
| | Aspirin | 45 ± 1 | 80 ± 8 * | 51 ± 2 * | 46 ± 1 | 45 ± 1 | < *0.001* |
| | A + $O_2$ | 49 ± 2 | 84 ± 6 * | 59 ± 2 * | 53 ± 3 | 51 ± 3 | < *0.001* |
| | Treatment P Value = *0.26*<br>Time*Treatment P Value = *0.87* Age*Treatment P Value = *0.30* | | | | | | |
| $K^+$ ($mmol.L^{-1}$) | Placebo | 4.79 ± 0.24 | 6.29 ± 0.55 * | 4.70 ± 0.23 | 4.66 ± 0.27 | 4.67 ± 0.21 | < *0.001* |
| | 40% $O_2$ | 4.93 ± 0.11 | 5.78 ± 0.15 * | 4.69 ± 0.11 | 4.86 ± 0.11 | 4.89 ± 0.17 | < *0.001* |
| | Aspirin | 5.03 ± 0.15 | 5.73 ± 0.23 * | 4.96 ± 0.13 | 4.65 ± 0.07 | 4.91 ± 0.07 | < *0.001* |
| | A + $O_2$ | 4.79 ± 0.20 | 5.75 ± 0.23 * | 4.80 ± 0.21 | 4.91 ± 0.24 | 4.97 ± 0.20 | < *0.001* |
| | Treatment P Value = *0.91*<br>Time*Treatment P Value = *0.06* Age*Treatment P Value = *0.42* | | | | | | |
| pH | Placebo ‡ | 7.38 ± 0.01 | 7.22 ± 0.02 * | 7.30 ± 0.01 * | 7.33 ± 0.01 | 7.35 ± 0.02 | < *0.001* |
| | 40% $O_2$ | 7.38 ± 0.00 | 7.19 ± 0.01 * | 7.29 ± 0.01 * | 7.33 ± 0.01 * | 7.34 ± 0.01 * | < *0.001* |
| | Aspirin ‡ | 7.38 ± 0.01 | 7.22 ± 0.03 * | 7.33 ± 0.02 * | 7.36 ± 0.01 | 7.38 ± 0.01 | < *0.001* |
| | A + $O_2$ | 7.37 ± 0.01 | 7.21 ± 0.02 * | 7.28 ± 0.02 * | 7.33 ± 0.01 * | 7.34 ± 0.02 * | < *0.001* |
| | Treatment P Value = *0.30*<br>Time*Treatment P Value = *0.54* ‡ represents Age*Treatment P Value = < *0.05* | | | | | | |
| Lactate ($mmol.L^{-1}$) | Placebo ‡ | 1.3 ± 0.2 | 4.2 ± 0.4 * | 3.9 ± 0.3 * | 3.3 ± 0.4 * | 3.0 ± 0.4 * | < *0.001* |
| | 40% $O_2$ ‡ | 1.3 ± 0.1 | 4.3 ± 0.3 * | 3.6 ± 0.3 * | 3.4 ± 0.3 * | 3.0 ± 0.3 * | < *0.001* |
| | Aspirin ‡ | 1.3 ± 0.1 | 3.7 ± 0.4 * | 3.4 ± 0.3 * | 3.0 ± 0.3 * | 2.4 ± 0.2 * | < *0.001* |
| | A + $O_2$ ‡ | 1.3 ± 0.1 | 3.7 ± 0.4 * | 3.7 ± 0.4 * | 3.3 ± 0.2 * | 3.0 ± 0.3 * | < *0.001* |
| | Treatment P Value = *0.77*<br>Time*Treatment P Value = *0.86* ‡ represents Age*Treatment P Value = < *0.05* | | | | | | |

Table 4.9 **$PO_2$, $PCO_2$, $K^+$, pH, and lactate concentrations in the venous efflux recorded before and after rhythmic handgrip contractions performed by older men.** * represent values significantly different from respective baselines while ‡ represents the age*treatment interaction.

| Older - Isometric Exercise | | Baseline | Post Exercise Responses | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | Venous Samples | | 0 s | 3 min | 5 min | 7 min | * represents Time effect P Value |
| $PO_2$ (mmHg) | Placebo | 50 ± 3 | 36 ± 4 * | 54 ± 3 | 62 ± 7 | 53 ± 5 | *0.001* |
| | 40% $O_2$ | 49 ± 5 | 37 ± 3 * | 60 ± 3 * | 59 ± 5 | 56 ± 5 | *0.001* |
| | Aspirin | 52 ± 2 | 34 ± 2 * | 59 ± 2 * | 56 ± 2 | 56 ± 3 | < *0.001* |
| | A + $O_2$ | 48 ± 5 | 33 ± 1 * | 61 ± 3 | 58 ± 4 | 55 ± 5 | < *0.001* |
| | Treatment P Value = *0.99*<br>Time*Treatment P Value = *0.61* Age*Treatment P Value = *0.06* | | | | | | |
| $PCO_2$ (mmHg) | Placebo | 44 ± 1 | 75 ± 10 * | 49 ± 2 | 45 ± 2 | 47 ± 2 | < *0.001* |
| | 40% $O_2$ | 47 ± 1 | 67 ± 4 * | 54 ± 2 | 48 ± 2 | 46 ± 1 | < *0.001* |
| | Aspirin | 42 ± 1 | 77 ± 8 * | 48 ± 1 | 46 ± 1 | 44 ± 1 | < *0.001* |
| | A + $O_2$ | 47 ± 2 | 74 ± 6 * | 53 ± 2 | 52 ± 1 | 46 ± 1 | < *0.001* |
| | Treatment P Value = *0.73*<br>Time*Treatment P Value = *0.58* Age*Treatment P Value = *0.41* | | | | | | |
| $K^+$ ($mmol.L^{-1}$) | Placebo | 4.73 ± 0.24 | 5.32 ± 0.35 * | 4.96 ± 0.37 | 5.07 ± 0.32 | 5.18 ± 0.29 | < *0.05* |
| | 40% $O_2$ | 4.84 ± 0.19 | 5.73 ± 0.30 * | 4.87 ± 0.18 | 4.93 ± 0.07 | 4.81 ± 0.14 | < *0.001* |
| | Aspirin | 5.09 ± 0.09 | 5.70 ± 0.23 * | 5.04 ± 0.15 | 5.11 ± 0.17 | 4.99 ± 0.16 | *0.02* |
| | A + $O_2$ | 4.83 ± 0.19 | 5.37 ± 0.22 * | 5.04 ± 0.19 | 5.22 ± 0.33 | 5.13 ± 0.22 | *0.002* |
| | Treatment P Value = *0.98*<br>Time*Treatment P Value = *0.87* Age*Treatment P Value = *0.38* | | | | | | |
| pH | Placebo ‡ | 7.39 ± 0.01 | 7.25 ± 0.03 * | 7.35 ± 0.01 * | 7.37 ± 0.01 | 7.37 ± 0.01 | < *0.001* |
| | 40% $O_2$ | 7.37 ± 0.01 | 7.26 ± 0.02 * | 7.31 ± 0.01 * | 7.35 ± 0.01 | 7.37 ± 0.01 | < *0.001* |
| | Aspirin ‡ | 7.41 ± 0.01 | 7.24 ± 0.04 * | 7.35 ± 0.01 * | 7.37 ± 0.01 | 7.39 ± 0.01 | < *0.001* |
| | A + $O_2$ | 7.38 ± 0.01 | 7.25 ± 0.03 * | 7.32 ± 0.01 * | 7.33 ± 0.01 | 7.36 ± 0.01 | < *0.001* |
| | Treatment P Value = *0.43*<br>Time*Treatment P Value = *0.70* ‡ represents Age*Treatment P Value = < *0.05* | | | | | | |
| Lactate ($mmol.L^{-1}$) | Placebo ‡ | 1.4 ± 0.2 | 3.4 ± 0.5 * | 3.0 ± 0.2 * | 2.9 ± 0.3 * | 2.7 ± 0.3 * | < *0.001* |
| | 40% $O_2$ ‡ | 1.3 ± 0.1 | 3.4 ± 0.3 * | 3.3 ± 0.2 * | 3.0 ± 0.3 * | 2.6 ± 0.2 * | < *0.001* |
| | Aspirin ‡ | 1.3 ± 0.1 | 3.7 ± 0.5 * | 3.1 ± 0.3 * | 2.8 ± 0.2 * | 2.3 ± 0.2 * | < *0.001* |
| | A + $O_2$ ‡ | 1.4 ± 0.1 | 3.6 ± 0.5 * | 3.3 ± 0.2 * | 3.3 ± 0.3 * | 2.8 ± 0.2 * | < *0.001* |
| | Treatment P Value = *0.84*<br>Time*Treatment P Value = *0.97* ‡ represents Age*Treatment P Value = < *0.05* | | | | | | |

Table 4.10 **$PO_2$, $PCO_2$, $K^+$, pH, and lactate concentrations in the venous efflux recorded before and after isometric handgrip contractions performed by older men.** * represent values significantly different from respective baselines while ‡ represents the age*treatment interaction.

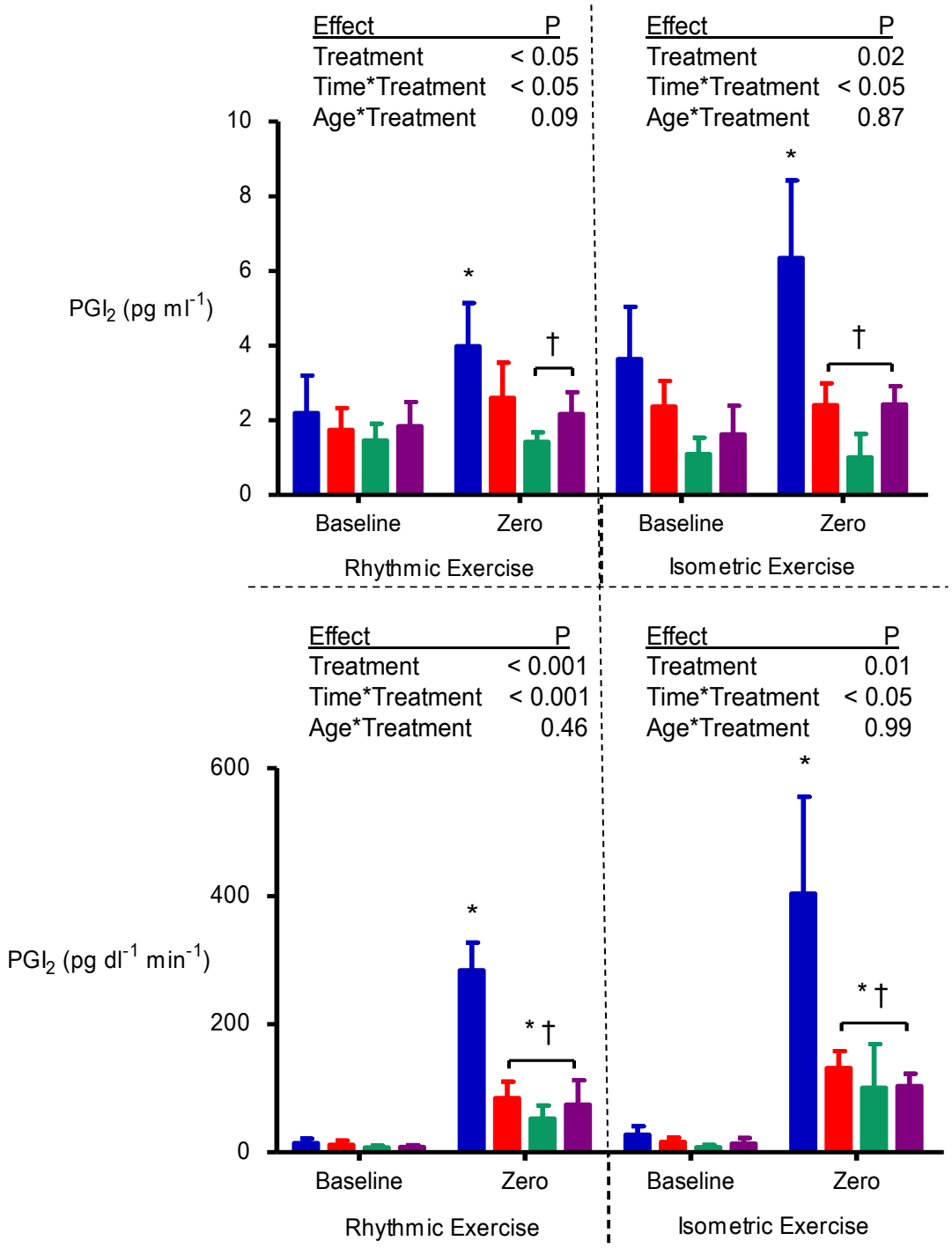



Figure 4.18 **$PGI_2$ (upper panel) measured in venous plasma samples and normalized $PGI_2$ (lower panel) values recorded before and after rhythmic and isometric handgrip contractions performed by older men.** Placebo is represented in blue while 40% $O_2$ supplementation is red, aspirin is green, and their combination ($A+O_2$) is shown in purple. * represent values significantly different from their respective baselines while † represents values significantly different from P treatment condition ($P < 0.05$). This includes all values within the brackets.

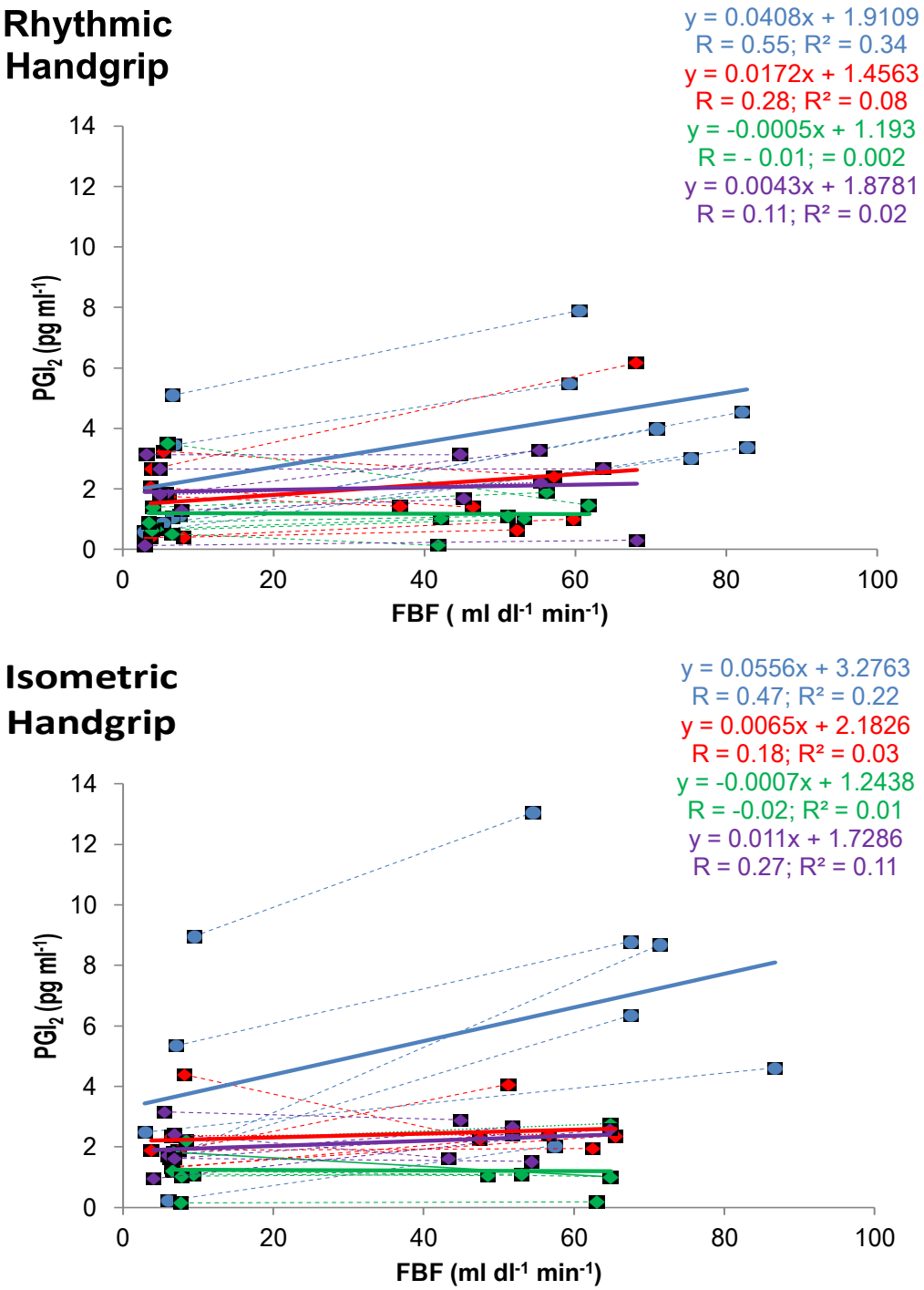



Figure 4.19 **Correlation between baseline and *post*-exercise FBF and $PGI_2$ (6-keto-$PGF_{1\alpha}$ ) concentrations for rhythmic (upper panel) and isometric (lower panel) exercise performed by older men.** P is represented in blue, 40% $O_2$ is red, A is green, and their combination (A+$O_2$) is shown in purple. Each individual's baseline and *post*-exercise response is shown by the dashed line while the solid line represents the line of best fit (regression analysis and $r^2$ values are provided for each treatment).

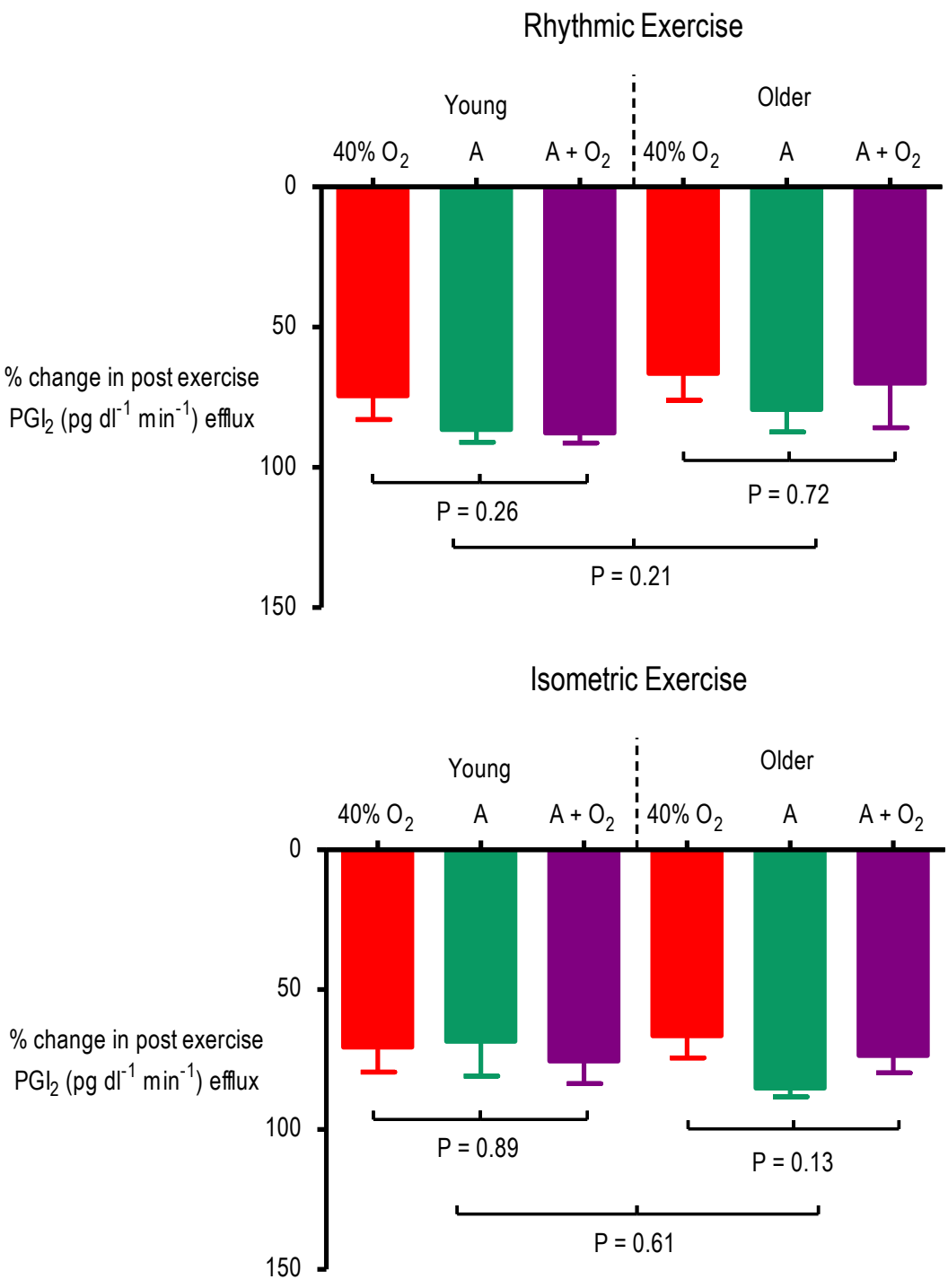



Figure 4.20 **Comparison of percentage reduction in $PGI_2$ efflux following rhythmic (upper panel) and isometric (lower panel) handgrip contractions performed by young and older men.** 40% $O_2$ supplementation is red, aspirin is green, and their combination (A+$O_2$) is shown in purple columns.

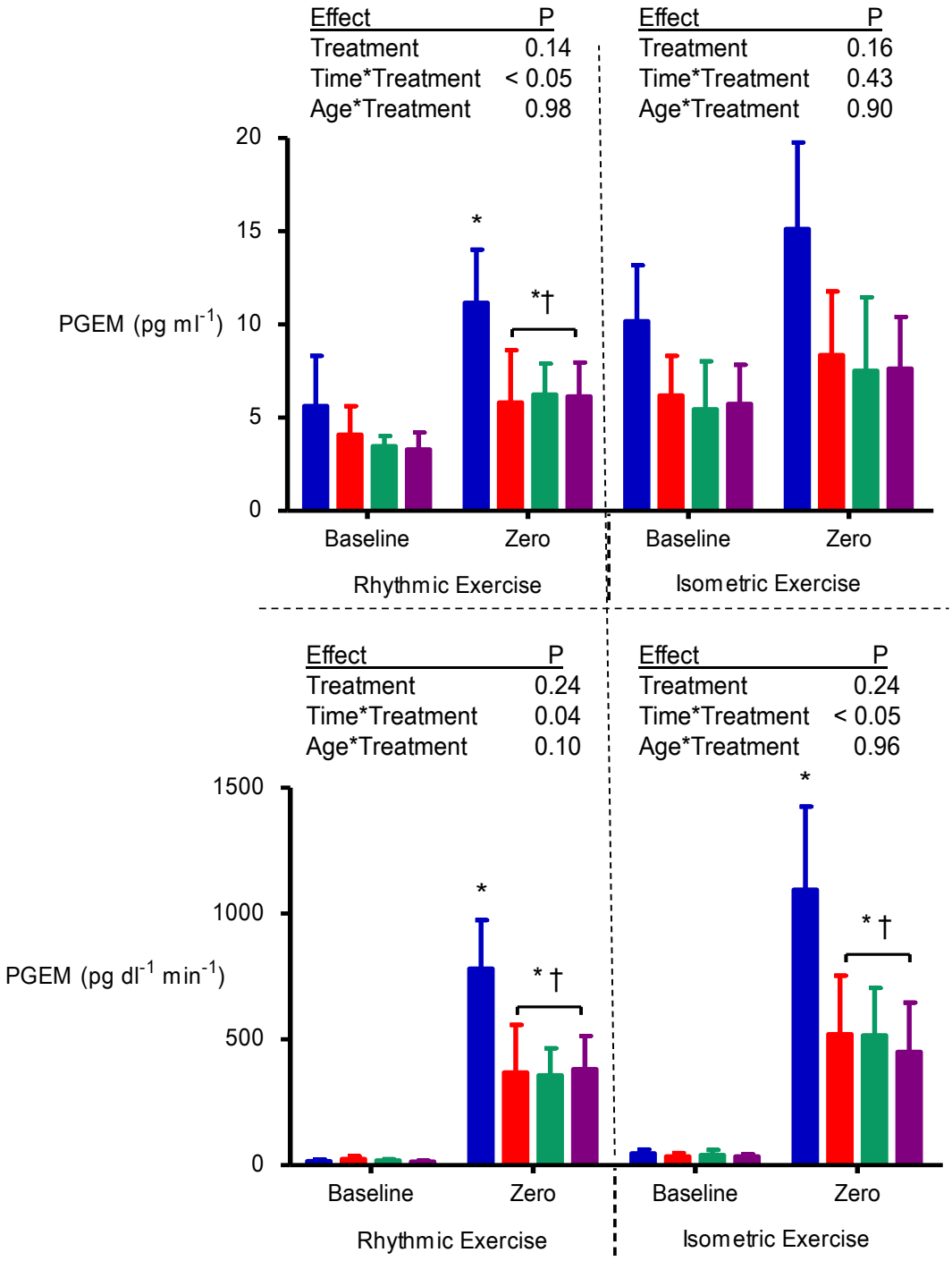



Figure 4.21 **PGEM (upper panel) measured in plasma sample of venous efflux and normalized PGEM (lower panel) values recorded before and after rhythmic and isometric handgrip contractions performed by older men.** Placebo is represented in blue while 40% $O_2$ supplementation is red, aspirin is green, and their combination ($A+O_2$) is shown in purple. * represent values significantly different from their respective baselines while † represents values significantly different from P treatment condition ($P < 0.05$). This includes all values within the brackets.

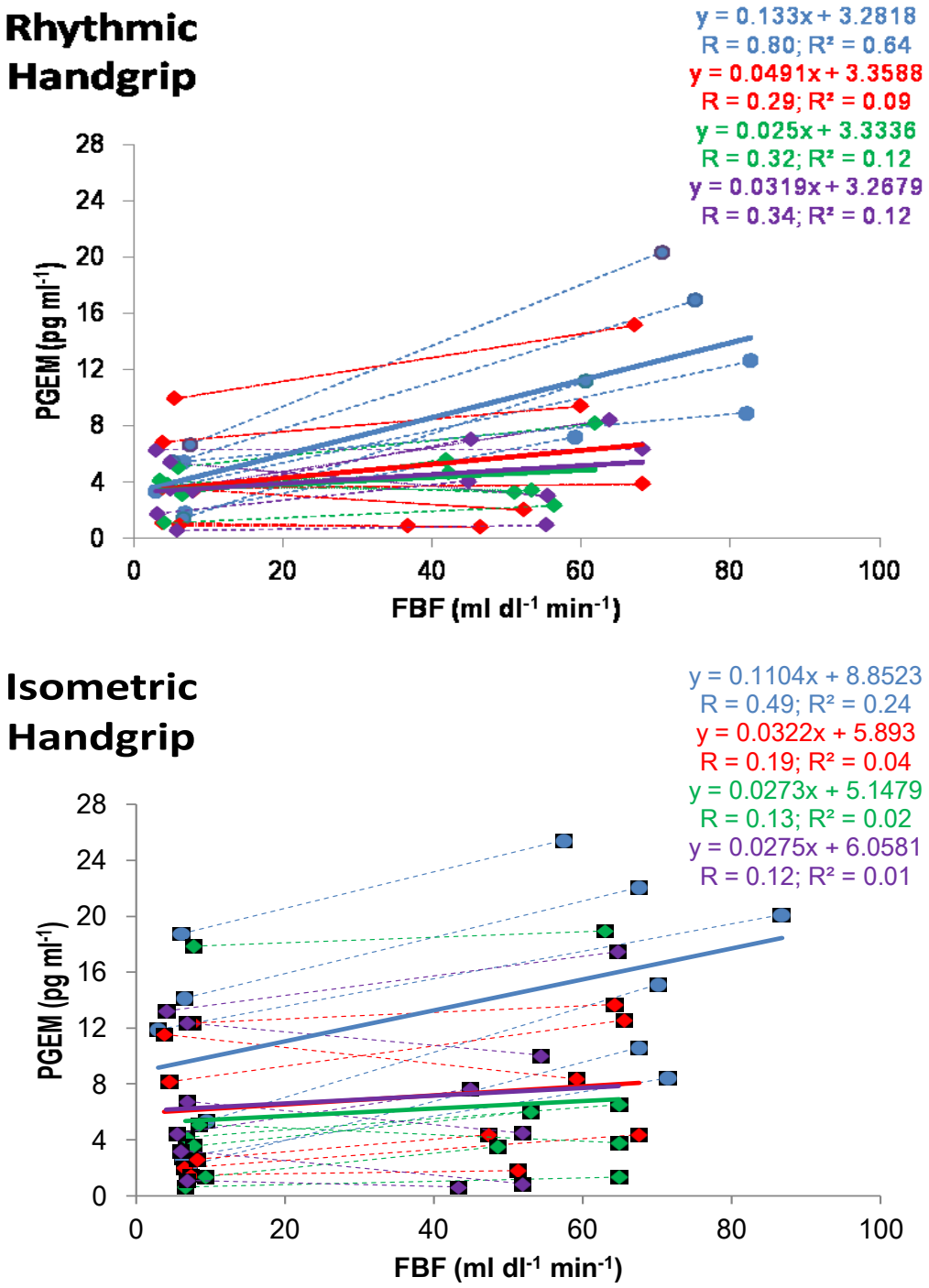



Figure 4.22 **Correlation between baseline and *post*-exercise FBF and PGEM concentrations for rhythmic (upper panel) and isometric (lower panel) exercise performed by older men.** P is represented in blue, 40% $O_2$ is red, A is green, and their combination (A+$O_2$) is shown in purple. Each individual's baseline and *post*-exercise response is shown by the dashed line while the solid line represents the line of best fit (regression analysis and $r^2$ values are provided for each treatment).

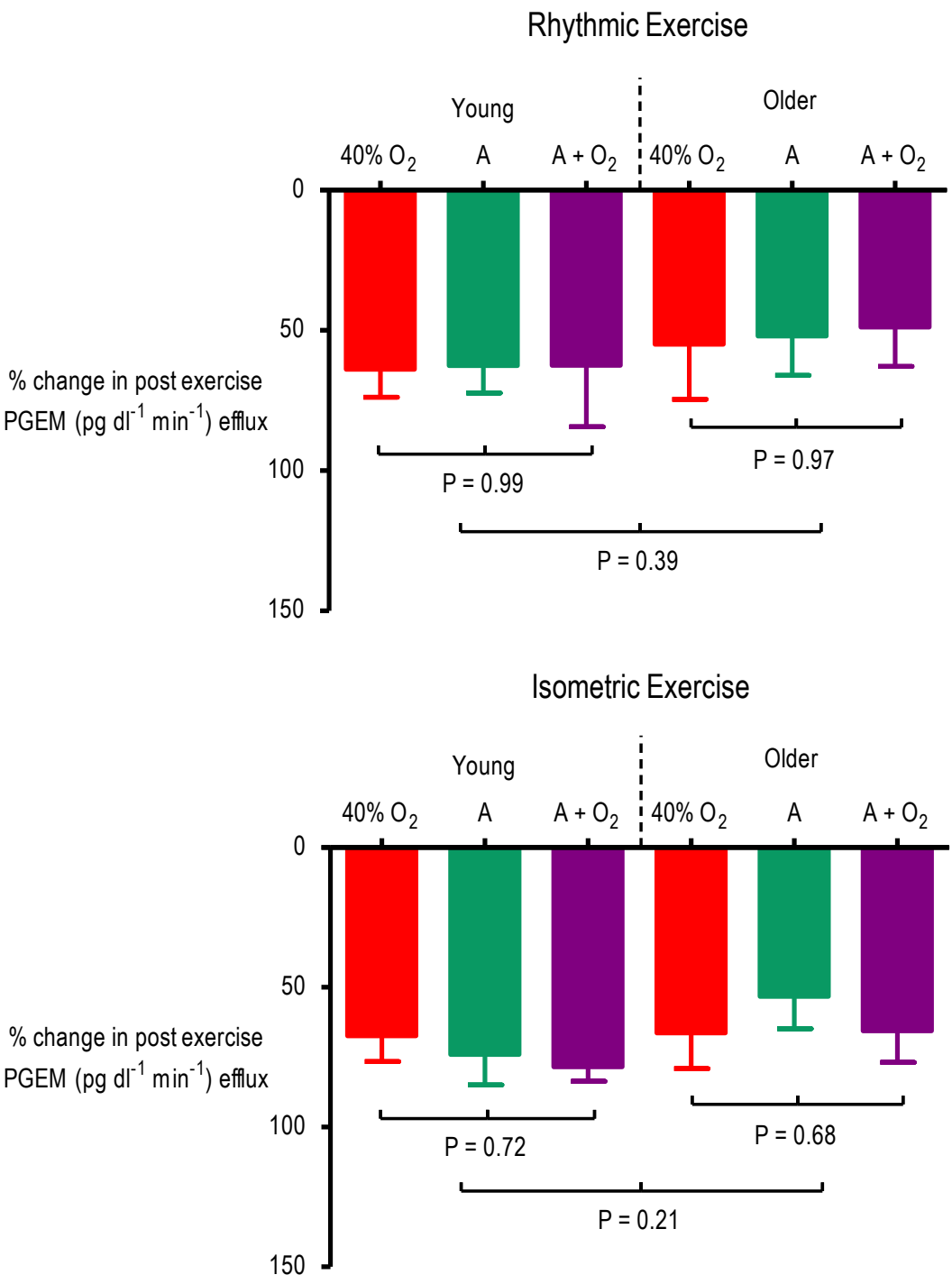



Figure 4.23 **Comparison of percentage reduction in PGEM efflux following rhythmic (upper panel) and isometric (lower panel) handgrip contractions performed by young and older men.** 40% $O_2$ supplementation is red, aspirin is green, and their combination (A+$O_2$) is shown in purple columns.

## 4.4 Discussion

There were several novel findings of the present study. Firstly, COX inhibition with aspirin, 40% $O_2$, and their combination substantially attenuated *post*-exercise hyperaemia associated with 60% MVC rhythmic *and* isometric handgrip contractions in both recreationally active young *and* older men. This attenuation was not different between treatments. Secondly, both rhythmic and isometric contractions substantially increased the venous efflux of $PGI_2$ (6-keto-$PGF_{1\alpha}$) and $PGE_2$ (PGEM) in young *and* older men; COX inhibition and/or 40% $O_2$ similarly attenuated this efflux. Finally, although there was no significant difference between young and old for the percentage attenuation in venous efflux of $PGI_2$ (6-keto $PGF_{1\alpha}$) and $PGE_2$ (PGEM) induced by 40% $O_2$ and COX inhibition, the accompanying attenuation of the exercise hyperaemia was significantly greater in young (~ 30%) than older men (~ 15%).

### 4.4.1 Systemic cardiovascular responses

It is well established that exercise leads to an increase in HR and MABP as recorded in the present study; responses which are attributable to central command and the reflex components mediated by peripheral inputs (200). A number of COX-related substances have been implicated in the reflex cardiovascular responses to exercise; including AA, $PGE_2$, and $TXA_2$ (200, 207, 262, 347). Therefore COX inhibition with aspirin could potentially have influenced exercise-related reflex changes in the systemic cardiovascular system. Moreover according to our original hypothesis production of PGs during exercise is $O_2$-dependent. Therefore 40% $O_2$ could have influenced these systemic reflex responses by changing the input from afferents in the contracting muscles. However, in agreement with our laboratory's previous work (123, 437), no such treatment effects were found regarding the changes in HR and MABP recorded in young men. Moreover, the interventional treatments also failed to influence exercise-related changes in

HR and MABP of the older men. The lack of effect on HR response is consistent with this being mainly centrally-mediated (5, 422); while the effect on vasoconstrictor component of exercise reflex may not have been apparent from MABP as this is dependent on both the cardiac output and TPR (163). The contributions of afferent neural inputs to the pressor reflex are addressed in more detail in the study described in Chapter 5 on the relationship between COX products, their $O_2$-dependency, and the exercise-related reflex vasoconstriction. The influence of age on this relationship is also one of the primary questions in Chapter 6. Therefore, the present discussion primarily focuses on the contributions of COX products and $O_2$-dependency to exercise hyperaemia and exercise-related changes in the efflux of different metabolites.

### 4.4.2 Exercise hyperaemia responses

#### 4.4.2.1 Control responses in young men

Before discussing the hyperaemia responses of rhythmic and isometric handgrip contractions, an important point that requires consideration is whether inflation of the venous occlusion cuff itself had an impact on the observed hyperaemia responses. Kirby et al. investigated the impact of external mechanical influences on the FVC by inflating a cuff around the forearm at different pressures (25 – 300 mmHg) (212). Their results revealed that the cuff pressures of 25, 50, 75, and 100 mmHg increased in FVC in a fairly linear manner. However from thereon, the observed hyperaemia remained quite stable. Thus, given the venous occlusion cuff was inflated around the upper arm to record FBF – a standard practice in the technique of venous occlusion plethysmography, it is possible this external pressure of ~ 50 mmHg on the upper arm had some myogenic impact on the baseline and *post*-contraction FVC changes recorded in the present study. However, any such effect was a consistent factor between treatments, and should not be the cause of any observed treatment effects. A second, related point that also requires consideration is whether these mechanical influences could affect the release of vasoactive

metabolites; in particular, PGs. It is possible they did influence the release of intravascular vasoactive ATP (76). However, independent NOS inhibition (32), and combined NOS and COX inhibition (356) did not attenuate the rapid component of the vasodilator response to muscle contraction. Therefore, it seems very unlikely that any increase in the vasoactive PGs observed in this study was the result of mechanical influences of cuff inflation; i.e., venous occlusion plethysmography cuff/s and/or Finapres cuff (Finapres cuff was on the non-exercising resting hand).

Both rhythmic and isometric handgrip contractions resulted in an increase in *post*-exercise FBF (hyperaemia), attributable to a reduction in vascular tone: vasodilatation. As might have been expected from previous studies in which $PGE_2$ and $PGI_2$ efflux were assessed during rhythmic exercise (198, 199), and the early study in which PGE-like substances were assayed following isometric contraction of the forearm (209), both types of contractions increased the venous efflux of $PGI_2$ (6-keto-$PGF_{1\alpha}$) and $PGE_2$ (PGEM) in the present study. As indicated in the Introduction, concentrations of $PGI_2$ and $PGE_2$ in muscle interstitium also increase during rhythmic exercise. This result suggests active participation of both vasodilator PGs to exercise hyperaemia of isometric *and* rhythmic contractions.

However, It is important to note that although a number of studies have reported exercise-related changes in the venous (302, 435) and interstitial PG concentrations (29, 198, 199, 224), the calculated values are not always comparable. This may reflect differences in the release of PG by the cannulated vessel during blood sampling (99, 302), *ex-vivo* (*in-vitro*) production of PGs (99), and/or the effect of different methodologies used to measure these PGs (99). In fact, when Dray et al. employed careful plasma extraction methodologies (i.e., anti-prostaglandin sera in cold vacutainers and careful removal of RBCs and platelets) to optimise measurement of human plasma $PGF_{\alpha}$, $PGE_1$, and $PGE_2$ concentrations (99), they measured values for 6-keto $PGF_{1a}$ of ~

12 and $PGE_2$ of ~ 4 pg/ml under baseline conditions; similar to those reported here. Therefore, it is reasonable to assume that the use of careful methodologies for sample collection (i.e., removal of 0.8 ml blood before each sample collection in ice-cold vacutainers, immediate addition of indomethacin, and supernatant plasma extraction after spinning at 2500 rpm at 4°C for 20 min; See Section 4.2.2), allowed the actual venous PG concentrations to be measured in the subjects who took part in the present study. Moreover, the range of "normal baseline PG concentrations" is quite wide (99, 447), which in itself allows for the variability in the reported PG concentrations data of different studies (198, 199, 302, 435). Lastly our results do concur with earlier studies (29, 198, 199, 302, 435, 447, 448) in that they are internally consistent (i.e., under placebo, venous efflux of PGs increased significantly following exercise).

Regarding the venous concentrations of metabolites ($K^+$, $H^+$, and lactate), their participation in active vasodilatation of either rhythmic or isometric handgrip exercise is questionable; the time course of the change in *post*-exercise venous concentrations of these metabolites remained completely unrelated to the changes in FBF and FVC.

#### 4.4.2.2 Effect of COX inhibition and breathing 40% $O_2$ in young men

Before discussing the effect of aspirin (600mg) on hemodynamic responses and intra-venous PG concentrations, it is important to mention here that a similar dose of aspirin has previously been shown to effectively inhibit the COX enzyme for 30 – 90 min (161). Moreover, a comparison between aspirin and indomethacin regarding their effectiveness in attenuating exercise hyperaemia during cycling revealed no difference between them (74). In the present study, COX inhibition attenuated the *post*-exercise hyperaemia of isometric handgrip contractions (60% MVC); results which agree with our group's previous observations (437). Moreover attenuation of *post*-exercise hyperaemia evoked by rhythmic handgrip contractions with COX inhibition in this study is also in agreement with previous observations (302, 303). However, in most previous

studies, the assessment of contribution of COX products to exercise hyperaemia was indirect: very few assayed PGs in same study in which blood flow was recorded and a COX inhibitor used. The measurements of venous $PGI_2$ (6-keto-$PGF_{1\alpha}$) and $PGE_2$ (PGEM) concentrations before and immediately after both types of handgrip contractions in present study clearly shows their release was indeed inhibited by aspirin and that the *post*-exercise hyperaemia responses were attenuated. Similar results were obtained by Kilbom and Wennmalm; indomethacin not only attenuated the hyperaemia responses but also significantly decreased the venous PGE concentrations. (209). Therefore, as the venous concentrations and efflux of PGs increase in response to either type of handgrip contraction and attenuate following COX inhibition, this study confirms their exercise-related release and is consistent with their ($PGE_2$ and/or $PGI_2$) active participation in the exercise hyperaemia response. In contrast, some previous studies have suggested that vasodilator PGs are not important mediators of exercise hyperaemia; at least following 10% MVC rhythmic handgrip contractions (359, 368). The results of the present study lend unequivocal support to vasodilator PGs being important contributors to exercise hyperaemia following isometric and rhythmic contractions at 60% MVC.

Win and Marshall's observation that 40% $O_2$ and aspirin similarly attenuated the *post*-exercise hyperaemia of isometric handgrip contractions (60% MVC) led to their proposal that the release of PGs during exercise was $O_2$-dependent (437). The present study confirmed this proposal, in that the efflux of both $PGE_2$ and $PGI_2$ following isometric contraction was attenuated by 40% $O_2$; and showed for the first time, that the release of these PGs induced by rhythmic handgrip contraction and the associated *post*-exercise exercise hyperaemia are also $O_2$-dependent. What makes the present findings really interesting is the observation that 40% $O_2$ attenuated the effluxes of both $PGE_2$ and $PGI_2$ caused by both types of handgrip contractions to values that were similar to those observed with COX inhibition. This finding suggests that the synthesis of

PGs during these contractions is explained by a fall in tissue $PO_2$; other stimuli for PG release such as shear stress are relatively less important. The finding that the combined use of $O_2$ and aspirin had no greater effect on either the *post*-exercise hyperaemia or the venous PG concentrations is also significant. It seems that the fall in $PO_2$ that occurs within forearm muscle during rhythmic and isometric handgrip contractions and the synthesis of $PGE_2$ and $PGI_2$ can be prevented by breathing supplementary $O_2$ at the modest concentration of 40%.

On the other hand, breathing 40% $O_2$ did not affect the increase in venous efflux of $K^+$ or lactate, or the fall in pH following either type of contraction. The lack of effect on $K^+$ is not surprising, if this is mainly due to efflux from contacting muscle fibres during action potentials (See Section 1.2.3.3.4). The lack of effect on lactate and pH contrasts with the finding of Fordy and Marshall; they were both attenuated when 40% $O_2$ was breathed during isometric contraction at 100% MVC (123). This disparity is probably the result of a greater fall in tissue $PO_2$ during maximal contraction that was ameliorated by breathing 40% $O_2$.

#### 4.4.2.3 Responses of the older men

The study performed by Schrage et al. raised interesting questions about the contribution of PGs towards exercise hyperaemia in older individuals (358). However, as the release of PGs is intensity-dependent (29, 198, 447, 448) and as they only used 10% MVC handgrip contractions and did not assay PG release, further investigations into the relationship between release of vasodilator PGs and their role in exercise hyperaemia of older individuals was warranted.

Rhythmic and isometric handgrip contractions performed at 60% MVC evoked a substantial *post*-exercise increase in FBF and FVC in older men, and interestingly, this increase was unaffected by aging. Although certain studies suggest the exercise-related increase in blood flow is attenuated with age (213, 322, 326), some have suggested that the hyperaemia responses of

older individuals are preserved with lifelong physical activity (87, 285, 305, 391, 395). Similar results have also been obtained in young and old, sedentary and exercise-trained rats (409).

Seen in this context the novel results of the present study were that the *post*-contraction hyperaemia in the older subjects was accompanied by a significant increase in the venous concentrations / efflux of vasodilator $PGE_2$ and $PGI_2$; COX inhibition attenuated the hyperaemia for both types of contraction. Moreover, as in young men, COX inhibition and 40% $O_2$ similarly attenuated venous the $PGE_2$ and $PGI_2$ efflux. This contrasts with the report of Schrage et al. (358) that the contribution of PGs to exercise hyperaemia is greatly attenuated or lost in older subjects. The present findings demonstrate that in fact a substantial contribution of PGs to exercise hyperaemia associated with medium-intensity exercise is maintained in older recreationally active men. This raises the possibility that the contribution of PGs is lost with sedentariness, rather than with ageing *per-se.* Indeed in support of this argument is the work performed by the Seals group which showed that not only does the central arterial compliance improve in older individuals who undergo a simple endurance training regimen of 30 min walking 3 – 4 days per week (395), but such regular aerobic exercise prevents the age-related decline in forearm's endothelium-dependent dilatation (87).

It must be acknowledged that Schrage et al. (358) only used a light level of rhythmic contraction (10% MVC) rather than 60% MVC, as used in the present study. Thus the disparity may partly be explained by the intensity of exercise, particularly as the release of PGs was shown to be exercise intensity-dependent (29, 198, 447, 448). However, Schrage et al. (358) reported that their older subjects *did not* perform regular physical exercise but only engaged in routine household tasks. Thus, an alternative explanation for their results could be the deterioration of endothelial function that occurs when relative-inactivity is combined with aging. Endurance exercise is associated with increased shear stress (403, 405, 406) which in itself has been linked

to increased mRNA expression of eNOS (439). Further, Trott et al. noticed that ACh-induced dilatation was attenuated in older sedentary rats but the use of superoxide-dismutase in these rats increased endothelial dilatation to values that were comparable with young (409). Therefore, it is possible that the contribution of PGs might have been compromised by the lack of regular exercise and/or by the increased oxidative-stress associated with aging (214).

The present finding showed that despite similar venous efflux of $PGE_2$ and $PGI_2$ in young and older men, the magnitude of their contribution to exercise-associated vasodilatation declined with age (~ 15 vs. 30%). This suggests age-associated changes in the action of vasodilator PGs, rather than an inability to synthesise PGs in response to exercise. As in young men, the combination of 40% $O_2$ and aspirin did not add further to the attenuation of exercise hyperaemia or venous PG efflux evoked by either type of contraction suggesting that the synthesis and release of PGs was completely $O_2$-dependent.

It should also be noted that the increase in venous lactate efflux was also smaller in older than young men; this was expected considering the age-related change in the predominant muscle type (i.e., slow-twitch vs. fast-twitch respectively) (49, 115). However as in the young subjects, breathing 40% $O_2$ had no significant effect on these effluxes.

### 4.4.3 Mechanism of action of 40% $O_2$ on PG generation

The observation that breathing 40% $O_2$ only *during* the period of exercise attenuates *post*-contraction hyperaemia (123) lends support to the argument that the stimulus for $O_2$-dependent release of PGs lies within the exercising muscles. However, exercise is not only associated with profound decline in intramuscular / interstitial $PO_2$ (143, 337-339), but similarly peri-venular $PO_2$ (226) also declines during this time. The observation of an increase in the $PGI_2$ concentrations when intravascular $PO_2$ declines (45, 46, 266) is consistent with the idea that this decline in peri-

venular / capillary structures during exercise is the source of $O_2$-dependent $PGI_2$ release. Therefore the present finding that 40% $O_2$ attenuated the release of $PGI_2$ to levels that were similar to those observed with COX inhibition raises the possibility that the increase $PO_2$ with 40% $O_2$ directly influenced / attenuated the release of vasodilator $PGI_2$ from venular endothelium: this does not exclude the possibility that $PGE_2$ is also released from venular endothelium.

An alternative explanation for the observed responses involves adenosine and/or ATP. Release of these compounds has been linked to hypoxia (24, 70, 71, 88, 142, 186, 244, 331) and exercise-intensity (142, 253). Based on the discussion in section 1.2.3.7 (interdependency and redundancy), it is clear that adenosine and ATP influence the production of PGs (286, 289, 304, 306). Thus, $O_2$ supplementation might be affecting the release of adenosine and/or ATP, thereby attenuating their contribution towards the release of vasodilator PGs. It is possible that the increased $PO_2$ inhibits the production of adenosine from any of its known sources (i.e., intra-muscular, interstitial, and ATP released by de-oxygenated RBCs). Moreover, increased $PO_2$ might attenuate the change in RBC profile (oxygenated to deoxygenated), thereby inhibiting their direct contribution towards the release of PGs.

Importantly, it is unclear whether adenosine and/or ATP have a direct impact on $PGE_2$ production. However, given that $PGE_2$ can be synthesised by skeletal muscle fibres (400), it is possible that breathing 40% $O_2$ attenuated synthesis and release from this site. Whether activation of COX in skeletal muscle fibres is $O_2$-dependent has not been tested. Figure 4.24 attempts to elucidate this complicated relationship and shows different, but not necessarily exclusive possibilities of attenuated PG release with 40% $O_2$.

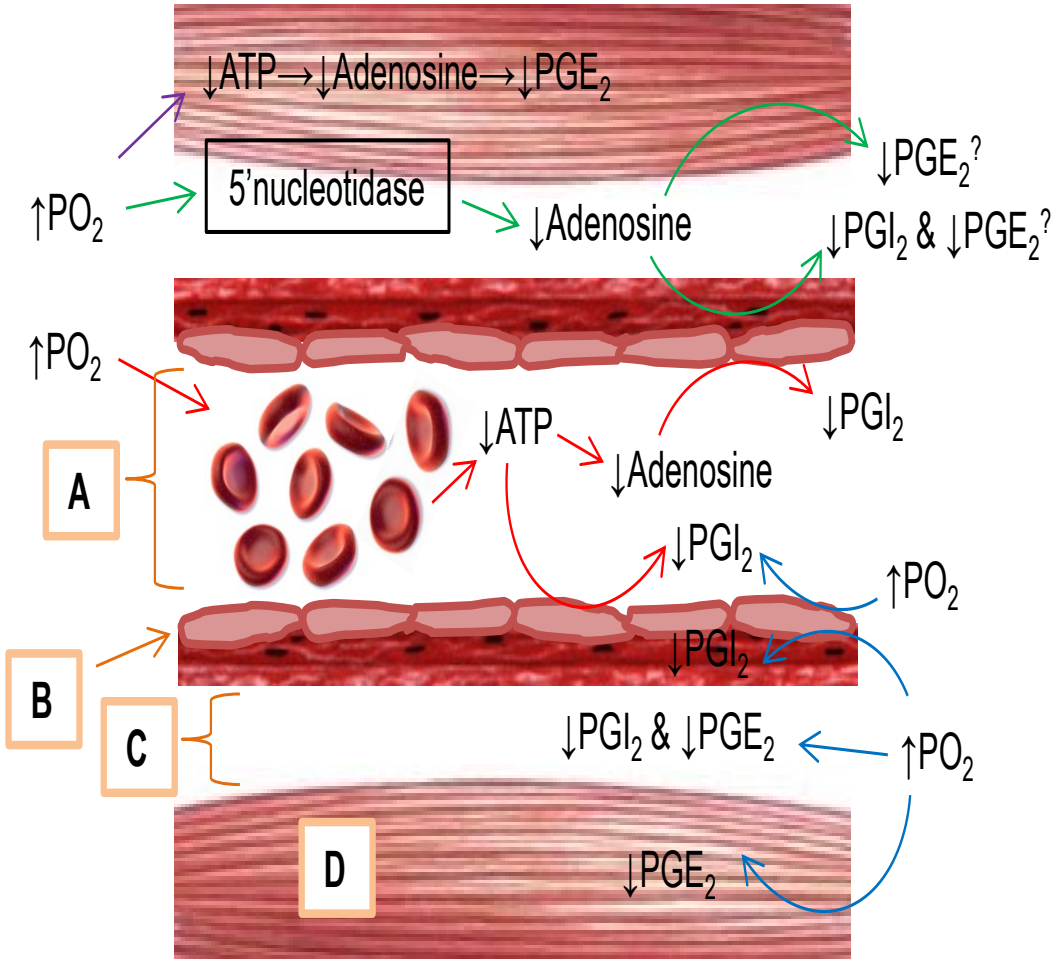



Figure 4.24 **Possible mechanisms by which 40% $O_2$ attenuates the release of vasodilator PGs.** Compartment A is intra-vascular, B is showing endothelium, C represents interstitium while D represents skeletal muscle. Different coloured arrows represent four distinct but not exclusive pathways that could be affecting the release of $PGI_2$ and $PGE_2$ during exercise. The blue → shows a pathway where the fall in $PO_2$ directly influences the release of $PGI_2$ and $PGE_2$ which is counteracted by 40% $O_2$. The red → pathway shows that as RBCs become deoxygenated, they release ATP which directly and/or via adenosine stimulates the endothelial release of $PGI_2$. The green → and purple → pathways shows that increase in $PO_2$ could be attenuating interstitial adenosine concentrations and thereby attenuating the release of vasodilator PGs.

In conclusion, this is the first study to show that vasodilator PGs ($PGI_2$ and $PGE_2$) are released in response to rhythmic and isometric handgrip contractions, in both, young and older men. Moreover, their release and contribution to exercise (*post*-exercise) hyperaemia is and remains $O_2$-dependent. Therefore, the hypotheses of this study are accepted.

### 4.4.4 Limitations and Future Work

A basic limitation to this work is that oral aspirin was used to COX enzyme: this rather blunt approach must have affected the systemic vascular responses and in turn, the responses of the exercising arm. Although intra-arterial COX inhibitor infusion during or just before commencement of exercise would have provided a more localized COX inhibition, this (i.e., arterial-line) would have made the protocol more risky and required medical cover. Further, the principal reason this approach was not adopted was because one of the PGs under investigation is primarily released by the skeletal muscle fibres (i.e., $PGE_2$) (400). It was not clear whether such a short lasting / abrupt infusion during or just prior to exercise, so as to avoid a systemic dose being given, would effectively lead to COX inhibition in the extra-vascular compartment.

Another limitation to this study is that venous samples of 6 randomly selected individuals were assayed for PGs. Although assay of these samples for PG concentrations revealed fairly consistent data; for a more thorough experimental approach, not only would it have been better to analyse venous samples from all individuals for PG concentrations, but it would have given temporal resolution if PGs had been measured at other time intervals. This approach could have further elucidated the apparently tight relationship already observed in this study between the $O_2$-dependent PGs and the exercise / *post*-exercise vasodilatation. Ideally, arterial samples would have aided in this understanding by allowing arterio-venous differences to be calculated. Furthermore, in order to examine the contribution of 40% $O_2$ to oxidative-stress, its markers (i.e., isoprostane, nitro-tyrosine, etc) could have been measured. However, these interventions were not done due to financial, time, and technical restraints.

Another limitation to this study is that the order of rhythmic and isometric handgrip contractions was not randomized, but arranged so that isometric contraction always occurred second. Ideally,

randomization should have been done but it was quickly observed that 3 min of isometric handgrip was more intense and fatiguing for participants than 3 min rhythmic contractions. Thus, the selected order of handgrip exercise bouts helped to ensure that the effect of the second exercise was not affected by the consequence of the first.

Separately, an important limitation is that all the hyperaemia responses presented here are *post*-exercise responses. Use of a duplex Doppler-ultrasound would have provided a continuous record of blood flow in the exercising arm; providing brachial artery diameter could be imaged successfully during isometric *and* rhythmic contractions at 60% MVC (see Chapter 3 Discussion). Interestingly, the data of Kagaya and Homma showed that brachial artery blood flow increased by ~ 60% from baseline during incremental isometric handgrip contractions ranging from 10 – 70% MVC (196). It was after contraction that the magnitude of *post*-contraction hyperaemia was proportional to the size / intensity of the contraction. Thus, it could be argued that the *post*-contraction hyperaemia was most likely, the component of vasodilatation induced by isometric handgrip contraction to be affected by the interventional treatments employed in the present study. However, it is the case that had successful imaging proved possible during rhythmic contractions at 60% MVC, despite the local tissue movement that inevitably occurs with medium-intensity rhythmic contraction, duplex Doppler-ultrasound could have elucidated the contribution of $O_2$-dependent PGs to the time course of vasodilatation *during* exercise. Moreover, the use of Doppler-ultrasound could also have allowed comparison of the influence of 60% MVC isometric handgrip contractions in restricting FBF in young and older men, as well as revealed any differences in the time course of the contribution of vasoactive PGs with age.

Lastly, a limitation of this study is partly related to experiments performed by Schrage et al. (358). As their subjects did not perform any regular exercise but only indulged in household tasks; an older group of healthy but sedentary individuals would have allowed comparison of the

relationship between PG release, and their contribution to the exercise / *post*-exercise hyperaemia of medium-intensity contractions in older people of different physical activity levels. Any future work should address these limitations.

# Chapter 5

# 40% $O_2$ and COX blockade attenuate reflex vasoconstriction evoked in resting leg by isometric handgrip contraction in young men

## 5.1 Introduction

Following the principal findings of Chapter 4, the next logical question was to investigate whether $O_2$-dependent COX products are involved in the reflex vasoconstriction of exercise?

It is established that exercise is accompanied by an increase in blood flow to the exercising muscles along with systemic cardiovascular responses: increased HR, MABP, MSNA, and peripheral vasoconstriction (72, 119, 172, 371, 421, 437). As mentioned in Chapter 1 (See Section 1.2.6), the two neural mechanisms contributing to these changes are central command (i.e., feed-forward) and muscle (afferent) / pressor reflex (i.e., feedback). Parallel activation of muscle contractile activity and the cardiovascular responses, defines central command; while the feedback mechanism is defined by contraction-induced mechano and/or metabo-afferent activation and thence, reflex sympathetic activation (173, 271). The thinly myelinated group III and un-myelinated group IV afferents are generally attributed with the respective mechanical and metabolic inputs of the exercise pressor reflex (172, 201, 203, 271).

A number of metabolites have been implicated as contributing factors to the reflex. These include bradykinin (201, 207, 261), $K^+$ (261), lactic acid (347, 372), and those involved in COX pathway; AA, $PGE_2$, and $TXA_2$ (207, 231, 262, 347). Recently, Leal et al. have shown that thromboxane-receptors are stimulated by both muscle stretch and 30 s of isometric contraction in freely perfused muscle; their inhibition attenuates the pressor response (231). This is an important finding considering not just $TXA_2$, but isoprostane, $PGE_2$, and $PGI_2$ can all stimulate the thromboxane-receptors (141). Consistent with the results of Chapter 4, concentrations of PGs ($PGE_2$ and $PGI_2$) increase in the interstitium of exercising muscle (79, 390, 435, 447, 448), and COX inhibition attenuates exercise hyperaemia by blocking the production of these PGs (74, 209, 437). Studies showing that AA and/or its subsequent products stimulate group III and IV

afferents (207, 262, 347) raise the possibility that these products might also contribute to the exercise pressor reflex / reflex vasoconstriction. For example, Rotto and Kaufman showed that the stimulation of group III and IV afferents in cats by arterial AA injections was almost completely abolished if indomethacin were given 30 min prior to AA (347). In another study, ~ 60% of group IV afferents located in triceps-surae were stimulated by 60 s of isometric contractions; a response which was abolished with either indomethacin or aspirin, suggesting that PGs facilitate activation of these afferents (346). Subsequent injection of 40 μg $PGE_2$ restored the response of group IV afferents to isometric contractions (346).

In humans, isometric handgrip contractions (25, 50, and 75% MVC) evoked reflex vasoconstriction in the relaxed contralateral arm when there was no electromyograph activity (72). Further, low level rhythmic handgrip contractions performed at 20% MVC for 3 min; a protocol that was designed to sensitise mechanoreceptors, evoked an increase in leg (peroneal nerve) MSNA activity which was abolished after COX inhibition with indomethacin (267). It was therefore argued that COX metabolites sensitise the mechanoreceptors that contribute to the exercise reflex. Subsequently, Cui et al. showed that progressive isometric handgrip exercise performed at 15% (1 min), 25% (1 min), and 30% MVC (until fatigue), followed by 2.5 min of PECO led to an increase in leg MSNA which was maintained during PECO; ketorolac attenuated these changes during exercise *and* during PECO (79). They therefore proposed that prostanoids contribute to the reflex activation of MSNA by stimulating metaboreceptors, as well as sensitising mechanoreceptors.

However, observations of a correlation between synthesis of COX products and reflex changes in MSNA and systemic cardiovascular responses are not a consistent finding. Doerzbacher and Ray (96) reported that oral administration of the COX inhibitor ketoprofen had no effect on the reflex increase in MSNA evoked either by isometric handgrip or rhythmic handgrip at 30% MVC

to exhaustion, even when PECO was maintained after exercise. As both, the release of PGs (29, 198, 447) and the increase in MSNA (158, 159, 421) during exercise are intensity-dependent, it seemed reasonable to investigate this relationship with handgrip contractions that are more intense (i.e., 60% MVC).

It has been shown that the pressor response to exercise is much greater when the arterial supply to the exercising limb is occluded, compared to unrestricted blood flow, suggesting either a direct role of $O_2$ or an $O_2$-dependency of metaboreceptor stimulating metabolites (5, 67). For example, Kaufman et al. showed that isometric contractions evoked in anesthetized cats under ischemic conditions compared to unrestricted blood flow resulted in greater stimulation of group IV afferents than group III afferents (although their activity also increased by ~ 12% under ischemia) (203). Similar results were obtained when rhythmic contractions were evoked in cats under ischemic and non-ischemic conditions (4, 174). The same group also showed that systemic hypoxia (3 – 5% $O_2$ for 3 – 3.5 min) led to greater stimulation of group IV afferents than group III at rest in anesthetized cats, despite no effect on venous pH and lactate efflux (174). Subsequent isometric contractions further increased the activity of group III and IV afferents (174).

These results are all consistent with the idea that activation of group IV afferents is COX and $O_2$-dependent. However, 100% $O_2$ has previously failed to attenuate the increase in peroneal MSNA of handgrip exercise (183, 361); possible reasons for this apparent disparity are discussed later in the Chapter. Nevertheless, following the results of Chapter 4, an investigation into the role of COX products and the $O_2$-dependency of metaboreceptor activation in exercise-evoked reflex vasoconstriction was warranted using COX inhibition and 40% $O_2$.

### 5.1.1 Aims and hypotheses

We hypothesized that the COX products (PGs and/or TX) produced in the exercising muscle during contractions at moderate intensity contribute to exercise-induced reflex vasoconstriction in resting limbs. We further hypothesized that their contribution to reflex vasoconstriction is $O_2$-dependent and would therefore be attenuated by breathing 40% $O_2$. We tested these hypotheses by recording the changes in vascular resistance of resting calf while subjects performed isometric handgrip contractions at 60% MVC for 2 min without and with 40% $O_2$ and COX inhibition. PECO was used to differentiate the effects of central command and mechanoreceptor activity from the effect of metaboreceptors.

## 5.2 Methods

Eleven young (18 – 21 years) men were recruited to participate in the study. All were healthy, recreationally active, non-smoking individuals. None were on medication for any medical conditions. All subjects were required to adhere to dietary, medicinal, and exercise restrictions as previously described in Chapter 2 (See Section 2.1). Administration of medical air or 40% $O_2$, and placebo or aspirin treatment along with measurements of MVC, TTI, HR, MABP, CBF, and CVR were done as described in Chapter 2.

### 5.2.1 Protocol

Subjects visited the laboratory on four occasions. The initial familiarization visit was used to complete health and activity questionnaire, consent form, familiarization of the subject with the protocol, and to record their MVC (See Section 2.3). The subsequent 3 experiment visits were carried out in a single-blind randomized manner. These visits involved administration of placebo (P) or aspirin (A) – both during air breathing and 40% $O_2$.

On experimental days, the handgrip dynamometer was set for the subject's MVC before the experiment began. On arrival, subject consumed the treatment drink (P or A; 600 mg) and then all recording equipment (CBF and MABP) was connected to the subject as previously described (See Section 2.5). As shown in Figure 5.1 and Figure 5.2, CBF was recorded from the ipsilateral leg. In order to ensure subjects did not move their leg / foot during the periods of rest or exercise, electromyograph activity was recorded with plate-electrodes connected via a dual bio-amp (FE135, AD Instruments) to the Power-lab. The electrodes were placed on tibialis-anterior and lateral-gastrocnemius; the area was always abraded, shaved, and cleaned before their placement.

After ~ 25 min of rest, the facemask was put on the subject and secured with elastic-straps which encircled the head to deliver medical air or 40% $O_2$. Subject was asked to breathe normally and after 5 min of gas inhalation, resting CBF measurements were made with venous occlusion plethysmography. The subject was then asked to perform an isometric handgrip contraction for 2 min at 60% MVC. As shown in Figure 5.3 (protocol schematic), CBF was measured at 1 min intervals for 6 min, beginning from the start of isometric handgrip contraction. However, instead of 2 and 4 min measurements, CBF was recorded at 1 min 45 s and 3 min 45 s. The gas flow was then turned off and the facemask was taken off while the subject rested for further 10 min. Following this, gas flow of either air or 40% $O_2$ was again turned on and resting CBF measurements were taken after 5 min. This was followed by a comparable isometric handgrip contraction and CBF measurements as just described. The only difference between the two periods of contraction was that PECO was applied following one of the contractions in a randomized manner. PECO was applied by inflating the sphygmomanometer cuff to > 240 mmHg around the upper arm of the exercising arm 5 s before the end of contraction; it remained inflated for 2 min 5 s. At the end of experiment, all recording equipment was disconnected and subjects were allowed to rest for 5 – 10 min before they left the laboratory.

### 5.2.3 Statistical analysis

Factorial ANOVA was used to identify time, treatment, exercise bout (i.e., NO PECO and PECO), and their interaction effects. The data of each treatment condition were also analyzed using 1-way repeated measures ANOVA to detect within time effects. Effect of exercise bout, treatment, and their interaction on exercise time and TTI were analyzed using 2-way ANOVA. Once a statistically significant effect was detected, Tukey's HSD (post-hoc test) was used to detect the exact point of statistical difference within and/or between conditions.

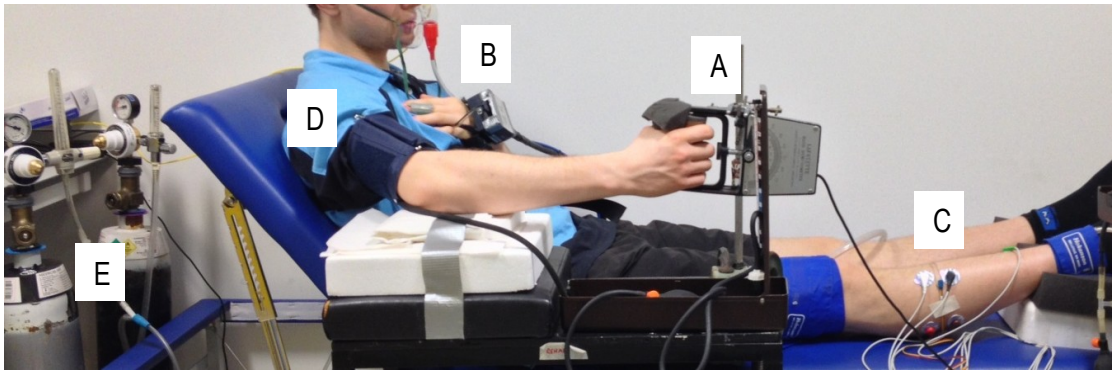



Figure 5.1 **Experiment setup showing arrangement of equipment**. Subjects performed isometric handgrip contraction using their dominant hand (A) while MABP and HR were recorded from contra-lateral hand (B). CBF was recorded from the ipsilateral side (C) using venous occlusion plethysmography. PECO (D) was applied using a sphygmomanometer cuff 5 s before cessation of exercise. Medical air and $O_2$ cylinders (E) were placed behind the subjects to keep them blinded to the treatment.

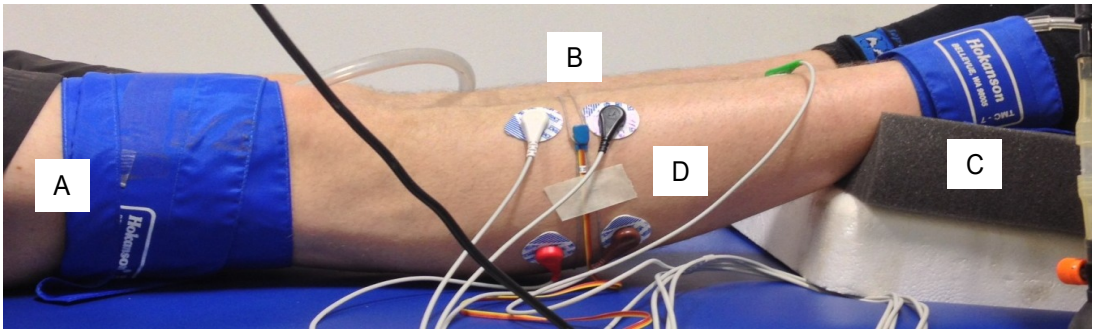



Figure 5.2 **Venous occlusion plethysmography and EMG electrodes**. Venous occlusion cuff (A) was inflated using an automatic rapid inflation system and the strain-gauge was placed around the widest part of the calf (B). In order to exclude foot circulation, a paediatric cuff was inflated above systolic pressure (C). Electrodes (D) were placed on the tibialis-anterior and lateral-gastrocnemius.

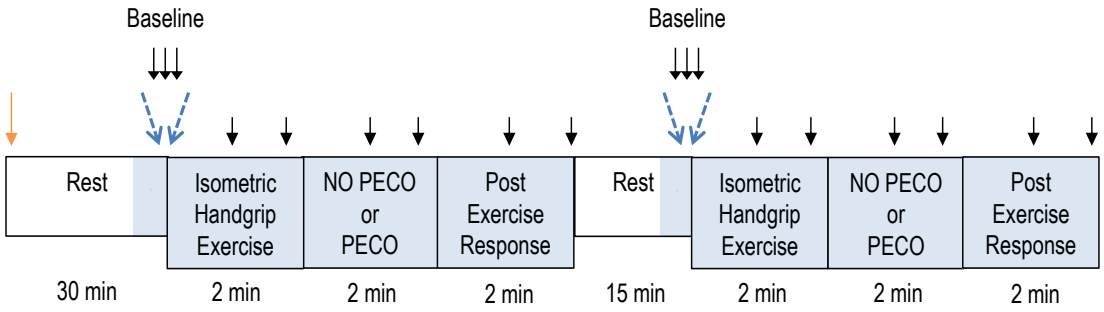



Figure 5.3 **Schematic diagram of the protocol**. Initial ↓ (orange) represents consumption of treatment drink (either P or with 600 mg A). ↓ (black) represents times at which CBF was measured and blue areas represent air or 40% $O_2$ inhalation. Air or $O_2$ flow was turned on 5 min before any CBF measurements were taken.

## 5.3 Results

Anthropometric data of individual subjects are shown in Table 5.1.

The TTI generated by subjects during isometric handgrip contraction is shown in Table 5.2; TTI were not different between treatments or between NO PECO and PECO conditions. All subjects managed to perform the isometric handgrip contraction at 60% MVC for the full 2 min and no difference was detected for duration between treatments during NO PECO or PECO conditions (Table 5.2).

As can be seen from Figure 5.4, HR increased significantly with contraction, but by 3 min *post*-contraction it returned to baseline during all treatments under NO PECO and PECO conditions (Figure 5.4; See above and below). Overall HR was different between treatment conditions; it was slightly higher with placebo than with aspirin and with 40% $O_2$ during both NO PECO and PECO (Figure 5.4). Further, MABP increased significantly during contraction in all treatment conditions of NO PECO and PECO (Figure 5.5). By 3 min (*post*-exercise) MABP had returned back to baseline during NO PECO; addition of PECO maintained the increase in MABP during all treatments (Figure 5.5; upper and lower panels) which returned to respective baselines upon its removal.

Table 5.3 and Table 5.4 show the absolute baseline CBF and CVR values during NO PECO and PECO conditions; no treatment effect was detected between them. Table 5.5 shows the absolute values for CBF and CVR before, during, and after isometric handgrip contractions performed at 60% MVC. Delta ($\Delta$) CVR was calculated by subtracting each CVR during contraction (and *post*-contraction) from the baseline CVR; these values are shown in Figure 5.6 and Figure 5.7. During NO PECO exercise bout, $\Delta$ CVR increased by ~ 12 and 21 RU at 1 min and 1 min 45 s respectively into contraction during placebo treatment. This increase in CVR was significantly

attenuated during contraction with both 40% $O_2$ and aspirin (Figure 5.6). During the *post*-exercise NO PECO period, $\Delta$ CVR was not different between treatments (Figure 5.6). However during PECO application, $\Delta$ CVR of placebo treatment still remained significantly elevated compared to both 40% $O_2$ and aspirin (Figure 5.7).

| | Age (yr) | Weight (Kg) | Height (m) | BMI (Kg/m$^2$) | MVC (N) | Calf C (cm) |
|---|---|---|---|---|---|---|
| | 21 | 80 | 1.76 | 25.83 | 245 | 37 |
| | 20 | 79 | 1.76 | 25.50 | 196 | 39 |
| | 20 | 64 | 1.61 | 24.69 | 147 | 36 |
| | 21 | 85 | 1.86 | 24.57 | 255 | 37 |
| | 18 | 65 | 1.82 | 19.62 | 216 | 30 |
| | 20 | 75 | 1.82 | 22.64 | 216 | 34 |
| | 19 | 60 | 1.68 | 21.26 | 177 | 34 |
| | 19 | 70 | 1.67 | 25.10 | 206 | 35 |
| | 20 | 59 | 1.70 | 20.42 | 245 | 32 |
| | 20 | 60 | 1.69 | 21.01 | 206 | 35 |
| | 21 | 80 | 1.79 | 24.97 | 196 | 42 |
| Mean | 19.91 | 70.64 | 1.74 | 23.24 | 209.58 | 35.55 |
| SEM | 0.28 | 2.87 | 0.02 | 0.69 | 9.56 | 0.99 |

Table 5.1 **Anthropometric data of subjects (n=11)**.

| | | P | 40% $O_2$ | A |
|---|---|---|---|---|
| TTI (N.s) | NO PECO | 12957.53±1096.85 | 13280.42±965.46 | 13046.89±908.72 |
| | PECO | 12664.50±1163.70 | 12991.93±1003.63 | 13281.40±889.08 |
| P Value | | Exercise bouts = *0.72* Treatment = *0.86* Exercise bout*Treatment = *0.97* | | |
| Time (s) | NO PECO | 120.55±0.42 | 121.06±0.33 | 120.77±0.35 |
| | PECO | 120.77±0.52 | 121.26±0.67 | 121.68±0.92 |
| P Value | | Exercise bouts = *0.34* Treatment = *0.55* Exercise bout*Treatment = *0.76* | | |

Table 5.2 **TTI and time recorded from NO PECO and PECO conditions while subjects performed isometric handgrip contractions at 60% MVC for 2 min**. TTI and time were not significantly different between treatments or the two exercise bouts (i.e., NO PECO and PECO).

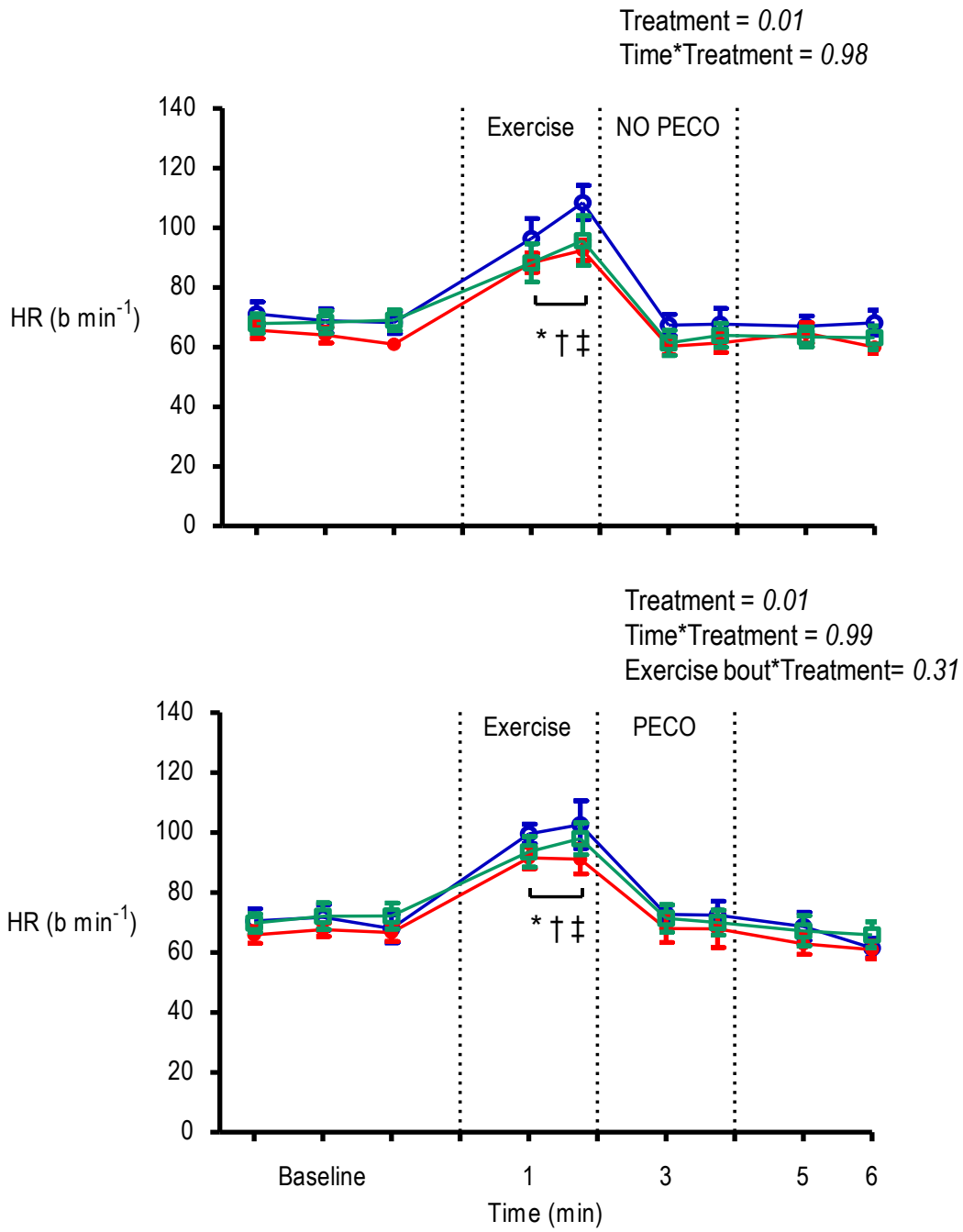



Figure 5.4 **HR responses to isometric handgrip contractions performed at 60% MVC for 2 min during NO PECO (upper panel) and PECO conditions (lower panel)**. Significant treatment effect was detected between placebo (blue), 40% $O_2$ (red), and aspirin (green; vs. placebo). */†/‡ show placebo, 40% $O_2$, and aspirin values significantly different from their respective baselines ($P < 0.001$; i.e., time effect). This includes all time points within the brackets.

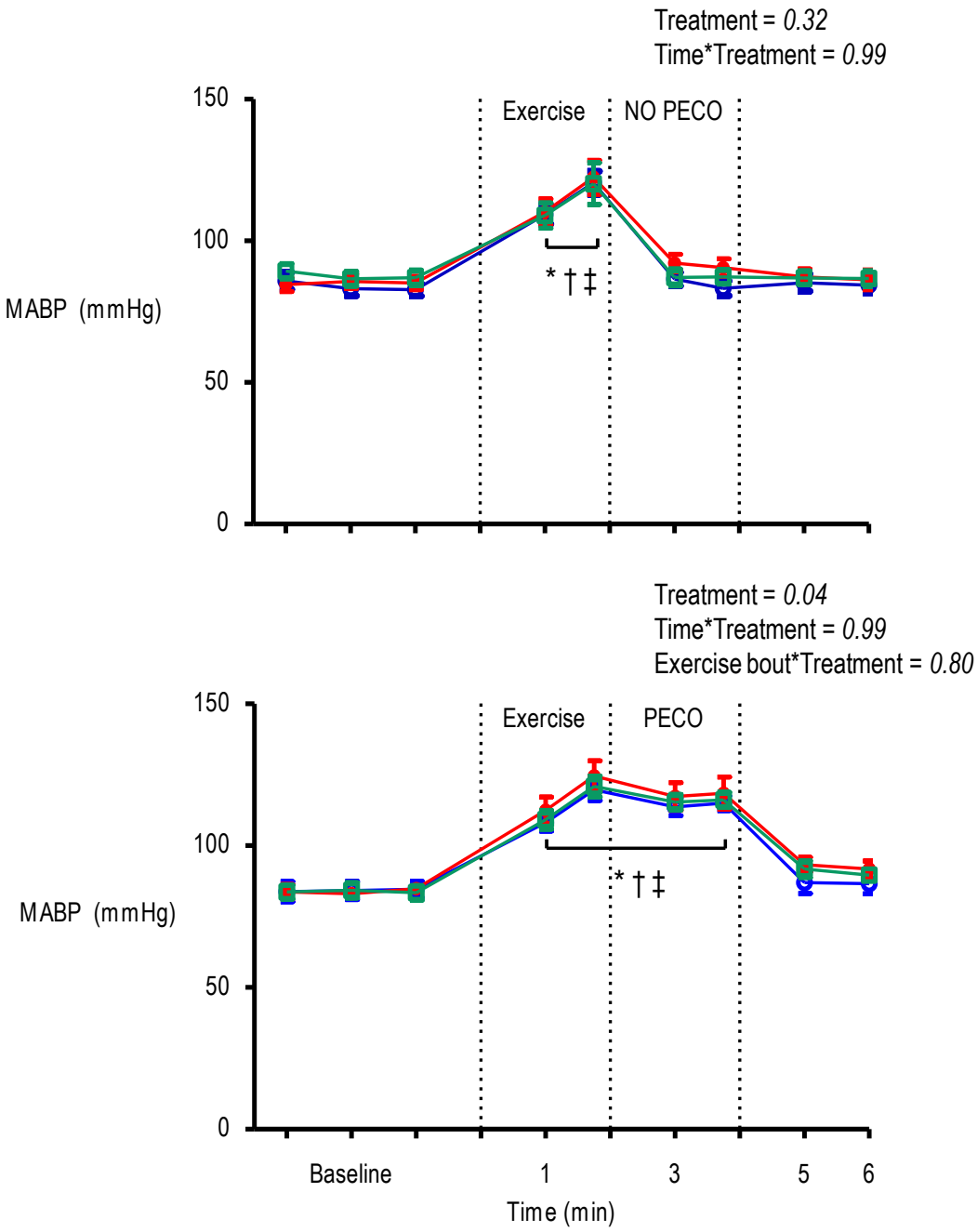



Figure 5.5 **MABP responses to isometric handgrip contractions performed at 60% MVC for 2 min during NO PECO (upper panel) and PECO conditions (lower panel)**. No treatment effect was detected between placebo (blue), 40% $O_2$ (red), and aspirin (green) during NO PECO but overall MABP was slightly higher with interventional treatments during PECO conditions ($P < 0.05$ vs. placebo). */†/‡ show values significantly different ($P < 0.001$) from respective baselines (placebo, 40% $O_2$ and aspirin, respectively). This includes all time points within the brackets.

| CBF (ml $dl^{-1}$ $min^{-1}$) | Treatment | Baseline 1 | Baseline 2 | Baseline 3 | Mean | P Value |
|---|---|---|---|---|---|---|
| NO PECO | P | 6.34±0.72 | 5.57±0.75 | 5.03±0.54 | 5.65±0.65 | *0.46* |
| | 40% $O_2$ | 4.21±0.72 | 3.93±0.49 | 4.23±0.59 | 4.12±0.57 | *0.97* |
| | A | 6.15±0.94 | 5.37±0.88 | 5.34±0.89 | 5.62±0.89 | *0.80* |
| P Value | Treatment = *0.23* | | | | | |
| PECO | P | 6.05±0.76 | 5.60±0.73 | 5.56±0.77 | 5.74±0.70 | *0.87* |
| | 40% $O_2$ | 4.94±0.87 | 4.70±0.87 | 4.44±0.75 | 4.65±0.78 | *0.91* |
| | A | 5.38±0.67 | 4.97±0.73 | 4.73±0.73 | 5.03±0.69 | *0.77* |
| P Value | Treatment = *0.58*<br>Exercise bout = *0.99* Exercise bout*Treatment = *0.74* | | | | | |

Table 5.3 **Baseline CBF responses during NO PECO and PECO conditions**. No time or treatment affect was found between them.

| CVR (RU) | Treatment | Baseline 1 | Baseline 2 | Baseline 3 | Mean | P Value |
|---|---|---|---|---|---|---|
| NO PECO | P | 15.73±2.24 | 17.65±2.20 | 18.37±1.87 | 17.25±1.20 | *0.66* |
| | 40% $O_2$ | 23.03±4.52 | 24.18±4.50 | 24.25±2.45 | 23.82±2.26 | *0.78* |
| | A | 18.01±2.47 | 19.90±2.54 | 19.59±2.49 | 19.17±1.40 | *0.85* |
| P Value | Treatment = *0.28* | | | | | |
| PECO | P | 16.27±2.12 | 18.12±2.58 | 18.17±2.84 | 17.52±1.41 | *0.83* |
| | 40% $O_2$ | 21.14±4.36 | 22.92±5.25 | 23.26±4.63 | 23.77±2.64 | *0.67* |
| | A | 18.02±2.41 | 20.36±2.80 | 21.04±2.63 | 19.81±1.48 | *0.70* |
| P Value | Treatment = *0.34*<br>Exercise bout = *0.77* Exercise bout*Treatment = *0.99* | | | | | |

Table 5.4 **Baseline CVR responses during NO PECO and PECO conditions**. No time or treatment effect was found between them.

| Condition | | Treatment | Mean Baseline | Exercise | | NO PECO / PECO | | Rest | | P Value |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 min | 1 min 45 s | 3 min | 3 min 45 s | 5 min | 6 min | |
| NO PECO | CBF (ml $dl^{-1}$ $min^{-1}$) | P | 5.65±0.65 | 4.53±1.13 | 3.84±0.85 | 4.87±0.42 | 4.71±0.38 | 5.07±0.40 | 5.05±0.52 | *0.59* |
| | | 40% $O_2$ | 4.12±0.57 | 5.03±0.90 | 5.01±0.80 | 4.00±0.48 | 3.87±0.24 | 4.53±0.59 | 4.23±0.56 | *0.70* |
| | | A † | 5.62±0.89 | 6.45±0.94 | 6.81±1.23 | 5.45±0.54 | 4.90±0.63 | 4.50±0.49 | 4.96±0.72 | *0.34* |
| | P Value | †Treatment = *0.01* Time*Treatment = *0.52* | | | | | | | | |
| | CVR (RU) | P | 17.25±1.20 | 29.81±4.99 * | 38.82±7.99 * | 19.61±2.53 | 18.98±1.94 | 17.64±1.40 | 18.94±2.37 | < *0.001* |
| | | 40% $O_2$ | 23.82±2.26 | 29.79±6.16 | 29.16±4.95 | 26.20±3.39 | 23.88±1.19 | 22.55±3.17 | 23.29±3.02 | *0.69* |
| | | A † | 19.17±1.40 | 19.20±3.12 | 19.30±2.74 | 18.17±1.77 | 19.44±2.23 | 21.62±3.03 | 21.25±3.12 | *0.97* |
| | P Value | † Treatment = *0.004* Time*Treatment = *0.08* | | | | | | | | |
| PECO | CBF (ml $dl^{-1}$ $min^{-1}$) | P | 5.74±0.70 | 4.22±0.82 | 3.87±0.89 | 4.18±0.63 | 3.87±0.34 | 5.23±0.77 | 5.12±0.73 | *0.42* |
| | | 40% $O_2$ † | 4.65±0.78 | 5.27±1.14 | 5.73±0.99 | 4.93±0.60 | 5.19±1.11 | 4.63±0.71 | 4.91±0.57 | *0.99* |
| | | A † | 5.03±0.69 | 7.25±1.43 | 6.90±1.21 | 5.80±0.69 | 6.41±1.03 | 4.91±0.55 | 4.91±0.44 | *0.34* |
| | P Value | † Treatment = *0.01* Exercise bout = *0.25* Time*Treatment = *0.41* Exercise bout*Treatment = *0.32* | | | | | | | | |
| | CVR (RU) | P | 17.52±1.41 | 29.88±3.42 * | 38.82±9.82 * | 31.27±4.21 * | 31.02±2.38 * | 21.26±3.57 | 20.47±2.75 | *0.01* |
| | | 40% $O_2$ | 23.77±2.64 | 28.10±5.58 | 25.53±3.96 | 26.38±4.32 | 28.93±4.24 | 25.62±4.31 | 23.93±3.28 | *0.92* |
| | | A † | 19.81±1.48 | 20.95±3.95 | 21.67±3.03 | 22.36±2.73 | 21.45±2.52 | 20.61±2.42 | 19.26±1.66 | *0.99* |
| | P Value | † Treatment = *0.003* Exercise bout = *0.07* Time*Treatment = *0.40* Exercise bout*Treatment = *0.29* | | | | | | | | |

Table 5.5 **CBF and CVR responses to isometric handgrip contractions performed at 60% MVC for 2 min during NO PECO and PECO conditions**. *,† represent time effect (i.e., vs. respective baseline) and over-all treatment effect (i.e., vs. placebo) respectively.

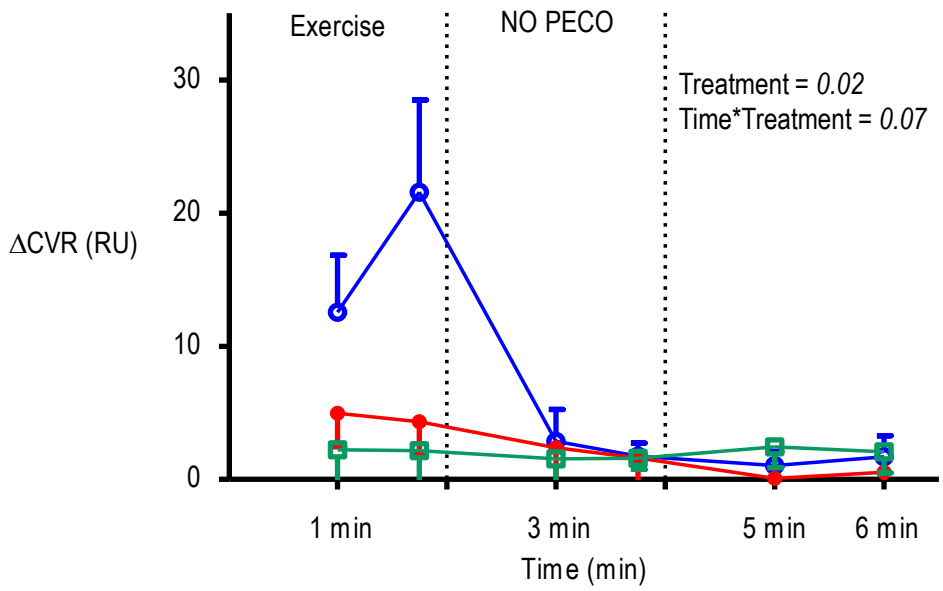



Figure 5.6 Δ **CVR responses to isometric handgrip contractions performed at 60% MVC for 2 min during NO PECO trial**. Treatment effect was detected during period of exercise between placebo (blue), 40% $O_2$ (red), and aspirin (green).

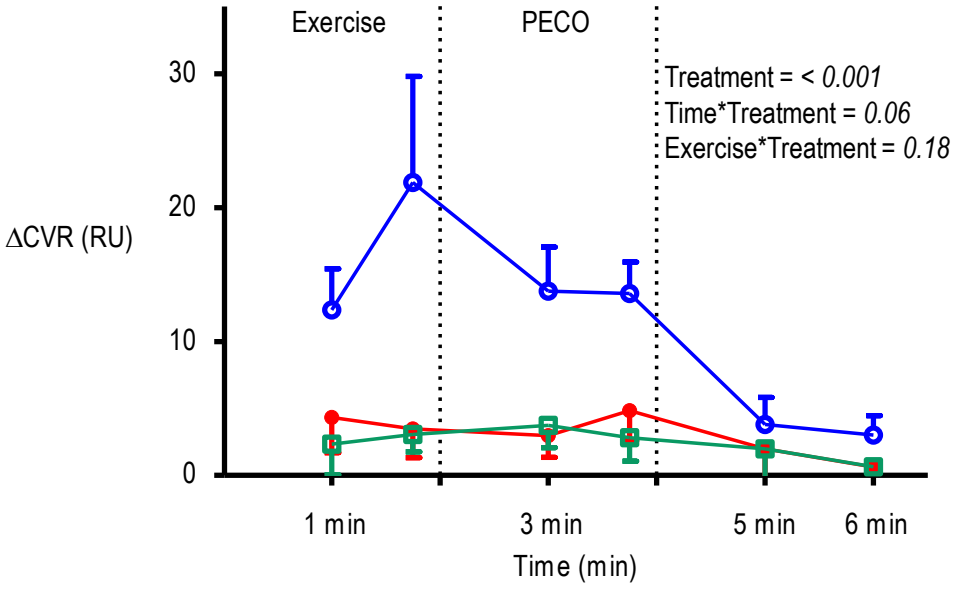



Figure 5.7 Δ **CVR responses to isometric handgrip contractions performed at 60% MVC for 2 min during PECO trial**. Treatment effect was detected during exercise and during PECO between placebo (blue), 40% $O_2$ (red), and aspirin (green).

## 5.4 Discussion

There are two novel findings of the present study. Firstly, the results of the study indicate that during isometric handgrip contraction, COX products produced in the exercising arm contribute towards exercise-evoked reflex vasoconstriction in the resting calf and secondly, that their contribution to this vasoconstriction is $O_2$-dependent.

### 5.4.1 Systemic cardiovascular responses

Concentrations of PGs in the skeletal muscle interstitium increase during exercise (198, 199, 302, 390, 435) and their presence contributes towards the exercise pressor reflex (79, 207, 231, 347). The exercise-related increase in HR is more widely believed to be under the influence of central autonomic pathways (119, 123). However, Leal et al. noticed that thromboxane-receptor activation, the same receptors that are stimulated by COX products (141), led to an increase in HR and MABP while their inhibition attenuated this response (231). Moreover, the isometric exercise-related increase in HR was attenuated by receptor inhibition but that associated with muscle stretch remained intact. The increase in MABP however was attenuated by receptor inhibition during both exercise and muscle stretch.

Based on such observations, COX blockade in the present study might have been anticipated to attenuate the systemic MABP, MSNA, and HR responses by inhibiting the release of COX products that stimulate afferents in contracting muscle. Therefore, the small attenuation of the increase in HR that occurred *during* muscle contraction following COX blockade and during 40% $O_2$ is consistent with the idea that mechano- and metaboreceptors are stimulated by PGs that are released in the contracting muscles (347). This does not take away from the observations of Fisher and White that the increase in HR tends to be greater with voluntary isometric contractions than those evoked by electrical stimulation (119).

It is somewhat surprising that attenuation of the HR response was not observed in the experiments of Chapter 4 when a comparable dose of aspirin and 40% $O_2$ were used. However, as the effect of aspirin and $O_2$ on the HR response was relatively small, the disparity may simply reflect variability between subject groups. The decrease in HR that occurred immediately after cessation of handgrip exercise both during NO PECO and PECO is consistent with the results of Chapter 4 and with the proposal that the influence of vagal tone on HR returns immediately after cessation of contractions (67, 255). It is not surprising that HR was not affected by aspirin or $O_2$ during this period.

The fact that the increase in MABP was not significantly different between treatments during the forearm contraction is also not surprising given it reflects the combined effects of changes in HR, stroke volume, and TPR (29, 74). This result corroborates the findings of the previous Chapter and other published data (74, 437). However during PECO, overall MABP was slightly higher with aspirin and 40% $O_2$. The slight increase during PECO trial possibly reflects a contribution of ROS generated within the hypoxic / ischemic arm to the pressor reflex (51, 62, 245).

### 5.4.2 Calf vasoconstriction

Baseline CBF and CVR were not affected by aspirin or 40% $O_2$. However, the idea that COX products contribute towards the exercise pressor reflex by stimulating muscle afferents is supported by our novel findings on the $\Delta$ CVR evoked by forearm contraction. The present findings showed that COX blockade with aspirin significantly attenuated the increase in CVR during the 2 min period of isometric handgrip exercise. PECO exercise bout's MABP and $\Delta$ CVR data shows that central command and/or mechanoreceptors contribute to exercise-evoked vasoconstriction. However, as the application of PECO excludes the contribution of mechanoreceptors and central command from the observed responses, the fact that the $\Delta$ CVR

was maintained during the 2 min of PECO and this effect was attenuated by aspirin indicates that COX products produced during exercise (390, 435, 447, 448) accumulate during this period, stimulate metaboreceptors (79, 346), and thereby contribute to the reflex vasoconstriction. The second novel finding that a similar attenuation of the increase in $\Delta$ CVR occurred with 40% $O_2$ is consistent with the finding of Kaufman et al. that not only were group III and IV afferents stimulated by AA (347) and their activity attenuated by COX blockade (346), but they were also stimulated under hypoxic and ischemic conditions (4, 174, 203). Taken together with the results of Chapter 4, the present results are consistent with the hypothesis that breathing 40% $O_2$ also attenuated the reflex vasoconstriction by inhibiting the production of $O_2$-dependent PGs in the contracting forearm.

Before accepting this hypothesis, alternative explanations for the present findings should be considered. Firstly, it might be argued that systemic COX blockade attenuated the calf vasoconstriction response by affecting the ability of an increase in resting calf's MSNA. However, previous studies have shown that PGs that are locally synthesised and released blunt the evoked sympathetic vasoconstriction; i.e., local PG concentrations and sympathetic vasoconstriction are inversely correlated with each other (362). Further, there is evidence that locally synthesised PGs inhibit the release of noradrenaline from sympathetic varicosities (385). Thus if either of these mechanisms were dominant during forearm contraction, then COX inhibition with aspirin would have been expected to augment, rather than attenuate the vasoconstriction in calf. Secondly, it might be argued that breathing 40% $O_2$ attenuated the calf vasoconstriction by unloading peripheral chemoreceptors and reducing their contribution to the reflex activation of MSNA (386, 387). Although this cannot be completely ruled out, the observation that $\Delta$ CVR was attenuated to similar extent with 40% $O_2$ and COX inhibition supports the contribution of $O_2$-dependent COX products to exercise-evoked reflex

vasoconstriction. Moreover, if hyperoxia were the sole factor responsible for attenuating peripheral sympathetic activity, reflex vasoconstriction during PECO with 40% $O_2$ would not have increased over time (3 min vs. 3 min 45 s; Figure 5.7).

It may be noted that in an apparent contrast with the present results, Houssiere et al. reported that breathing 100% $O_2$ throughout isometric handgrip at 30% MVC augmented the evoked increase in systemic MABP and MSNA (peroneal nerve) (183). Further, Seals et al. showed that breathing 100% $O_2$ decreased MSNA at rest but not the increase evoked by rhythmic handgrip exercise (0.5 Hz; 50% MVC) (361). However, breathing 100% $O_2$ has been shown to increase ROS generation and to decrease baseline blood flow and vascular conductance at least in part by decreasing the vasodilator contributions of NO and PGs (75, 256). Moreover, centrally-generated and reflex changes in sympathetic activity are known to be influenced by ROS molecules (51). Therefore, it seems likely that the effects of 100% $O_2$ on exercise-evoked increase MSNA reported by Houssiere et al. (183) and Seals et al. (361) reflect the combined outcome of increased ROS concentrations acting centrally and peripherally.

If it is assumed that breathing 40% $O_2$ in the present study did not increase ROS generation, the most obvious interpretation of the results is as hypothesised that $O_2$-dependent PGs that are released in the contracting forearm, stimulate muscle afferents, including mechano and metaboreceptors to contribute to the reflex increase in MSNA that causes vasoconstriction in the resting calf. The issue of whether 40% $O_2$ generates ROS is addressed in Chapter 8.

In summary, Chapter 4 showed that PG production during exercise is $O_2$-dependent. The results of the present Chapter extend these observations to provide indirect evidence that COX products released during handgrip contraction contribute to reflex vasoconstriction in the ipsilateral leg in an $O_2$-dependent manner. Therefore, the hypotheses proposed are accepted.

### 5.4.3 Limitations and Future Work

The principal limitation to this study is that the conclusions are based only on evoked changes in CVR. Although, Chapter 4 shows that the production of vasodilator PGs in the exercising limb is indeed $O_2$-dependent, nonetheless, had venous samples been taken in the present study, they could have shed light on actual concentrations of PGs and TXs in both the exercising and resting limbs. Moreover, as this study proposes that the observed vasoconstriction response was a reflection of an attenuated exercise-evoked increase in MSNA; neural recordings from the resting leg (peroneal) during NO PECO and PECO would have further elucidated the role of $O_2$-dependent COX products to exercise-evoked vasoconstriction in the resting muscles. Any future studies should address these issues.

Lastly, it was observed that the increase MABP and $\Delta$ CVR decreased following cessation of voluntary isometric exercise *during* PECO. Nonetheless, they still remain elevated during *post*-exercise PECO compared to the *post*-exercise NO PECO; reflecting the contribution of metaboreceptors and perhaps some minor contribution from the metabolite sensitized mechanoreceptors (79, 267, 347, 372). However, had the protocol been repeated with additional electrically-stimulated muscle contractions, such experiments would have separated the contribution of central command from the mechano- and metaboreceptor contributions of the observed responses *during* exercise. These additional experiments would have helped further clarify the influence of $O_2$-dependent COX products on exercise-evoked vasoconstriction.

# Chapter 6

# Effects of 40% $O_2$ and COX blockade on muscle oxygenation and haemodynamics in exercising and resting arms of young and older men

## 6.1 Introduction

The results of previous two Chapters raised a number of questions.

It was discussed previously that exercise was associated with a decline in intra-muscular (337-339) and peri-venular (226) $PO_2$. As the release of PGs is associated with both, the decline in $PO_2$ (45, 46, 264, 266) and exercise (198, 199, 302, 390, 435), it seemed logical that the exercise-related increase in vasodilator PGs might be $O_2$-dependent. The results of Chapter 4 indeed showed that to be true; the venous efflux of both $PGE_2$ and $PGI_2$ evoked by either rhythmic or isometric handgrip contractions performed at 60% MVC was $O_2$-dependent. However, these observations raised questions regarding tissue oxygenation / local circulation under the treatment conditions. The profound decline in muscle oxygenation from ~ 34 to ~ 3 mmHg observed by Richardson et al. occurred within a few seconds of dynamic knee-extension exercise in both trained and untrained individuals (337-339). However, when muscle contraction is isometric in nature, an exercise model which is traditionally understood to compromise tissue perfusion (22, 234), more questions are raised regarding the local tissue oxygenation / perfusion during this period. Such exercise might be expected to further enhance the decline in local tissue oxygenation. However, the results of Chapter 4 failed to observe any major difference in the efflux of $O_2$-dependent $PGE_2$ and $PGI_2$ between the two exercise types. This must imply that besides an increase in muscle-metabolism / $VO_2$ (211), tissue perfusion increases during isometric exercise such that it provides adequate oxygenation for the period of exercise.

Fordy and Marshall had noticed attenuation in the *post*-exercise hyperaemia of isometric contraction with 40% $O_2$ only when it was breathed *during* the period of exercise (123). Moreover, as lactate efflux was attenuated if subjects breathed 40% $O_2$ *during* isometric exercise, their results not only imply that tissue perfusion is relatively maintained during isometric

exercise but in contrast to the earlier conclusion, this hyperaemia is not sufficient to meet the demands to tissue oxygenation. Together with the results of Chapter 4 it can be assumed that 40% $O_2$ alleviates this "decline" in tissue oxygenation. However, if the influence of 40% $O_2$ on attenuating exercise-related PG production is to be better understood, this relationship clearly needed further investigating.

A separate question is raised regarding the affect of COX inhibition on tissue oxygenation and local circulation. COX inhibitors attenuate exercise and *post*-exercise hyperaemia (73, 74, 100, 209, 437), an effect that by itself would be expected to cause an even greater decline in tissue oxygenation, than during air breathing conditions. Some evidence for this assumption lies in the observation that vasoconstriction brought on by vasopressin and noradrenaline is associated with an increase in arteriolar $VO_2$ (443). However this issue is complicated by the observations that COX inhibition attenuates skeletal muscle mitochondrial respiration (28, 222, 270, 281). Therefore, if exercise-related $O_2$-dependent production of COX products is to be understood, the impact of COX inhibition on isometrically exercising muscle's oxygenation / $VO_2$ and its perfusion clearly warrant investigation.

Lastly, the results of Chapter 4 showed that despite some decline in the contribution of $PGE_2$ and $PGI_2$ to exercise hyperaemia with aging, their venous efflux was not attenuated. This is a particularly interesting observation considering the age-associated change in the predominance of skeletal muscle fibre types (i.e., change in the proportion and/or size of fibres favouring slow-oxidative type) (408). Aniansson et al. (8) recorded no change in the vastus-lateralis and biceps-brachii enzymatic activity of triose-phosphate-dehydrogenase, 3-hydroxy-CoA-dehydrogenase, and citrate-synthase in older men who remained active over the years. This suggests that under the particular circumstances of isometric exercise, $VO_2$ of healthy recreationally active older individuals ought to be greater. This is supported by the Kime et al. who noticed that the

exercise-related $VO_2$ was greater in oxidative fibres (211). However maintenance of the predominance of type-1 fibres with age, even in legs, is not a consistent finding (129). Separately, it is unclear whether age itself equally affects the activity patterns of human arms and legs, but aging has been correlated to the decrease in activity / preference of the dominant arm, compared to the non-dominant one (197). Therefore, it is possible that age-associated decline in activity is not just restricted to legs; a hypothesis indirectly supported by the observation that in men, age associated decline in unit muscle mass strength occurs similarly in arms and legs (240). Therefore, if $O_2$-dependent production of vasodilator PGs is to be better appreciated in older, recreationally active individuals, the effect of exercise on local oxygenation and tissue perfusion required investigation.

The results of Chapter 5 also raised their own questions that require addressing. It is clear from previous studies that metabolites, particularly COX products stimulate the mechano and metaboreceptors to elicit reflex increase in sympathetic vasoconstriction (207, 261, 262, 267, 347, 372). However, the results of the previous two Chapters indicate that the COX products of exercise are produced in an $O_2$-dependent manner and then elicit an $O_2$-dependent reflex vasoconstriction response, at least in young men. These results raise a question regarding the tissue perfusion profile in resting muscles going through the exercise-evoked vasoconstriction.

Another question is raised regarding the exercise-evoked vasoconstriction in healthy older men. Studies have indicated that plasma concentrations of noradrenaline (sympathetic neurotransmitter) increase with age (190, 446), raising the possibility there may be an age-related exaggeration in the exercise pressor reflex. For example, Taylor et al. noticed that during dynamic cycling exercise at 45, 65, and 85% $VO_2$-max, the vasoconstriction evoked in the forearm and the venous efflux of noradrenaline were greater in older than younger men (399). Similarly, Momen et al. showed that fatiguing isometric handgrip contractions at 40% MVC led to

an increase in renal vascular resistance in older subjects that was two-fold greater than that observed in young subjects; application of PECO maintained ~ 50% of this response (275, 276). However, other investigations have revealed conflicting results. Roseguini et al. observed no difference in the calf vasoconstriction evoked by 30% MVC isometric handgrip between young and older men (342) while Ng et al. failed to observe any age-related difference in the unit increase in MSNA evoked by fatiguing isometric handgrip at 40% MVC (299). On the other hand, Markel et al. observed attenuated increases in peroneal MSNA in older individuals compared with young, when they performed incrementally ischemic rhythmic handgrip contractions at 30% MVC (247).

It is possible that the results obtained in some of these studies reflect the intensity-dependent production (29, 198, 447) of mechano / metaboreceptor stimulating (207, 262, 267, 347) COX products. Indeed, Hansen et al. also showed that in contrast to rhythmic handgrip exercise performed at 45% MVC, lower intensity contractions did not evoke sympathetic activation in peroneal nerve (158). Thus, it may be that the presence or magnitude of a mechano / metaboreceptor-mediated exercise pressor reflex in the elderly is dependent on the intensity of exercise performed. Therefore, extending the results of Chapter 5, the study described in this Chapter sought to investigate the contribution of $O_2$-dependent factors and COX products to the vasoconstriction evoked by isometric handgrip contractions performed at 60% MVC in both age groups.

### 6.1.1 NIRS

Over the years NIRS has become an important tool for measuring tissue perfusion and oxygenation in a non-invasive manner. The technique was forged in 1930s by Millikan who developed the dual-wavelength oximeter for muscles (268) and Jobsis who noticed the

differential absorption of light by haemoglobin (189). The principle of the technique is similar to the conventional photo-plethysmography, pulse-oximetry and laser-Doppler; calculations are made based on the difference between emitted and detected light which reflects the property / quantity of the absorbing material. The range over which NIRS operates is between 700 - 1100 nm, allowing deeper penetration of photons into the tissue (189, 292). Importantly, it is within this range that haemoglobin and myoglobin are detected; their concentration and oxygenation state determines light absorption (i.e., the difference between emitted and detected light) (292). The principal mathematical foundation for the technique is the Beer-Lambert law; light passing through a compound is absorbed, decreasing the intensity of the detected light (152, 292, 308). However as light is also scattered in tissue and the exact path-length of a photon is not always known, the NIRS equipment uses algorithms of modified Beer-Lambert law (308). The modern NIRS machines such as the one used in the present study (NIRO 200NX; Hamamatsu Photonics) also employ spatially-resolved spectroscopy, method which reliably measures the relative concentrations by using the two light detection probes that are a known distance apart from one another (152). In the present study, the light emission and detection probes used were 4 cm apart and as the penetration depth of the NIRS light is roughly half the distance between them (152, 416), this allowed for penetration depth of ~ 2 cm. Recordings were made of the tissue oxygenation index (TOI) which reflects tissue $O_2$ saturation, total haemoglobin index (THI) which provides an index of local blood flow and deoxy-haemoglobin (HHb) which reflects tissue $O_2$ uptake (145, 427).

### 6.1.2 Aims and hypotheses

Apart from examining the effect of the physical act of contraction on muscle perfusion; the present study investigated the affect of interventional treatments on muscle haemodynamics and tissue oxygenation *during* a period of isometric handgrip contraction performed at 60% MVC.

The effect of such isometric contraction on exercise-evoked vasoconstriction in young and older men has not been compared. Thus, the study also investigated the contribution of $O_2$-dependent COX products to this vasoconstriction in the contralateral (resting) arm in both age groups.

Firstly, in relation to the results of Chapter 4, we hypothesized that 40% $O_2$ and aspirin would similarly attenuate the hyperaemia that occurs *during* isometric handgrip contraction in both age groups, but would perhaps have different effects on tissue oxygenation; i.e., $VO_2$ responses during COX inhibition would be attenuated despite a decrease in tissue perfusion. Moreover, we hypothesized that isometric handgrip contractions at 60% MVC does not completely occlude the muscle perfusion of the contracting muscle in either age group. Lastly, we hypothesized that despite different tissue oxygenation profiles, exercise-evoked vasoconstriction in resting arms of both young and older men is similarly mediated by $O_2$-dependent COX products.

## 6.2 Methods

Ten young (18 – 25 years) and eleven older (65 – 78 years) men were recruited; all were recreationally active non-smoking adults (two older subjects quit smoking over forty years ago). Medication history included occasional use of salbutamol by two young subjects, occasional use of cetirizine by one older subject, and the use of statins and low-dose aspirin by two older men. Supplemental medications included use of multivitamins by two older subjects, glucosamie by one older subject while seven young and ten older men reported consuming either oily-fish or fish-oil supplements at least once a week. All subjects were required to adhere to dietary, medicinal, and exercise restrictions previously described in Chapter 2 (See Section 2.1): older subjects on statins and low-dose aspirin voluntarily offered and agreed to go without medication for 10 days prior to experiments. Subjects were also asked to stop taking multivitamins / fish-oil for at least 7 days prior to the experiments. Administration of air or 40% $O_2$, and placebo or aspirin along with measurements of MVC, TTI, HR, MABP, FBF, and FVC were done as described in Chapter 2.

### 6.2.1 Protocol

Subjects visited the laboratory on three occasions. The initial visit was used to complete the health and activity questionnaire, consent form, to familiarize the subject with the experimental protocol, and to record the subject's MVC (See Section 2.3). The following two experimental visits were carried out in a single-blind, partially randomized manner: the randomized visits were placebo (P) and aspirin (A); with the 40% $O_2$ always following the air breathing handgrip trial (See Figure 6.1 for protocol's schematic).

On experimental days, handgrip dynamometer was set up for the subject's MVC. On arrival, the subject consumed the treatment drink (P or A; 600 mg). This dose of aspirin has previously been

shown to affectively inhibit the COX enzyme for 30 – 90 min (161). After cleaning the skin with alcohol wipes and allowing the area to dry, NIRS probes were then placed on the cleaned forearm sites over flexor digitorum-superficialis of both exercising and resting arms to record local muscle oxygenation and hemodynamic responses: black plastic sheets were used to protect the probes from external light (Figure 6.2 and Figure 6.3). The recording equipment for HR and MABP was then connected to the subject's non-dominant hand (See Chapter 2). After ~ 25 min of rest, the facemask was put on the subject to deliver medical air and made secure with elastic-straps around the head. The subject was asked to breathe normally and after 5 min, they performed isometric handgrip contractions for 2 min at 60% MVC. Following the cessation of exercise, the recordings of systemic cardiovascular and muscle hemodynamic variables were continued for a further 2 min. Gas flow was then ceased and the facemask was removed; the subject rested for a further 25 min. Following this, gas flow of 40% $O_2$ was turned on and after 5 min was followed by a comparable isometric handgrip contraction. *Post*-exercise responses were again measured for a further 2 min; following which all recording equipment was disconnected. The subject rested for 5 – 10 min before leaving the lab. Figure 6.4 shows an example of NIRS and ABP trace recordings while a subject performed isometric handgrip exercise at 60% MVC for 2 min.

### 6.2.2 Statistical analysis

NIRS and TTI data were collected in 5 s sections; THI and HHb reflect relative changes from an arbitrary point-zero (0 s), which lies immediately prior to the start of 1 min of baseline recording. TOI was expressed in percentage where 1 V represents 100% saturation, THI was expressed in arbitrary units (AU) where 1 V represents 1 AU, and HHb was expressed in µmol.cm where 1 V of NIRS signal represents 200 µmol.cm. TTI of the full 2 min of contraction was also collected as explained earlier. Factorial mixed-model ANOVA was used to identify time, treatment, forearm,

age, and their interaction effects. The data of each treatment condition were also individually analyzed using 1-way repeated measures ANOVA to detect within time effects. Once a statistically significant effect was detected, Tukey's HSD was used as a post-hoc test to detect the exact point of statistical difference within and/or between conditions.

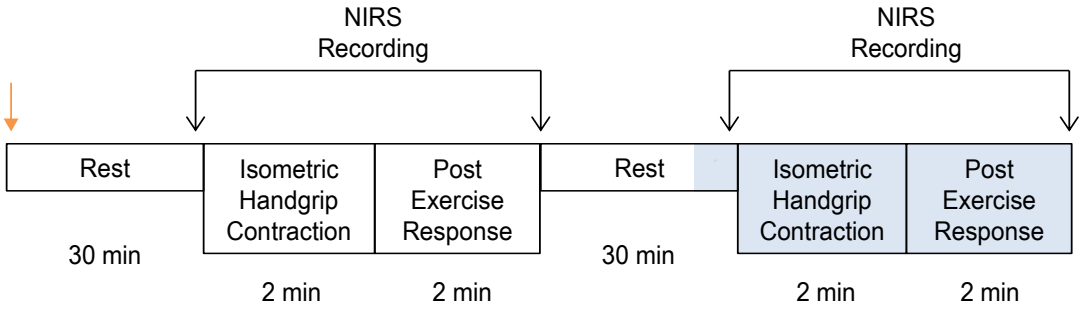



Figure 6.1 **Schematic diagram of the protocol.** Initial ↓ (orange) represents the consumption of treatment drink (P or A), black ↓ represents the duration during which cardiovascular and hemodynamic responses were measured (1 min baseline, 2 min *during* exercise, and 2 min *post*-exercise); air treatment always preceded the 40% $O_2$ trial (blue). Gas flow through the mask was always turned on 5 min prior to the start of isometric handgrip contractions (60% MVC; 2 min).

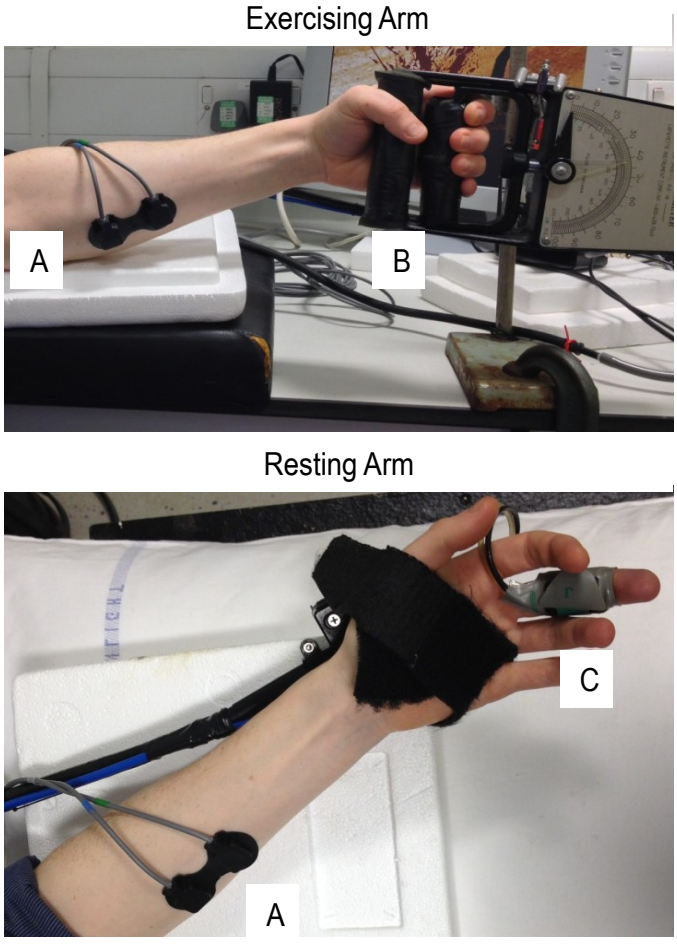



Figure 6.2 **Arrangement of the NIRS probes (upper and lower panel).** NIRS probes (A) were placed over the flexor digitorum-superficialis on both exercising and resting forearms. Isometric handgrip contractions (60% MVC; 2 min) were performed using a handgrip dynamometer (B) while systemic cardiovascular responses were measured from the resting hand (C) using Finapres.

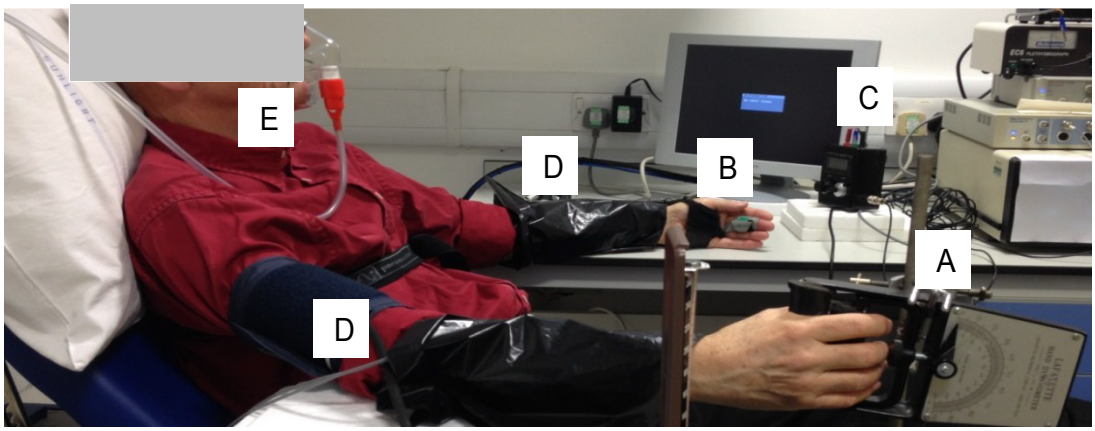



Figure 6.3 **Experiment setup showing the arrangement of the equipment.** Subjects performed isometric handgrip contraction using their dominant hand (A) while MABP and HR were recorded from contra-lateral hand (B); both arms rested at the level of the heart. Voltage monitor (C) was used as a visual aid to help with the generating and maintain the appropriate level of force. Muscle hemodynamic responses were measured using NIRS; probes were covered using black sheets (D). Air or 40% $O_2$ were delivered via facemask (E) to keep the subjects blinded to the treatment.

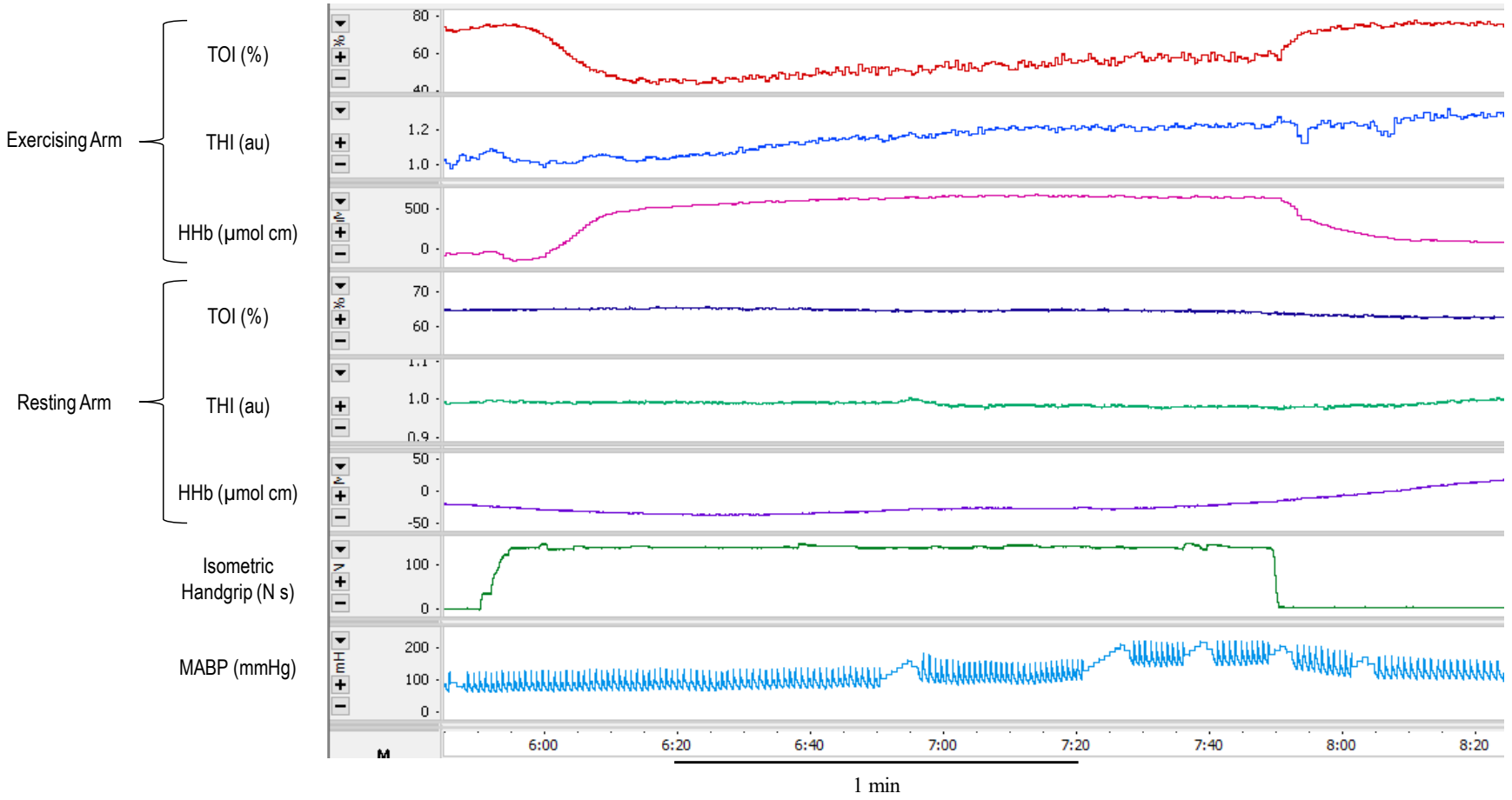



Figure 6.4 **An original trace showing NIRS, isometric handgrip exercise tension, and ABP.** This example raw trace shows NIRS (TOI, THI, and HHb) traces from the exercising and resting forearm (flexor digitorum-superficialis) while the subject performed isometric handgrip contraction at 60% MVC for 2 min; ABP is recorded from the resting hand. The gaps in the ABP trace show regular auto-calibration of the Finapres monitor.

## 6.3 Results

Anthropometric data of the young (22.30±0.65 years) and older (71.64±1.54 years) men are shown in Table 6.1.

Average MVC is shown in Table 6.1; all subjects performed handgrip contractions for the required 2 min. TTI was not affected by time and/or treatments (Figure 6.5) but the TTI of older men was lower than young men (Figure 6.5). HR and MABP increased with exercise; after cessation of contraction, HR returned to baseline quicker than MABP. Baseline HR and MABP were not affected by treatments in either age group but the absolute increase in HR and MABP was less in older men after aspirin (Table 6.2).

### 6.3.1 Exercising arm

Changes in TOI, THI, and HHb of young and older men performing isometric handgrip contractions are shown in Figure 6.6 and Figure 6.7 respectively. In young men, at the onset of contraction there was an immediate decrease in TOI to reach its lowest level at ~ 35 s and a concomitant increase in HHb to reach a peak at ~ 1 min 15 s; both variables remained more or less steady after that during contraction and returned towards their baseline after cessation of contraction. The exercise-related decrease in TOI and the increase in HHb were similarly attenuated by interventional treatments and were significantly different from placebo (Figure 6.6). THI increased gradually *during* the period of isometric contraction indicating increased muscle perfusion and remained elevated during the 2 min of *post*-contraction. All three interventional treatments similarly attenuated this increase (Figure 6.6).

In older men, onset of contraction decreased TOI, reaching its lowest point at ~ 45 s while HHb peaked around ~ 1 min 10 s; the values remained stable for rest of the contraction and similar to

that seen in young, only returned back to baseline after contraction ceased (Figure 6.7). Similarly, the decrease in TOI and the increase in HHb were equally attenuated by all three treatments. The THI responses were also similar to those recorded in young. However these changes were smaller than in young subjects (Figure 6.7).

### 6.3.2 Non-exercising arm

The changes in TOI, THI, and HHb of non-exercising arm of young and older men are shown in Figure 6.8 and Figure 6.9 respectively. Under placebo, muscle contraction decreased THI in the contralateral arm of young men, indicating decreased muscle perfusion; this response became significantly different from its respective baseline at ~ 20 s into the handgrip contraction and was similarly attenuated by the interventional treatments (40% $O_2$, aspirin, and their combination). During this period, $VO_2$ also decreased, reflected by a decrease in HHb values while TOI showed a parallel increase.

In older men, handgrip contraction was also accompanied by a small decline in muscle perfusion of the non-exercising arm and this was attenuated by the 3 treatments (Figure 6.9). However, when compared to the response seen in young men, this decline in muscle perfusion was significantly smaller even though the older men were performing the same *relative-intensity* (i.e., 60% MVC) contractions (Figure 6.9). There was also a small decrease in HHb which was attenuated by the three treatments; TOI did not change in response to exercise.

| | Young Subjects | | | | | | | Older Subjects | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Age (yr) | Weight (Kg) | Height (m) | BMI ($Kg/m^2$) | MVC (N) | Dominant FAC (cm) | Non-Dominant FAC (cm) | Age (yr) | Weight (Kg) | Height (m) | BMI ($Kg/m^2$) | MVC (N) | Dominant FAC (cm) | Non-Dominant FAC (cm) |
| | 25 | 54 | 1.67 | 19.36 | 245 | 24 | 24 | 68 | 67 | 1.83 | 20.01 | 196 | 24 | 24 |
| | 22 | 81 | 1.81 | 24.72 | 275 | 27 | 27 | 66 | 73 | 1.83 | 21.80 | 275 | 26 | 26 |
| | 25 | 79 | 1.8 | 24.38 | 314 | 27 | 27 | 78 | 64 | 1.82 | 19.32 | 118 | 23 | 23 |
| | 21 | 67 | 1.76 | 21.63 | 226 | 23 | 23 | 68 | 77 | 2.03 | 18.69 | 147 | 26 | 26 |
| | 18 | 62 | 1.76 | 20.02 | 235 | 23 | 23 | 65 | 93 | 1.92 | 25.23 | 157 | 29 | 29 |
| | 23 | 70 | 1.8 | 21.60 | 314 | 26 | 26 | 76 | 77 | 1.8 | 23.77 | 324 | 25 | 25 |
| | 22 | 90 | 1.92 | 24.41 | 177 | 26 | 26 | 77 | 61 | 1.71 | 20.86 | 177 | 24 | 24 |
| | 23 | 86 | 1.87 | 24.59 | 294 | 26 | 26 | 76 | 89 | 1.88 | 25.18 | 196 | 28 | 28 |
| | 21 | 67 | 1.75 | 21.88 | 343 | 26 | 26 | 66 | 75 | 1.76 | 24.21 | 255 | 27 | 27 |
| | 23 | 80 | 1.8 | 24.69 | 216 | 26 | 26 | 76 | 74 | 1.75 | 24.16 | 167 | 27 | 27 |
| | | | | | | | | 72 | 88 | 1.91 | 24.12 | 255 | 27 | 27 |
| Mean | 22.30 | 73.60 | 1.79 | 22.73 | 263.89 | 25.40 | 25.40 | 71.64 | 76.18 | 1.84 | 22.49 | 206.01 | 26.00 | 26.00 |
| SEM | 0.65 | 3.60 | 0.02 | 0.66 | 16.64 | 0.48 | 0.48 | 1.54 | 3.12 | 0.03 | 0.73 | 18.99 | 0.56 | 0.56 |

Table 6.1 **Anthropometric data of young (n=10) and older (n=11) men.**

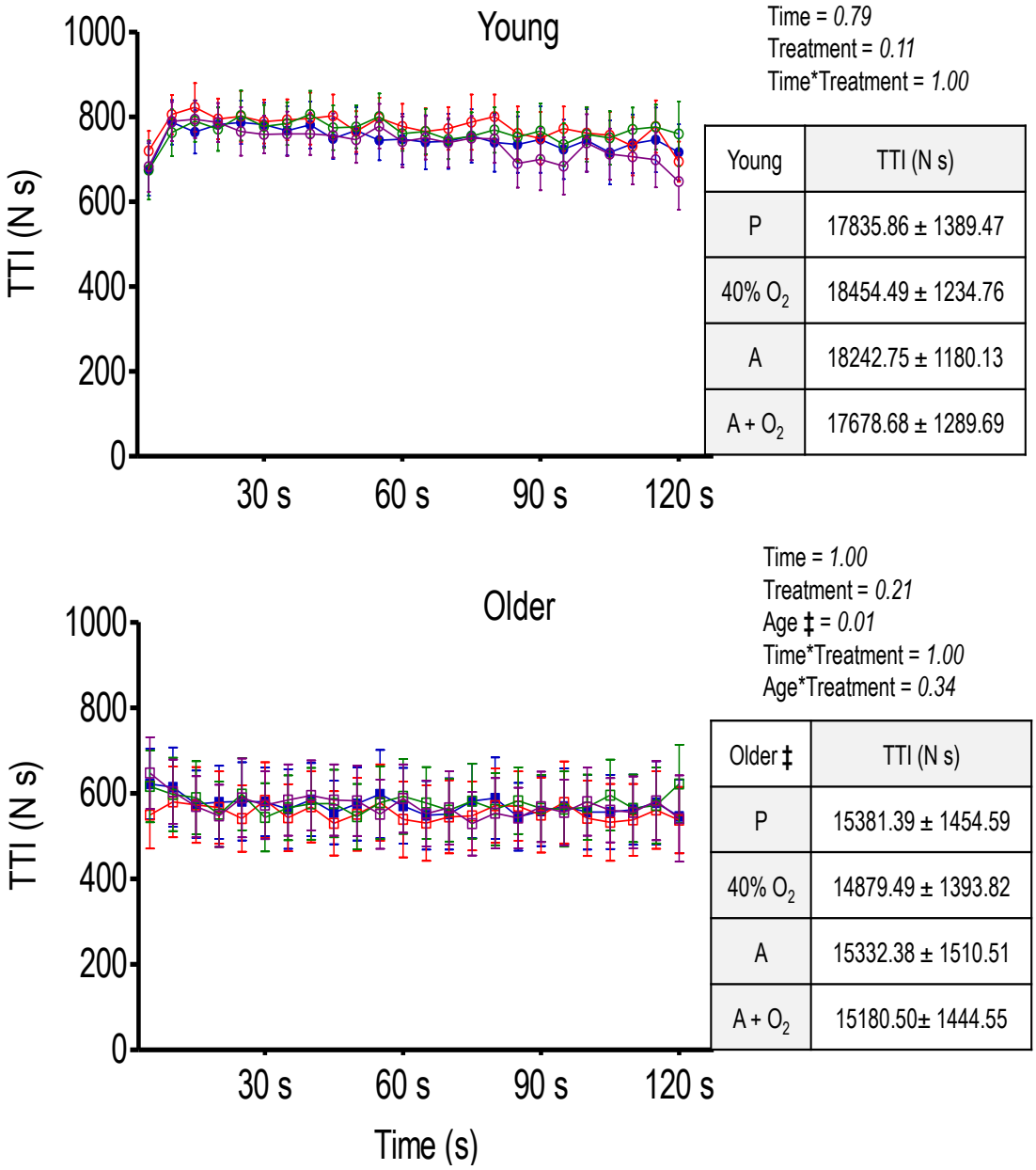



Figure 6.5 **TTI recorded for isometric handgrip contractions performed by young and older men.** Placebo is represented in blue while 40% $O_2$ is red, aspirin is green, and their combination ($A+O_2$) is purple. No treatment effect was detected but † represents significant difference between age groups. All subjects performed handgrip contraction for the full 2 min.

| Age | | Treatment | Baseline | Exercise | | | | | Post Exercise Responses | | | | | P Value (Within) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | - 60 s | 5 s | 30 s | 60 s | 90 s | 120 s | 125 s | 150 s | 180 s | 210 s | 240 s | |
| Young | HR (b min$^{-1}$) | P | 61 ± 3 | 76 ± 4 * | 76 ± 7 * | 76 ± 5 * | 80 ± 6 * | 82 ± 4 * | 68 ± 2 | 57 ± 2 | 57 ± 2 | 57 ± 2 | 52 ± 5 * | < *0.001* |
| | | 40% $O_2$ | 60 ± 2 | 75 ± 5 * | 76 ± 6 * | 78 ± 5 * | 77 ± 6 * | 88 ± 5 * | 71 ± 3 | 55 ± 1 | 55 ± 2 | 51 ± 4 | 56 ± 3 | < *0.001* |
| | | A | 61 ± 3 | 74 ± 4 * | 79 ± 4 * | 80 ± 5 * | 85 ± 4 * | 82 ± 6 * | 66 ± 5 | 55 ± 5 | 58 ± 4 | 59 ± 3 | 59 ± 3 | < *0.001* |
| | | A + $O_2$ | 59 ± 2 | 73 ± 5 * | 82 ± 5 * | 84 ± 4 * | 88 ± 5 * | 90 ± 9 * | 75 ± 6 * | 52 ± 4 | 53 ± 2 | 56 ± 3 | 52 ± 5 | < *0.001* |
| | | P Value | Treatment = *029* Time*Treatment = *0.69* | | | | | | | | | | | |
| | MABP (mmHg) | P | 79 ± 2 | 83 ± 2 | 95 ± 3 * | 106 ± 4 * | 114 ± 4 * | 127 ± 7 * | 115 ± 8 * | 86 ± 4 * | 86 ± 4 * | 86 ± 4 * | 87 ± 5 * | < *0.001* |
| | | 40% $O_2$ | 84 ±2 | 87 ± 3 | 100 ± 4 * | 112 ± 4 * | 122 ±5 * | 132 ± 4 * | 117 ± 3 * | 91 ± 2 * | 91 ± 3 * | 91 ± 3 * | 90 ± 2 | < *0.001* |
| | | A | 80 ± 3 | 89 ± 3 * | 96 ± 3 * | 108 ± 4 * | 120 ± 4 * | 132 ± 5 * | 116 ± 5 * | 90 ± 4 * | 89 ± 4 * | 88 ± 4 * | 86 ± 3 | < *0.001* |
| | | A + $O_2$ | 87 ± 4 | 93 ± 3 | 103 ± 3 * | 117 ± 4 * | 126 ± 4 * | 131 ± 11 * | 118 ± 7 * | 96 ± 5 * | 93 ± 4 | 93 ± 4 | 93 ± 4 | < *0.001* |
| | | P Value | Treatment = *0.08* Time*Treatment = *0.96* | | | | | | | | | | | |
| Older ‡ | HR (b min$^{-1}$) | P | 62 ± 4 | 67 ± 5 | 68 ± 4 * | 69 ± 4 * | 70 ± 4 * | 69 ± 3 * | 64 ± 3 | 62 ± 4 | 62 ± 3 | 65 ± 2 | 62 ± 3 | < *0.05* |
| | | 40% $O_2$ | 60 ± 4 | 65 ± 2 | 67 ± 3 | 68 ± 3 * | 69 ± 5 * | 71 ± 4 * | 64 ± 2 | 60 ± 3 | 61 ± 3 | 60 ± 3 | 61 ± 3 | *0.002* |
| | | A | 64 ± 2 | 70 ± 2 * | 68 ± 5 | 68 ± 3 * | 72 ± 2 * | 70 ± 3 * | 64 ± 3 | 61 ± 2 | 60 ± 2 | 59 ± 3 | 60 ± 4 | *0.001* |
| | | A + $O_2$ | 61 ± 3 | 68 ± 2 * | 66 ± 3 | 66 ± 5 | 68 ± 4 * | 69 ± 3 * | 63 ± 4 | 62 ± 3 | 63 ± 4 | 60 ± 3 | 59 ± 5 | < *0.05* |
| | | P Value | Treatment = *0.26* ‡ Age = <*0.001* Time*Treatment = *0.84* Age*Treatment = *0.83* | | | | | | | | | | | |
| | MABP (mmHg) | P | 87 ± 5 | 97± 4 | 99 ± 4 * | 104 ± 4 * | 109 ± 5 * | 109 ± 6 * | 105 ± 5 * | 89 ± 4 | 88 ± 3 | 91 ± 3 | 90 ± 4 | < *0.001* |
| | | 40% $O_2$ | 92 ± 4 | 97 ± 4 | 103 ± 4 * | 107 ± 5 * | 112 ± 7 * | 116 ± 7 * | 110 ±8 * | 94 ± 5 | 96 ± 5 | 93 ± 5 | 94 ± 4 | < *0.001* |
| | | A | 89 ± 4 | 96 ± 3 * | 98 ± 4 * | 98 ± 5 * | 105 ± 6 * | 110 ± 6 * | 103 ± 7 * | 87 ± 3 | 89 ± 3 | 88 ± 3 | 88 ± 4 | < *0.001* |
| | | A + $O_2$ | 88 ± 4 | 94 ± 4 * | 96 ± 5 * | 100 ± 4 * | 108 ± 6 * | 115 ± 7 * | 110 ± 7 * | 93 ± 5 | 91 ± 4 | 93 ± 4 | 92 ± 5 | *0.002* |
| | | P Value | Treatment = *0.06* ‡ Age = *0.02* Time*Treatment = *0.88* Age*Treatment = *0.05* | | | | | | | | | | | |

Table 6.2 **HR and MABP recorded from young and older men.** No treatment effect was detected between placebo, 40% $O_2$, aspirin, and A + $O_2$; * represents significant difference from respective baseline; ‡ represents difference between age groups ($P < 0.05$).

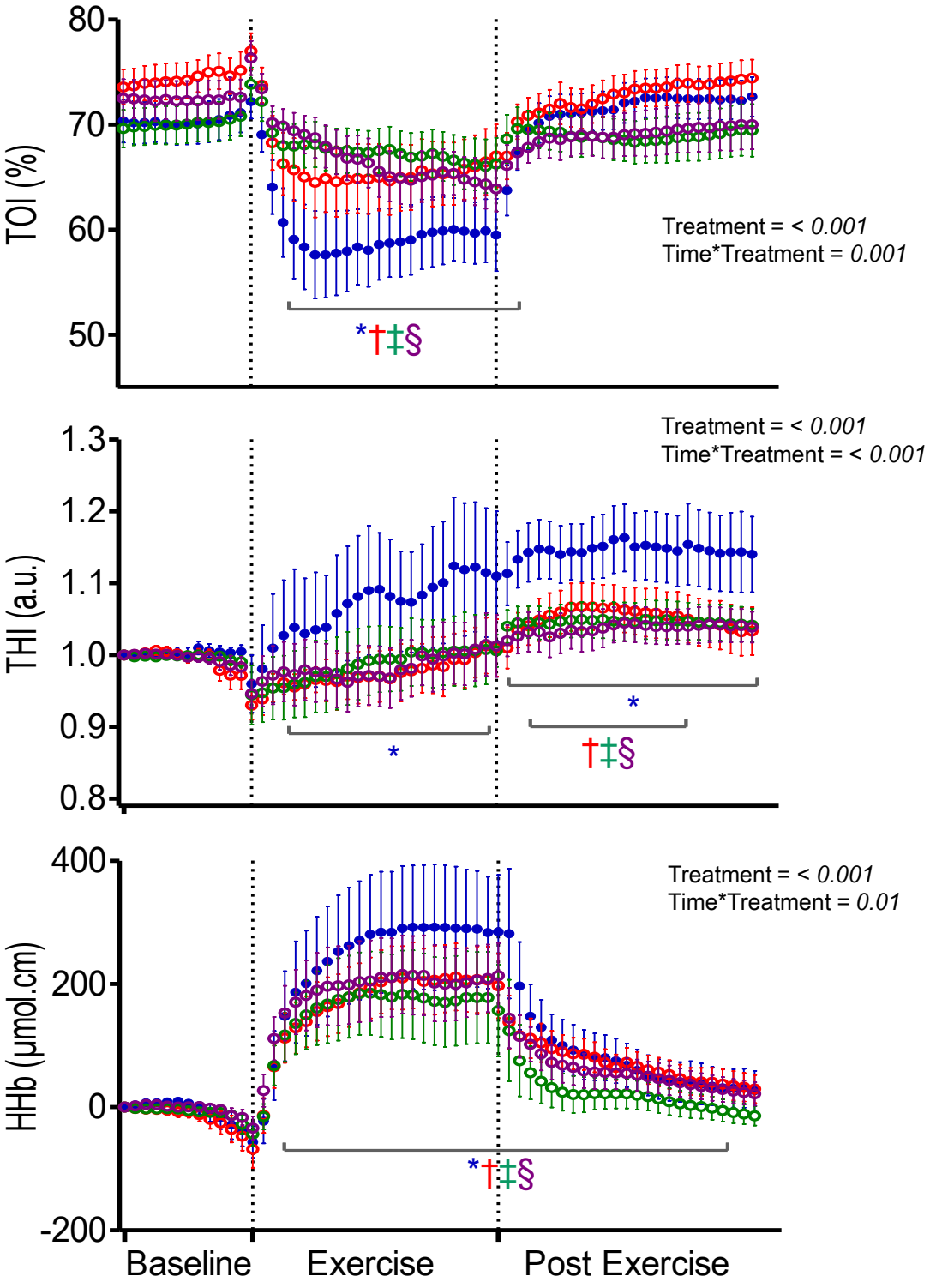



Figure 6.6 **Changes in TOI, THI, and HHb recorded from exercising arm of young men.** Placebo is represented in blue, 40% $O_2$ is red, aspirin is green, and their combination (A+$O_2$) is purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.05$).

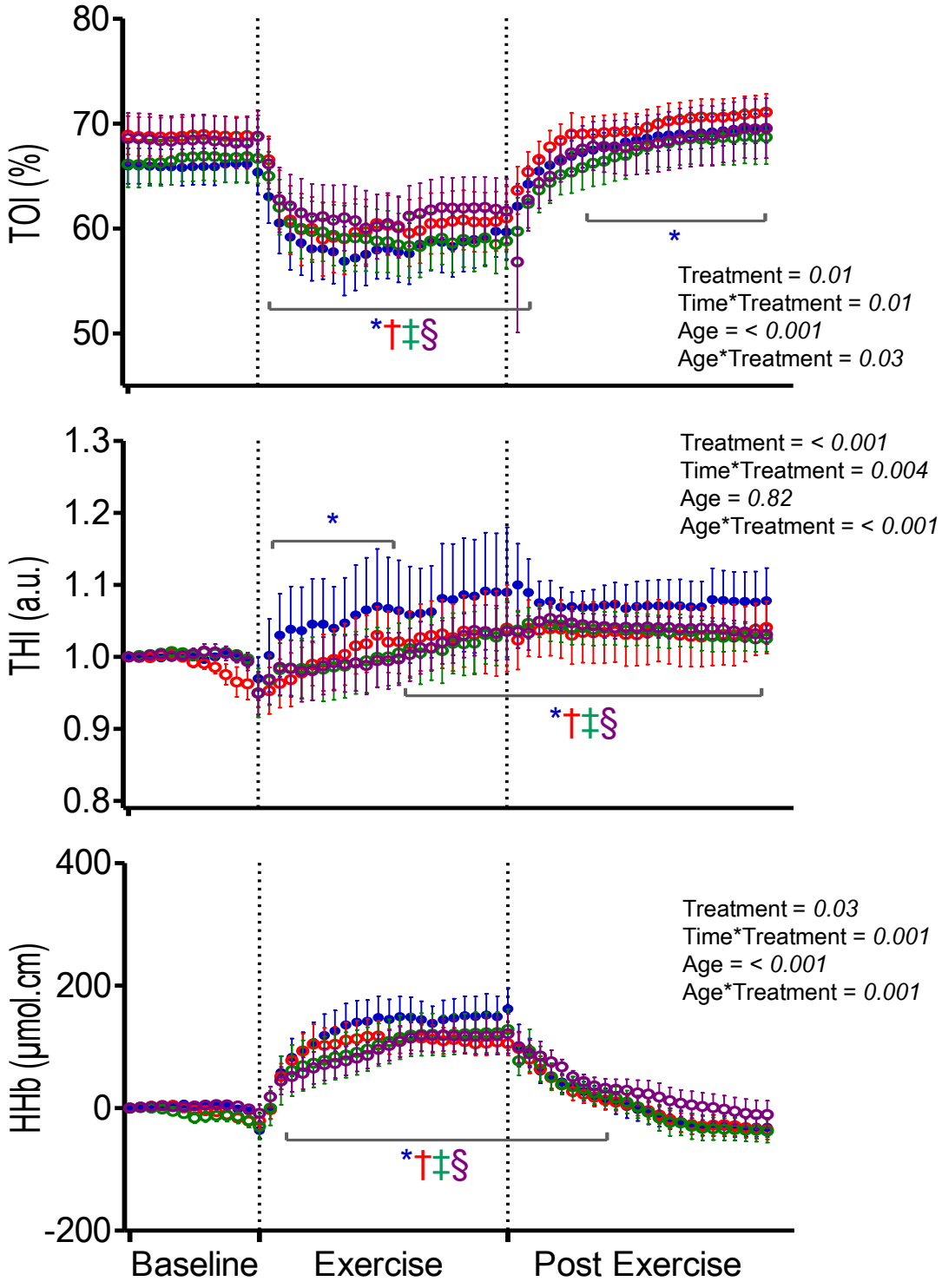



Figure 6.7 **Changes in TOI, THI, and HHb recorded from exercising arm of older men.** Placebo is represented in blue, 40% $O_2$ is red, aspirin is green, and their combination (A+$O_2$) is purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.05$).

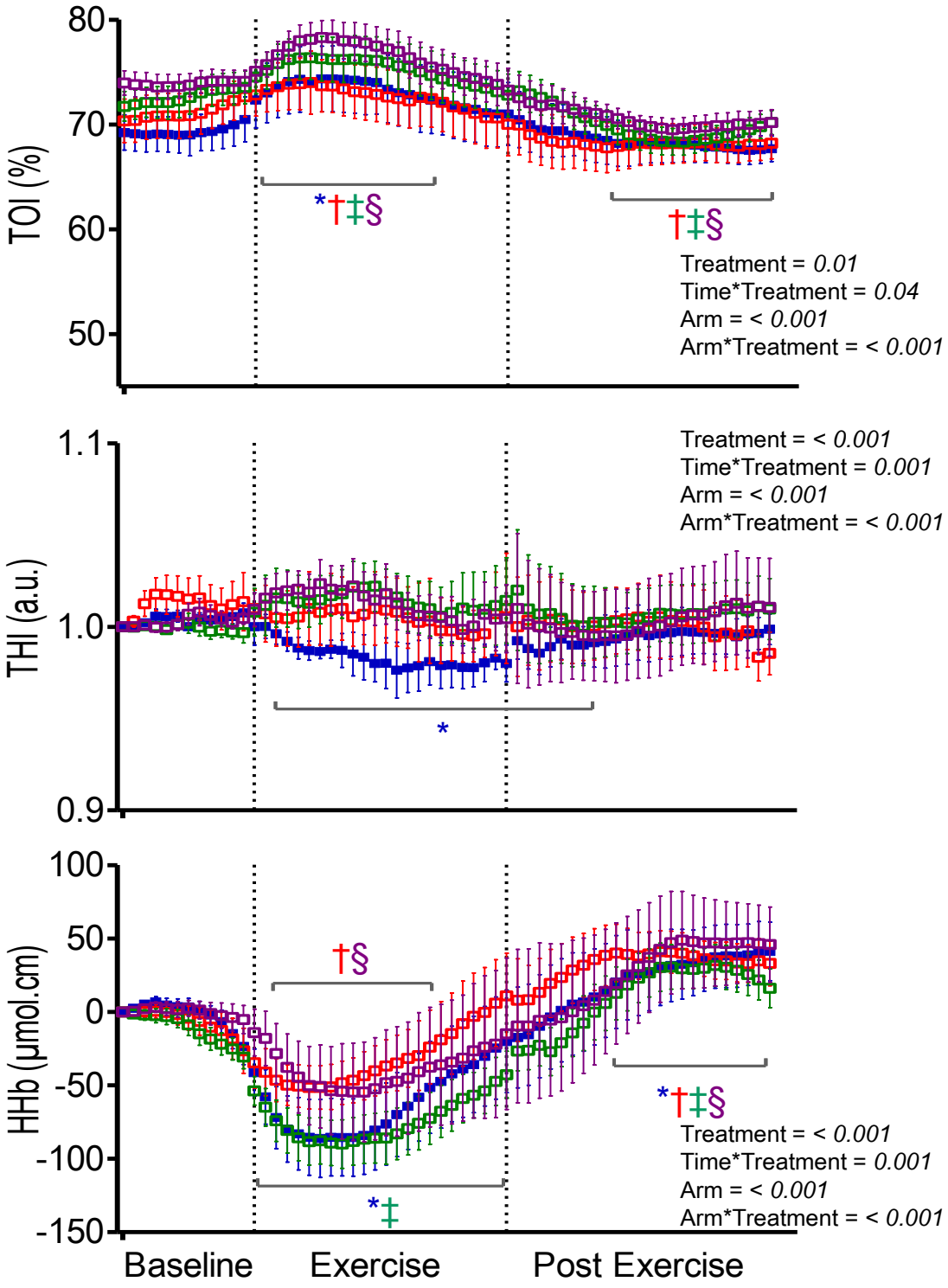



Figure 6.8 **Changes in TOI, THI, and HHb recorded from non-exercising arm of the young men.** Placebo is represented in blue, 40% $O_2$ in red, aspirin (A) is shown in green, and their combination (A+$O_2$) in purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.05$).

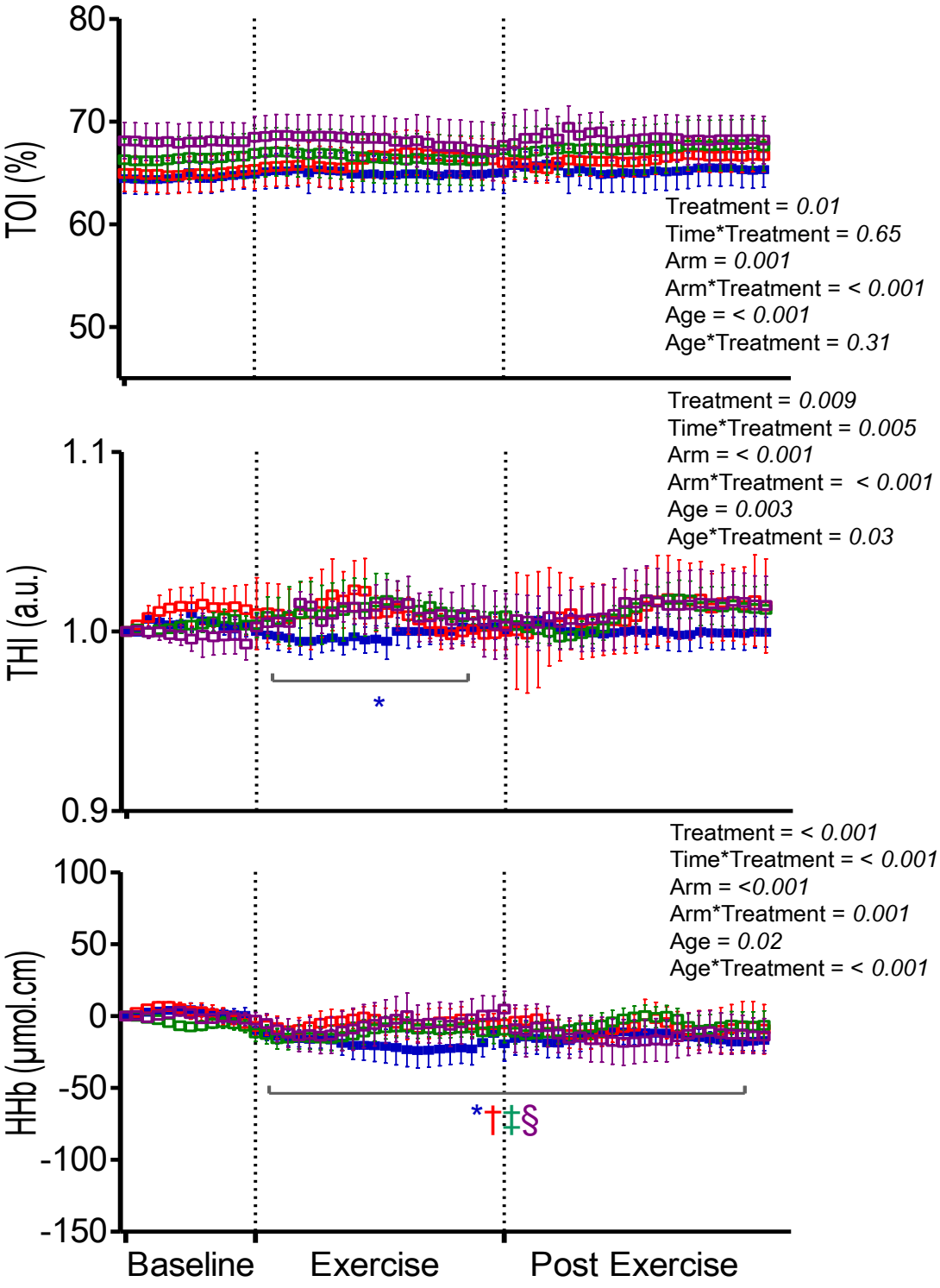



Figure 6.9 **Changes in TOI, THI, and HHb recorded from non-exercising arm of the older men.** Placebo is represented in blue, 40% $O_2$ is red, aspirin is green, and their combination (A+$O_2$) is purple. */†/‡/§ represents time effect for placebo, 40% $O_2$, aspirin, and A + $O_2$ (i.e., vs. respective baselines in each case; $P < 0.05$).

## 6.4 Discussion

This study produced several novel findings. Firstly, isometric handgrip contraction at 60% MVC for 2 min does not occlude muscle perfusion in either age group. Secondly, as the treatments similarly attenuated the increase in perfusion *during* contraction, this study showed for the first time that $O_2$-dependent COX products are involved in the exercise hyperaemia that occurs *during* isometric handgrip contraction in both age groups. Concerning the non-exercising arm, this study shows that the exercise-evoked vasoconstriction in the resting arm was similarly attenuated by COX inhibition or 40% $O_2$. Thus, $O_2$-dependent COX products are involved in the exercise-evoked vasoconstriction in both young and older men. Lastly, as TOI and HHb responses increased while THI was in decline in the non-exercising (resting) arm, this study has also unexpectedly revealed that isometric exercise decreases muscle metabolism (i.e., $VO_2$) in the resting arm *during* exercise.

### 6.4.1 Responses in the exercising arm of young men

Studies in the past have suggested that isometric handgrip contractions at 20% MVC decrease muscle perfusion (234), while at around 60% MVC they can completely occlude flow (22). However, our group have in the past observed attenuation in post-exercise hyperaemia with 40% $O_2$ when isometric contractions have been performed at 60 (437) or 100% (123) MVC, suggesting the presence of some muscle perfusion. Indeed, the fact that attenuation of *post*-exercise hyperaemia with 40% $O_2$ occurred only if it were breathed *during* exercise suggests this (123). Moreover the Doppler-ultrasound data from Kagaya and Homma shows that the brachial artery blood flow increased to the same level *during* isometric handgrip contractions of different incremental intensities (i.e., between 10 – 70% MVC); implying some restriction on blood flow, but no indication of occlusion (196). The THI results of the present study show that muscle

perfusion is not only maintained during isometric handgrip contraction of 60% MVC, but also shows a constant / persistent increase in perfusion (i.e., exercise hyperaemia). These observations were made from NIRS probes that were placed over the flexor digitorum-superficialis, but as the NIRS light used in this study penetrated ~ 2 cm of tissue (152), it can be reasonably proposed that isometric handgrip contractions at 60% were accompanied by a hyperaemic response. Importantly, this does not signify completely unhindered muscle perfusion, but just an overall increase *during* 60% MVC isometric contraction.

These same recordings showed that isometric exercise is accompanied by a profound decline in tissue oxygenation with a substantial increase in muscle $VO_2$ (indicated by a simultaneous increase in HHb). This observation agrees with Van-Beekvelt et al. who also observed a substantial increase in muscle $VO_2$ evoked by only 10% MVC isometric handgrip contractions (416). However as the THI kept increasing considerably while the HHb stabilised at ~ 1 min, this suggests continuous increase in muscle $VO_2$ during exercise. Previous studies of our group (123, 437), including those of Chapter 4, indicated that 40% $O_2$ supplied during isometric handgrip exercise ameliorated tissue oxygenation and thereby, attenuated the exercise / *post*-exercise hyperaemia. The results of the present Chapter show that to be true as not only was the increase in THI attenuated by 40% $O_2$ but tissue oxygenation also improved; the fall in TOI decreased, accompanied by an attenuated increase in HHb. It is possible that the attenuated increase in HHb observed with 40% $O_2$ was the result of decreased $VO_2$. However, there is no reason to believe that 40% $O_2$ *per-se* would decrease $VO_2$ in the exercising muscle when a similar amount of isometric work was performed between placebo and $O_2$ trials.

It was also suggested in the introduction to this Chapter that COX inhibition might augment the decline in tissue oxygenation by attenuating exercise hyperaemia. There was also a possibility that COX inhibition with aspirin would inhibit skeletal muscle metabolism (28, 222, 270, 281). The

results of the present study show an attenuated decline in TOI and increase in HHb; and therefore seem to favour the second possibility. However these explanations do not explain why the increase in HHb with combination of aspirin and 40% $O_2$ was similar to their individual effects. Nonetheless, as the increase in THI was equally attenuated by the two treatments alone or combine, these results, with those of Chapter 4, strongly indicate that $O_2$-dependent COX products are indeed mediating the exercise and *post*-exercise hyperaemia of isometric handgrip contraction. Moreover, the added observation of similar attenuation of the changes in THI and HHb suggests that COX products are released due to the decline in tissue oxygenation during isometric exercise, and that their production is inhibited by added $O_2$ and separately by decreased metabolism secondary to COX inhibition with NSAIDs (28, 222, 270, 281).

### 6.4.2 Responses in the exercise arm of older men

Before discussing the responses obtained from older subjects, it is important to mention that although an age-related difference in the muscle mass can be expected, which might itself influence tissue perfusion through the exercising muscle; however, as shown in the anthropometric data (Table 6.1), the forearm circumference of subjects was not different between the age groups making the comparison between them relatively simple.

The results obtained from the older men during placebo showed a similar increase in tissue perfusion *during* isometric handgrip contraction. However the magnitude of this response appears to be attenuated with increased age. Age is generally associated with a proportional shift towards oxidative muscle metabolism (408), which would decrease the concentrations of vasodilator metabolites of glycolysis. However the age-related increase in $VO_2$ should produce more $O_2$-dependent vasodilators. Therefore on this basis, the increase in HHb was expected to be greater than that observed in the young. However, some longitudinal studies have observed

an age-related decline in the $VO_2$ kinetics (16, 23, 81), perhaps reflecting decreased $O_2$ delivery to the working muscles as capillary to fibre ratio also declines with age (129). Moreover, Coggan et al. observed an age-related decline in the activity of succinate-dehydrogenase, citrate-synthase, and β-hydroxyacyl-CoA-dehydrogenase in gastrocnemius samples of individuals who indulged in normal to low-intensity recreational activities (64). The discrepancy between the findings of Coggan et al. (64) and Aniansson et al. (8) perhaps reflects the activity status of their volunteers and indeed there is some evidence suggesting this; i.e., the age-related decrease in $VO_2$ kinetics is limited to muscles that are normally inactive (61). These observations are in agreement with our findings and those of Bell et al. (23) in showing that supplementary $O_2$ *per-se* does not improve $VO_2$ in older individuals.

Despite these observations it is clear from the present study that isometric handgrip contractions at 60% MVC do elicit some strain on the tissue oxygenation that is alleviated by 40% $O_2$, such that the increase in THI is attenuated by supplementary $O_2$. The effect of COX inhibition in older men on the exercise hyperaemia of isometric contraction was similar to that in young men. Therefore, the similar attenuation in THI during isometric handgrip exercise with the two treatments alone or combine, confirms, and extends the findings of Chapter 4. The finding that the magnitude of exercise hyperaemia that was attenuated with use of 40% $O_2$ and/or COX inhibition is negatively affected by aging is also in agreement with conclusions of Chapter 4.

### 6.4.3 Responses in the resting arm of young men

In young men, isometric handgrip contractions evoked a decline in the perfusion of resting muscles which was similarly inhibited by 40% $O_2$, aspirin, and their combination. These results not only show $O_2$-dependent vasoconstriction in the resting contralateral arm but add to the results of Chapter 5 in showing for the first time that combination of aspirin and 40% $O_2$ equally

attenuated the decrease in THI. The mechanism by which these later effects occur has already been the subject of discussion in the previous Chapter.

The findings on $O_2$ saturation, tissue perfusion, and HHb data obtained in the resting arm are of particular interest here (Figure 6.8). Despite a decrease in the THI of the resting arm under placebo condition, HHb values also declined sharply during contraction, reflecting an exercise-related decrease in the $VO_2$ of the resting muscles. Despite presence of some evidence in animals to suggest that $VO_2$ is related to the $O_2$ delivery; i.e., the decline in $O_2$ delivery beyond a critical point leads to decrease in $VO_2$ (107, 108, 355), the observations made in this study are independent of this relationship. This fall in HHb remained as such for the initial ~ 55 s of isometric exercise before starting to recover while THI was still in decline (See Figure 6.8).

Importantly, the relationship between tissue oxygenation and sympathetic vasoconstriction has been the subject of investigation in previous studies. Hansen and colleagues evoked sympathetic vasoconstriction in the resting muscles by the use of lower body negative pressure and observed a decline in tissue oxygenation / perfusion; i.e., greater decline in oxygenated haemoglobin and some increase in HHb (59, 157, 158). Although this shows that tissue oxygenation declines with sympathetic vasoconstriction, $VO_2$ is still maintained during this time, reflected by an increase in HHb. However, the relationship between tissue oxygenation of resting muscles during exercise and sympathetic vasoconstriction observed in this study is novel; it shows that the decline in tissue oxygenation / perfusion during exercise in resting muscles is accompanied by attenuation in its $VO_2$. The exact reason for this attenuation at the moment is not known. However this decline in $VO_2$ in the resting muscles is unrelated to $O_2$ delivery; i.e., 40% $O_2$ does not inhibit this response. The finding that 40% $O_2$ inhibited the decline in THI and only partly inhibited the decline in HHb may be a reflection of the fact that 40% $O_2$ increased $O_2$

availability to the working muscle fibres from plasma instead of being off-loaded from haemoglobin; rather than an attenuated decline in $VO_2$ with 40% $O_2$ *per-se*.

However, COX inhibition which attenuated the decline in tissue perfusion during isometric handgrip contractions also resulted in a decrease in HHb suggesting a decrease in $VO_2$. This was not completely surprising and could partly be explained by the effect of COX inhibition on muscle metabolism (28, 222, 270, 281). Although why the magnitude of this decrease in HHb was similar to that observed during placebo and why was the recovery was delayed is not clear. However, the combination of aspirin and 40% $O_2$ alleviated the decline in HHb during exercise, but the effect of COX inhibition on HHb and presumably on $VO_2$ was still evident; i.e., the recovery was delayed with COX inhibition. These observations indicate that the decline in $VO_2$ is not just the result of inhibited muscle metabolism secondary to COX inhibition with NSAIDs but is a separate novel finding; a conclusion that is strengthened by the observation of increase in TOI exactly when HHb was declining. Therefore, these present findings indicate an isometric exercise-related / mediated decline in muscle metabolism of resting muscles which is independent of COX inhibition mediated decline in muscle metabolism; and that may be partially $O_2$-dependent.

### 6.4.4 Responses in the resting arm in older men

Similar to young men, isometric exercise was accompanied by decrease in muscle perfusion of the resting arm of older men. However the data presented here shows that this vasoconstriction was less intense in the older group; thereby, contrasting with those who show an age-related increase in the pressor reflex (275, 276, 399). However, it should be noted that some researchers have observed results that are similar to those of the present study (247, 299). Moreover, $\alpha_1$-adrenergic vasoconstriction evoked by tyramine which stimulates endogenous

noradrenaline release is attenuated in healthy sedentary men (91). Therefore, this decrease in sympathetic vasoconstriction might be the reason for the age-related attenuation in the magnitude of exercise-evoked vasoconstriction observed in this study.

Nonetheless, the observations of Markel et al. are particularly relevant; they used rhythmic handgrip contractions at 30% MVC with incremental ischemia of the exercising arm to investigate their effects on peroneal MSNA (247). Their results showed that while resting and non-ischemic MSNA were higher in older individuals, muscle ischemia attenuated MSNA in older individuals. This appears to contrast with the evidence showing metabolites stimulate group III and IV afferents (207, 261, 262, 347, 372); but they proposed that their response were a reflection of increased oxidative metabolism by the older skeletal muscles, or perhaps reflected a decreased $VO_2$. Taken together with results obtained in the exercising arm of older individuals in the present study, it seems likely that their results and those presented here are explained by an age-related decline in $VO_2$. This raises questions regarding the $O_2$-dependent contribution of COX products to exercise hyperaemia and exercise-evoked vasoconstriction. Nonetheless, the data presented here show that the vasoconstriction evoked in response to isometric handgrip contraction of older men was similarly attenuated by 40% $O_2$, aspirin, and their combination; thus, indicating a reflex vasoconstriction response that is mediated by $O_2$-dependent COX products as in young men.

However, if the exercise-related $VO_2$ is different between both age groups, a question is raised regarding the similar efflux of $O_2$-dependent $PGE_2$ and $PGI_2$ in young and older men (Chapter 4). The answer to this question perhaps lies in the observations made by Tang and Vanhoutte that the gene-expression of $PGE_2$-synthase and prostacyclin-synthase along with other enzymes of COX pathway are all increased with age (396). Another consideration that needs bearing in mind

is that if the exercise-evoked efflux of $O_2$-dependent COX products does not change with age (as shown in Chapter 4), their contribution to exercise-evoked vasoconstriction is attenuated.

The present study further showed that although an exercise-related decline in $VO_2$ of the resting muscles did occur in the older men, it was clearly also attenuated by age (See THI and HHb changes in young vs. old; Figure 6.8 and Figure 6.9). These observations have to be separate from the generalized decrease in $VO_2$ kinetics of the older individuals, as firstly it does not explain the decrease in HHb of the resting arm during isometric exercise but further, it also fails to explain the responses in young men. Separately, but rather surprisingly, the exercise-related decline in $VO_2$ of the resting arm of older men was completely inhibited by 40% $O_2$, aspirin, and their combination. This observation lends its support to the idea that this decrease in $VO_2$ of resting muscles is not similar to that observed with COX inhibition.

In conclusion, the novel findings of the present study confirm that $O_2$-dependent COX products are important mediators of exercise and *post*-exercise hyperaemia of isometric contraction and that of exercise-evoked vasoconstriction in resting muscles in both young and older men. However, their contribution to both exercise hyperaemia and vasoconstriction decreases with age. This study also presented novel findings that exercise is associated with a decrease in resting muscle's metabolism; a response that in itself is attenuated with age. The hypotheses of this study are accepted.

### 6.4.5 Limitations and Future Work

A principal limitation of this study lies in the fact that NIRS does not distinguish between cutaneous and deeper perfusion / oxygenation profiles (177, 377-379). In fact, recently, Sørensen et al. have shown that ~ 30% of NIRS signals reflect cutaneous perfusion / oxygenation profiles (377, 378). This is despite the fact the spatially-resolved spectroscopy

suppresses the influence of surface layers (125). As the homogenous models used for development of NIRS equipment cannot fully account for the heterogeneous composition of the forearm, a possibility has to be considered that the responses observed and presented here reflect the combined responses of both cutaneous and muscular compartments.

The observation that cutaneous sympathetic activity increases with exercise (232, 423, 424) then becomes an important issue, for it is possible that the NIRS responses of this study may have been influenced by cutaneous vasoconstriction caused by the increase in cutaneous sympathetic activity. However, firstly, it is important to mention that a comparison between the Fick method and NIRS technique showed that despite its limitations, NIRS is an effective tool for measuring perfusion and oxygenation / $VO_2$ profiles in exercising muscle (416). Therefore, it seems fair to assume that despite some contamination from the surface layers, the data reported here do predominantly reflect responses of perfusion and oxygenation profiles of deeper muscle tissue. Secondly, the responses recorded in the resting arm during exercise of the contra-lateral arm are relevant, for Vissing et al. showed that the increase in cutaneous sympathetic activity that occurs during exercise is mainly attributable to central command, with little or no reflex contribution from the muscle metaboreceptors (423). Metabolites and COX products sensitize and/or activate both mechano- and metaboreceptors (3, 267, 347, 372), and as both $O_2$ and COX inhibition attenuated the exercise-induced fall in THI in the resting arm, it is fair to assume that the observed response was predominantly reflex rather than central command in nature. In other words, it is likely the resting arm's responses predominantly reflected vasoconstriction in muscle rather than skin; and that the interventions affected exercise-evoked vasoconstriction induced in skeletal muscle. This proposal / argument is supported by the observation that the decrease in resting arm's THI increased overtime, becoming significantly different from baseline after ~ 15 to 20 s of continuous isometric contraction (Figure 6.8). However, this proposal could be

investigated / elucidated by recording cutaneous perfusion with laser-Doppler flowmetry, for if any cutaneous vasoconstriction occurring in the resting arm persists during treatment with $O_2$ or COX inhibition, then this would add weight to the idea that the NIRS data are attributable to responses in the skeletal muscle. A future study that incorporates recordings of both skin and MSNA, and uses NIRS equipment could also help clarify the contribution of $O_2$-dependent and PG-mediated mechanisms to exercise-evoked vasoconstriction.

Lastly, although the data presented here of exercising flexor digitorum-superficialis suggests hyperaemia rather than obstruction of tissue perfusion; however, it is possible that some perfusion was hindered. Thus, future work with combined use of Doppler-ultrasound with NIRS could help further clarify the oxygenation / perfusion profile of exercising forearm.

# Chapter 7

# 40% $O_2$ attenuates exercise hyperaemia of twitch contractions; the role of adenosine $A_{2A}$-receptors

## 7.1 Introduction

Following the results of the previous three Chapters and the knowledge that adenosine has been implicated in the production of vasodilator PGs, questions arise regarding the $O_2$-dependent nature of adenosine's contribution to the exercise hyperaemia.

The enzyme 5'nucleotidase produces adenosine intracellularly and on either side of the vascular wall (interstitial or intravascular) from its nucleotides (112, 164, 314, 315). Exercise increases interstitial and venous concentrations of adenosine which remain elevated during the initial minutes of *post*-exercise rest (18, 237). Ballard et al. electrically-evoked contractions (4 Hz) in dog gracilis-muscle while collecting samples from the arterial supply and venous drainage to assay for adenosine (18). They deduced that ~ 15% of the hyperaemia in the 1$^{st}$ min of exercise was dependent on adenosine's vasodilator contributions. By the 5$^{th}$ min, this contribution had reached ~ 40% and from then on remained stable. Moreover, adenosine was also a significantly important factor in the *post*-exercise hyperaemia (18). Using Wistar rats, Ray and Marshall reported similar exercise and *post*-exercise related vasodilator contributions of adenosine by observing the effect of selective inhibition of adenosine $A_{2A}$-receptors (333).

In humans, Hellsten et al. observed a near 5 fold increase in the concentrations of interstitial adenosine and its precursor nucleotides with 10 W knee-extension exercise; modest further increases with greater workload (~ 50 W) (165). Nonetheless, rate of increase in interstitial adenosine and blood flow were found to have a good correlation ($r = 0.98$); causally implicating adenosine in vasodilatation of exercise. The observation of modest increase in interstitial adenosine at higher workload however suggests that the contribution of other intensity-dependent metabolites increases at these intensities; for example, vasodilator PGs (29, 198, 447, 448). The results of the previous Chapters show that PGs work in an $O_2$-dependent manner

to cause dilatation during exercise. Indirect evidence regarding adenosine leads to similar conclusions. For example, Costa et al. observed a larger increase in interstitial adenosine concentrations if 15% MVC handgrip contractions were performed under ischemia (70). Consistent with these results are the observations that venous efflux of adenosine is greatly increased during systemic hypoxia (71, 274).

In addition, Mortensen et al. showed that in humans, the attenuation of exercise hyperaemia following combined NO and COX inhibition was not added to by further inhibition of adenosine-receptors with theophylline (289). Although theophylline and aminophylline are weak adenosine-receptor antagonists with no selectivity between receptor subtypes; and inhibit phosphodiesterase activity (249), Mortensen et al.'s (289) data did suggest that adenosine's contribution to exercise hyperaemia may be mediated by or dependent on NO and/or PGs. Similar conclusions were reached by Nyberg et al. when they noticed that adenosine infusion in either interstitial or intravascular compartments increased concentrations of vasodilator PGs (306).

Thus, taken together, questions are raised regarding the relationships between adenosine and PGs, their $O_2$-dependency, and their importance during the early and later part of exercise hyperaemia. Moreover, it was appropriate to carry out this study on rats as this allowed strong competitive adenosine-receptor antagonists to be used. As indicated above, adenosine's contribution to exercise hyperaemia is understood to be mediated by $A_{2A}$-receptors rather than by any other adenosine-receptor (324, 333). Therefore an $A_{2A}$-receptor inhibitor was used.

### 7.1.1 Aims and Hypotheses

This study aimed to investigate the direct and indirect relationship between tissue oxygenation, $O_2$-dependent COX products and adenosine in relation to exercise-related vasodilatation. We

hypothesized that adenosine's direct contribution to exercise hyperaemia is $O_2$-dependent; i.e., supplementary $O_2$ would ameliorate tissue oxygenation causing similar attenuation in exercise hyperaemia as that observed with adenosine-receptor inhibition. We also hypothesized that further COX inhibition would not add to this attenuation in exercise hyperaemia.

## 7.2 Methods

Experiments were performed on eight young male Wistar rats (252 – 300 g) in accordance with the UK legislation (Animals Scientific Procedures Act 1986): Home Office project license 30/2894; personal license 40/10794. Anaesthesia was induced with Isoflurane (3.5% with $O_2$); surgical level was marked by the absence of pedal withdrawal-reflex. A jugular vein was cannulated to deliver intra-venous Alfaxan (20 - 23 mg/kg/h) for the maintenance of anaesthesia; adequacy judged by absence of pedal withdrawal-reflex and stability of ABP (335). At the end of the experiment, animals were euthanized using an anaesthetic (Euthatal) overdose and confirmed by cervical dislocation.

### 7.2.1 Surgical Preparation

The surgical preparations used have previously been employed by our group (333, 335). Briefly, the rat breathed spontaneously via a cannula in the trachea; a t-tube connected to this allowed the percentage of inhaled $O_2$ to be controlled (air or 40% $O_2$). A cannula placed in the brachial artery was connected to a pressure transducer which was calibrated against a manual sphygmomanometer to monitor the ABP; MABP and HR were recorded online from this data. The right femoral artery was isolated, its blood flow (FABF) was measured continuously using a peri-vascular flow-probe (0.5 V; Transonic systems Inc.) connected to a flow-meter (TI06, small animal flow-meter; Transonic systems Inc.). The tail artery was cannulated for administration of pharmacological agents.

The extensor digitorum-longus (EDL) was chosen for the study as the blood flow through this mixed-muscle is similar per gram of tissue as the whole hindlimb, both during rest and exercise (12). Moreover, our group have previously used it to show that the adenosine released during exercise, acts specifically on the $A_{2A}$-receptors to induce vasodilatation (333). The right EDL was

isolated, tendons sectioned distally and attached to the isometric force transducer (TRI-201 Letica Scientific, Spain) using an inextensible suture. The ankle was immobilized using a clamp so that the developed tension could be recorded; baseline level was 0.05 N (5 g). The right sciatic nerve was isolated, sectioned proximally, and attached to a hook-electrode.

Data were collected on an Apple computer using Power-lab (AD Instruments, USA): femoral artery vascular conductance (FAVC) was derived online by dividing FABF by ABP. Approximately 60 min were allowed for stabilization before beginning of the experimental protocol.

### 7.2.2 Protocol

Maximal twitch of EDL was established by stimulating the sciatic nerve with increasing voltage (1 – 6 V). Maximal twitch voltage at 4 Hz for 5 min (1200 pulses; pulse-duration 0.1 s) was used to evoke all muscle contractions. Our group's previous work in rats has shown that when employing successive stimulations, the responses to the first stimulation differed from those evoked by subsequent stimulations (333, 335). Similar results were also obtained in cats (324). Therefore Stimulation-2 was used as the control (placebo) and was initiated 30 min after cessation of Stimulation-1. Simulation-3 was evoked ~ 35 min later following 5 min of breathing 40% $O_2$ through the tracheal cannula; and subsequent stimulations were all done during 40% $O_2$ supplementation. 30 min after cessation of Stimulation-3, the control cardiovascular responses to intra-vascular adenosine (1.2 mg/kg/min for 5 min) infusion were recorded; followed by $A_{2A}$-receptor inhibitor ZM241385 (0.05 mg/kg). 15 min later, $A_{2A}$ inhibition was confirmed by another intra-vascular infusion of adenosine. This was followed Stimulation-4. 15 min later, diclofenac was infused (1 mg/kg) for COX inhibition, and after further 20 min, Stimulation-5 was applied. These doses of pharmacological agents have previously been shown to be effective (331, 333,

335). Figure 7.1 and Figure 7.2 briefly show the schematic diagrams of the surgical preparation and experimental protocol.

### 7.2.3 Statistical analysis

Cardiovascular / hemodynamic data were averaged over 60 s. Adenosine infusion data are shown as 1 min pre-infusion, 5 min during, and 1 min *post*-infusion; while muscle contraction data are shown as 1 min before, 5 min during, and 7 min *post*-contraction. 2-way repeated-measures ANOVA was used to identify time, treatment, and their interaction effects. Each treatment's data were further analyzed using 1-way repeated measures ANOVA. Once a statistically significant effect was detected, Tukey's HSD was used to detect the exact point of statistical difference within and/or between conditions.

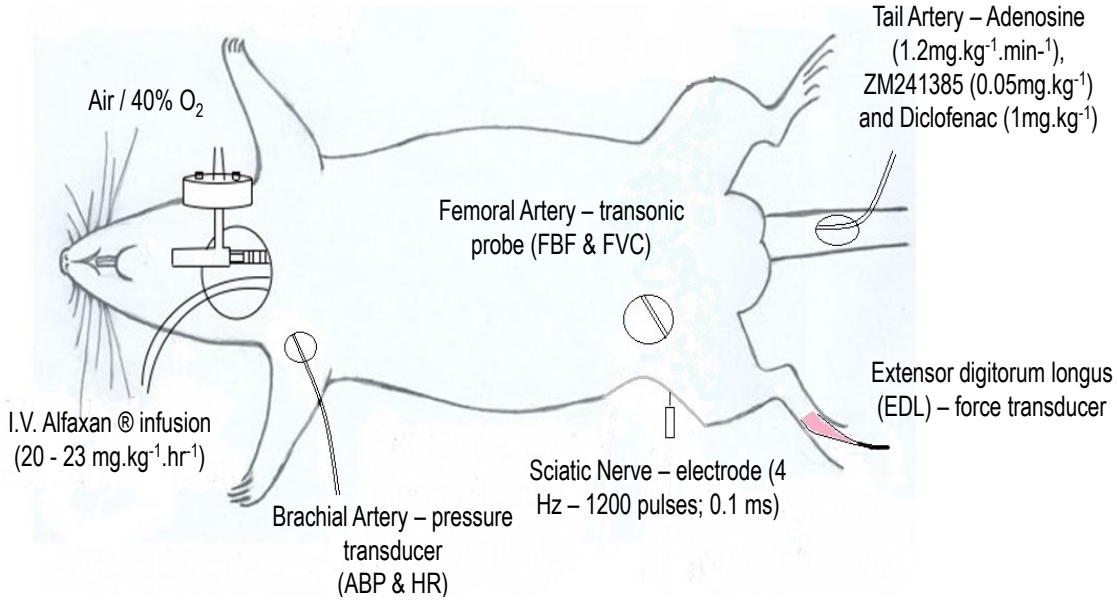



Figure 7.1 **Schematic diagram of surgical preparations for protocol.**

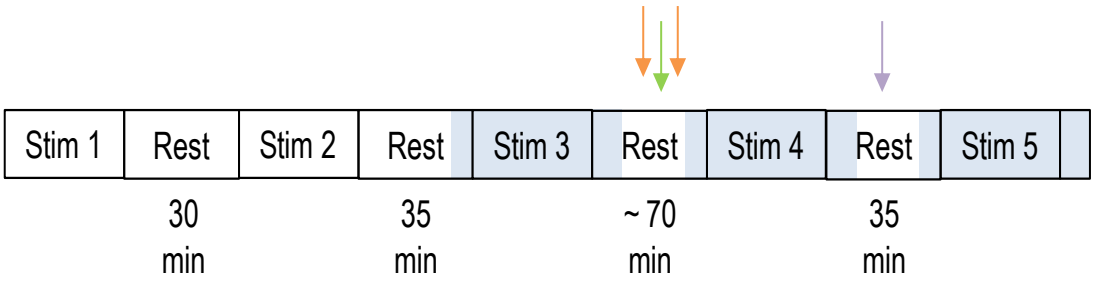



Figure 7.2 **Schematic diagram of the protocol.** Stimulation-2 is the control stimulation (air + no pharmacological agents) while the subsequent stimulations were carried out under hyperoxia (40%; blue area). $O_2$ supplementation was always started for 5 min before stimulation and carried out for 7 min while the *post*-contraction responses were being recorded. Orange ↓ represents adenosine infusion, green ↓ represents ZM241385 infusion and purple ↓ represents diclofenac infusion. Each stimulation lasted 5 min (4 Hz – 1200 pulses; 0.1 s).

## 7.3 Results

Before examining the affects of different interventions on exercise hyperaemia, Figure 7.3 shows that adenosine $A_{2A}$-receptor inhibition with ZM241385 substantially attenuated the increase in FAVC induced by intra-vascular adenosine infusion.

Sciatic nerve stimulation at 4 Hz for 5 min produced rhythmic twitch contractions of the hindlimb. Table 7.1 shows that time and/or treatments did not influence the generated TTI. HR and MABP were also not affected by the treatments (Figure 7.4) and were maintained throughout, allowing FAVC conductance to follow the changes in FABF (Figure 7.5). Twitch contractions increased FABF and FAVC significantly under placebo conditions (Figure 7.5). This increase in FABF and FAVC both during and after hindlimb exercise was significantly attenuated by 40% $O_2$; during exercise this attenuation was ~ 20% compared to control. The combination of hyperoxia and $A_{2A}$-receptor inhibition following ZM241385 administration did not add to the attenuation of the exercise-related increases in FABF and FAVC. Moreover, similar responses were observed following added COX inhibition with diclofenac. During Stimulations 4 and 5, the attenuation in the increase in FAVC was ~ 18% and ~ 19% respectively.

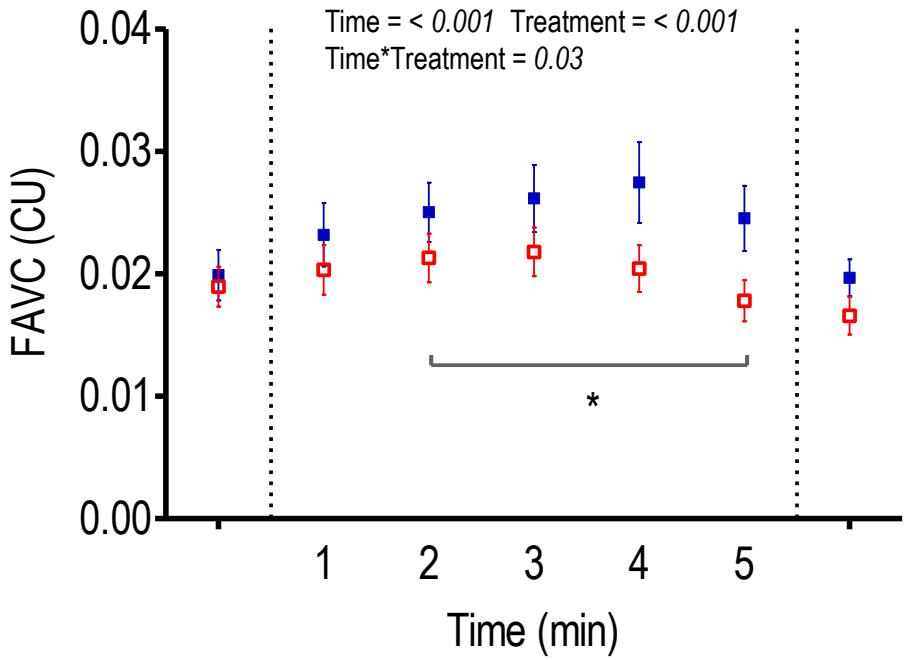



Figure 7.3 **Hyperaemia responses following intra-vascular adenosine infusion.** FAVC increased with adenosine infusion under control condition (blue) but this increase was attenuated following $A_{2A}$-receptor inhibition with ZM241385 (red). * represents significant interaction effect; includes all the data points within bracket.

| Treatment | TTI (N.s) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1st min | 2nd min | 3rd min | 4th min | 5th min | P Value (within) | All 5 min |
| Placebo | 4.52±0.56 | 4.60±0.45 | 4.62±0.46 | 4.60±0.43 | 4.44±0.45 | *0.93* | 22.73±2.22 |
| 40% $O_2$ | 4.48±0.44 | 4.68±0.41 | 4.57±0.45 | 4.55±0.43 | 4.63±0.45 | *0.33* | 22.49±1.80 |
| 40% $O_2$ + ZM241385 | 4.40±0.51 | 4.57±0.45 | 4.57±0.46 | 4.47±0.49 | 4.41±0.38 | *0.29* | 23.06±2.58 |
| 40% $O_2$ + ZM241385 + Diclofenac | 4.35±0.51 | 4.45±0.54 | 4.44±0.53 | 4.39±0.57 | 4.44±0.55 | *0.85* | 23.01±3.01 |
| P Value | Time = *0.99* Treatment = *0.95* Time*Treatment = *1.00* | | | | | | |

Table 7.1 **TTI responses of rhythmic twitch contractions.** Sciatic nerve was stimulated at maximum twitch contraction voltage at 4 Hz for 5 min. Neither time nor treatments had an effect on the measured force of contraction.

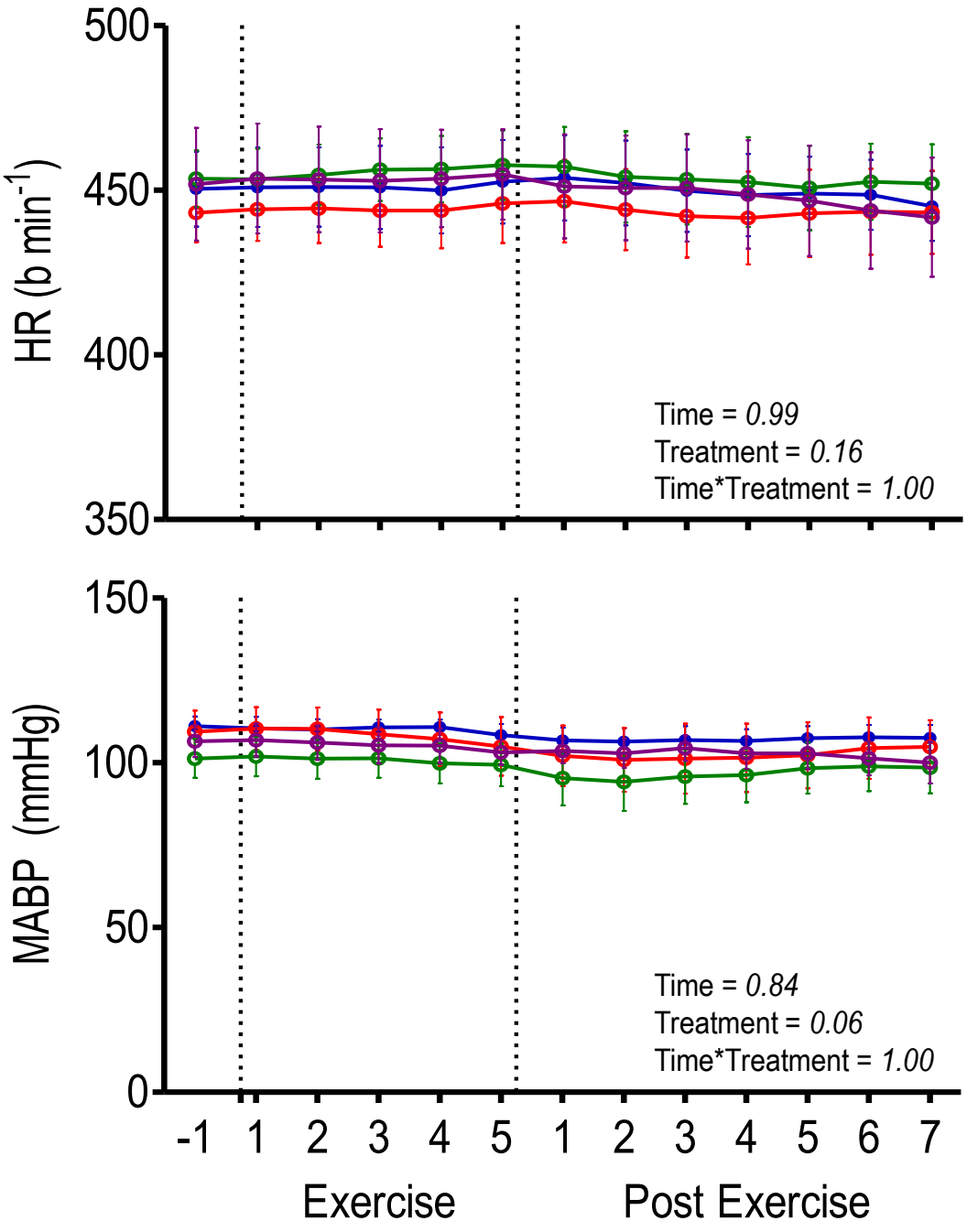



Figure 7.4 **HR (upper panel) and MABP (lower panel) recorded before, during, and after rhythmic twitch contractions of the hindlimb.** No treatment affect was detected between placebo (blue), 40% $O_2$ (red), 40% $O_2$ + $A_{2A}$-receptor inhibition (green), and 40% $O_2$ + $A_{2A}$-receptor inhibition + COX inhibition (purple) for HR and MABP.

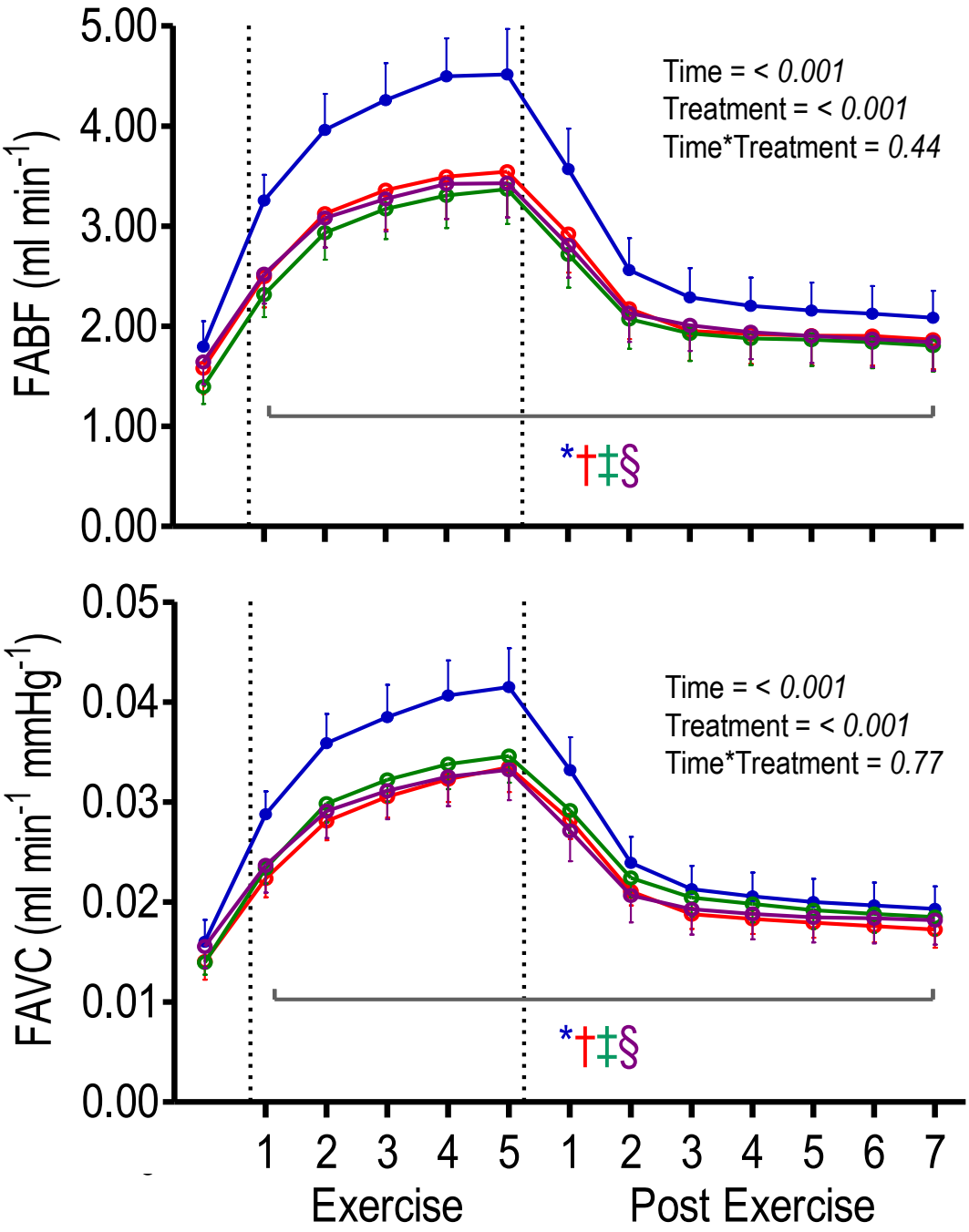



Figure 7.5 **FABF (upper panel) and FAVC (lower panel) recorded before, during, and after rhythmic twitch contractions of the hindlimb.** Placebo is represented in blue, 40% $O_2$ is red, 40% $O_2$ + $A_{2A}$-receptor inhibition is green, and 40% $O_2$ + $A_{2A}$-receptor inhibition + COX inhibition is shown in purple; FABF and FAVC increased with twitch contractions during all conditions. Interventional treatments equally attenuated FABF and FACV compared to placebo response. */†/‡/§ represents time effect for placebo, 40% $O_2$, 40% $O_2$ + $A_{2A}$-receptor inhibition, and 40% $O_2$ + $A_{2A}$-receptor inhibition + COX inhibition, respectively (i.e., vs. respective baselines; $P < 0.01$).

## 7.4 Discussion

Our group have used a similar exercise protocol before in rats and showed that systemic and limb specific cardiovascular / hemodynamic responses of electrically-evoked EDL twitch contractions for 5 min each are not different between stimulations 2 to 5 (i.e., no time or stimulation effect was detected) (333, 335). Therefore, time control experiments were not performed in the present study. Moreover, TTI during stimulation-2 to stimulation-5 in the present study were consistently comparable with our group's earlier work (333, 335). This allows for a simpler comparison between vascular responses evoked by different stimulations. As demonstrated in Figure 7.3, a substantial inhibition of the dilatation induced by adenosine infusion was achieved with the highly selective $A_{2A}$-receptor inhibitor (ZM241385) (323).

### 7.4.1 Exercise responses

During placebo, by the end of low frequency twitch contractions, FAVC had increased by ~ 3 fold; values that are comparable to that achieved in earlier work (18, 324, 333). Hyperoxia or the addition of pharmacological agents did not affect the baselines; and any exercise-related changes in HR and MABP were minor. However, hyperoxia attenuated the exercise hyperaemia during the period of contractions by ~ 20%, as well as the *post*-contraction hyperaemia. This decrease was greater than that previously observed with lone $A_{2A}$-receptor inhibition; in that it mainly affected the *post*-contraction hyperaemia (333). However, the addition of ZM241385 to hyperoxia in the present study caused no further attenuation of exercise hyperaemia. Thus, taken together with the results of Ray and Marshall (333), the present results suggest that although adenosine participates late in the exercise hyperaemia, 40% $O_2$ attenuates hyperaemia from the very beginning of the contraction. Moreover as the combination of 40% $O_2$ and ZM241385 did not add to this attenuation and revealed an effect that was very similar to $O_2$

alone (stimulation-3), it implies that adenosine's contribution to exercise hyperaemia is $O_2$-dependent; i.e., added $O_2$ inhibits adenosine's contribution to hyperaemia. The fact that subsequent COX inhibition had no further effect in the presence of 40% $O_2$ and ZM241385 indicates that any contribution of $O_2$-dependent PGs had already been prevented.

The data here shows that the attenuation in hyperaemia occurs *during* both *exercise and post-exercise* periods. This observation confirms and extends the observations of the previous chapters; i.e., attenuated hyperaemia responses of previous chapters reflected attenuated exercise hyperaemia, not just the *post*-exercise hyperaemic response. This study also confirms the findings of Chapter 6 in showing that attenuation in exercise hyperaemia with 40% $O_2$ occurs from early periods of exercise. The results of the previous Chapters strongly suggest that $O_2$-dependent metabolites that attenuate exercise hyperaemia belong to the COX pathway, and as 40% $O_2$ also attenuates this hyperaemia early on during contraction (here and in Chapter 6), an obvious possibility is that the COX pathway is activated early during exercise to generate dilator PGs. Indeed, it could be that early in the exercise, adenosine neither contributes to exercise hyperaemia nor mediates the release of vasodilator PGs; i.e., these early COX products are produced independently of adenosine. Alternatively, looking at the results of Ray and Marshall (333), and those of Ballard et al. (18), it is possible that adenosine released early does not directly participate in the exercise hyperaemia but rather is the main precursor of further vasodilator metabolites. Indeed, Nyberg et al. have observed increased NO and PG concentrations in both interstitial and intravascular compartments with adenosine infusion (306). Moreover, Mortensen et al. have also revealed an interdependent relationship between these three metabolites (289).

In conclusion the results of this study show for the first time that adenosine as well as vasodilator COX products contribute to exercise hyperaemia in an $O_2$-dependent manner. As such, the

hypotheses of this are accepted. However, whether these metabolites work independent of one another or participate in an interdependent mediation of exercise hyperaemia is a question that remains unanswered.

### 7.4.2 Limitations and future work

An important limitation to this study is that efficacy of COX blockade with diclofenac was not actually tested. However, the concentration used in this study has previously been used by our group; and was found to effectively attenuate the adenosine evoked increase in $PGI_2$ (331). However, in the absence of a test of efficacy in the present study, we cannot be sure that diclofenac achieved effective COX inhibition to block the contribution of $O_2$-dependent PGs to exercise hyperaemia.

This issue is important because the changes in THI recorded in the exercising arm before and after COX inhibition shown in the previous Chapter clearly demonstrated that vasodilator PGs are involved in exercise hyperaemia from the beginning of handgrip contraction (60% MVC). A similar pattern of change in the hyperaemia was observed with hyperoxia. As argued above, the results of the present Chapter indicate that adenosine and vasodilator COX products act in an $O_2$-dependent manner to regulate exercise hyperaemia. Nonetheless, it would have been better if a series of COX inhibition experiments had been performed using a rat model. Such experiments on their own and in combination with 40% $O_2$, and $A_{2A}$-receptor inhibition would have further aided the understanding of exercise hyperaemia responses of $O_2$-dependent adenosine and PGs. They could have shown whether COX inhibition in rats also attenuates exercise hyperaemia from the very beginning of exercise and/or whether the contributions of adenosine and PGs to exercise hyperaemia are interdependent. Moreover, analysis of PGs and adenosine from arterial and venous samples would have also helped with this understanding.

Although such experiments were planned and indeed attempted, technical and time limitations prevented their completion.

Additionally, it is important to remember that Ray and Marshall (333) suggested that adenosine's contribution to exercise hyperaemia is mediated only by $A_{2A}$-receptors and that these are on the smooth muscle rather than endothelium; i.e., adenosine released only from the skeletal muscles contributes to the exercise hyperaemia. This conclusion was mainly based on their finding that the $A_{2A}$-dependent component of exercise hyperaemia was not mediated by NO (335); and therefore, they concluded that adenosine was acting from the interstitium on the smooth muscle $A_{2A}$-receptors, rather than from the intraluminal side on endothelial adenosine-receptors. Our group had previously shown that during systemic hypoxia, the adenosine component of hypoxic dilatation is mediated by endothelial $A_1$-receptors, which stimulate NO synthesis and release (34, 334, 373). Moreover, the observations that interstitial adenosine concentrations remain unaffected by systemic hypoxia (273), and that the endothelium acts as effective barrier between intra and extra-vascular compartments for adenosine and its nucleotides, contributed to this understanding (237, 249, 333). However, as they did not use a specific $A_1$-receptor inhibitor (for example, DPCPX) in their study (333), their results may not actually show the complete contribution of adenosine to exercise hyperaemia. Therefore a study clearly needs to be performed with specific $A_1$-receptor inhibition during exercise. As the intravascular concentrations of adenosine increase during systemic hypoxia (273) and $A_1$-receptors are involved in hypoxic dilatation (334), their contribution to $O_2$-dependent hyperaemia during exercise also needs investigating. Moreover, as adenosine increases concentrations of other metabolites (289, 306, 331), intravascular adenosine's relationship to PGs would also need investigating.

Lastly, ATP has recently been shown to influence the production of NO and vasodilator PGs (304). Moreover, as RBCs work as $O_2$ sensors and the change in ligand structure of RBCs from

oxygenated to deoxygenated results in ATP release (111, 112), a future study also needs to investigate the relationship between hyperoxia, ATP (purigenic-receptors), adenosine ($A_1$ and $A_{2A}$-receptors), and vasodilator COX products in relation to exercise hyperaemia.

# Chapter 8

# Acetylcholine (ACh) induced endothelial dilatation is blunted by 60% and 100% $O_2$, but not by 40% $O_2$

## 8.1 Introduction

Following the results of the past 4 Chapters, it was important to determine whether breathing 40% $O_2$ enhances oxidative-stress to a level that affects endothelium-dependent dilatation; and thereby, the endothelium-dependent component of exercise hyperaemia.

Furchgott and colleagues discovered that ACh-evoked vasodilatation is endothelium-dependent (133, 134). The exact mechanism by which ACh produces its dilatation remains unclear, but its presence in the cutaneous circulation is known to stimulate the NOS, COX, and non-NOS and COX vasodilator pathways (182, 206, 301, 350). The relevance of ACh-induced endothelium-dependent dilatation to this thesis lies in the observations that hyperoxia interferes with the processes that mediate its vasodilatation (263, 291, 349, 350, 442). For example, Messina et al. observed that removal of the endothelium, or COX inhibition with indomethacin eliminated the vasoconstriction evoked by increased $PO_2$ (263). Further, Yamazaki et al. showed that NOS and COX inhibition attenuated the decrease in cutaneous blood flow induced when their subjects breathed 100% $O_2$ (442); indicating that 100% $O_2$ disrupted the production and/or action of the endothelium derived vasodilators (NO and $PGI_2$). $CO_2$ values during this time remained unaffected. Similar conclusions were drawn by Rousseau et al. when they noticed that ACh-induced increase in cutaneous circulation was attenuated by ~ 30% with 100% $O_2$; this response was partially restored by oral Vitamin-C which is an anti-oxidant (350). Moreover, COX inhibition abolished the difference in ACh-induced responses between air and 100% $O_2$ breathing indicating that hyperoxia acts directly and/or indirectly to interfere with the vasodilating function of PGs.

ROS, are produced by non-enzymatic (398) and enzymatic pathways; i.e., via NADPH-oxidase, xanthine-oxidase, mitochondrial respiration, lipoxygenase, COX, cytochrome-P450 pathway,

NOS uncoupling, and lipid-peroxidation (48, 69, 320, 398, 419, 420, 449). Their relevance to ACh-induced dilatation is evident from the observations of Rubanyi and Vanhoutte (353). They noticed that the *in-vitro* ACh-induced dilatation of canine coronary and femoral arteries increased with superoxide-dismutase. Further, Cooke et al. noticed that methacholine-induced vasorelaxation of mesenteric vessels from superoxide-dismutase knockout mice was significantly attenuated relative to the wild-type mice: immunohistochemistry showed substantial increase in peroxynitrate formation in the knockout mice (66).

The importance of these observations to this thesis is increased by the studies that link different levels of hyperoxia to increased oxidative-stress. Turrens et al. observed a linear increase in ROS in isolated lung mitochondria from 0 to 60% $O_2$ (415). Beyond 60%, the increase in ROS was much more dramatic and steep. *In-vivo*, McNulty et al. showed that coronary blood flow and conductance decreased substantially (by ~ 30 – 40%); ACh-mediated dilatation was inhibited and the efflux of nitro-tyrosine increased when patients with stable coronary disease breathed 100% $O_2$ for only 15 min (256). As the deleterious effects of hyperoxia on hemodynamics are linked to oxidative-stress, a study was warranted to find out if a relatively safe range of $O_2$ concentration exists; i.e., is ACh-induced endothelium-dependent dilatation similarly affected by lower hyperoxia levels (i.e., 40 and 60%) as it is with 100% $O_2$?

Similar to the observations made for limb blood flow (358), aging is associated with a decline in the contributions of vasodilator PGs to cutaneous circulation (182). Holowatz et al. (182) observed that the peak ACh-induced increase in cutaneous blood flow was not different between age groups. However in contrast to young subjects, baseline cutaneous blood flow was augmented in older individuals by COX inhibition, and the ACh-induced dilatation was also attenuated less by COX inhibition in the older individuals. This suggests a decreased role of vasodilator PGs with ageing. Moreover, they showed that reflex cutaneous vasodilatation

induced by body heating of older individuals was enhanced by ascorbate microdialysis; so implicating ROS (181). This finding is consistent with much evidence that oxidative-stress is implicated in the very process of aging. Studies show that ROS causes greater damage to the aging DNA and in a vicious cycle, damaged DNA produce more ROS (114, 118, 259). As oxidative-stress within the cardiovascular system is a key component of the age-associated decline in endothelial function (181, 214, 417), it was particularly important to determine whether breathing different $O_2$ concentrations affected endothelium-dependent dilatation in the older individuals. As ascorbate is a potent ROS scavenger (13, 14, 54, 181, 233), the burden of oxidative-stress on cutaneous circulation with different $O_2$ concentrations was investigated using oral Vitamin-C.

### 8.1.1 Aims and hypotheses

The primary aim to the study was to determine whether endothelium-dependent dilatation would be similarly affected by breathing different supplementary $O_2$ concentrations? The study aimed to address this question in both young and older individuals. Based on the results of the previous four Chapters, it was hypothesized that 40% $O_2$ would not attenuate endothelium-dependent dilatation in either age group, but that higher $O_2$ concentrations would.

## 8.2 Methods

### 8.2.1 Acknowledgment

Before proceeding, it is important to acknowledge that the experiments on young subject were conducted by Florentyna D'Souza for her 3rd year Biomedical Science project (82). However, the data presented in this thesis were separately extracted from the original traces and analyzed. All experiments on older subject were carried out independently.

Twenty four young (19 – 25 years) men and twenty seven older (60 – 76 years) men and women were recruited; all were recreationally active non-smoking adults (8 older subjects were ex-smokers; quit at least 25 years ago). The female subjects had reached menopause at least 15 years before the study.

The medication history for the 40% $O_2$ group included occasional use of salbutamol by 2 young subjects for asthma, alfazosin-hydrochloride by 1 older male for benign prostatic-hyperplasia and mesalazine for maintenance of remission from ulcerative-colitis by a further older male. Another older male was previously a patient of aplastic-leukaemia. In the 60% $O_2$ group, 2 older men were using statins while 1 had a history of discontinued use of ramipril and bendroflumethiazide. In the 100% $O_2$ group, 1 subject was prophylactically using low-dose aspirin while another was using statins and clopidogrel for prevention of cardiovascular events. The responses of all these individuals appeared consistent with others in their respective groups. Multivitamins were used occasionally by 1 young and 6 older subjects, while omega-3 supplements were used occasionally by 1 young and 4 older subjects. All subjects were required to adhere to dietary, medicinal, and exercise restrictions previously described in Chapter 2 (See section 2.1). Subjects were also asked to stop taking multivitamins / fish-oil and NSAIDs for at least 7 days prior to the experiment. This included voluntary restriction of vasoactive drugs for ~ 2 weeks prior to the

experiments. Administration of air or $O_2$ and the recordings of HR and MABP were done as described in Chapter 2. In addition, cutaneous red cell flux (RCF) was monitored continuously by using a laser-Doppler probe as described below.

### 8.2.2 Laser-Doppler

The basic principles behind Laser-Doppler technique are similar to those used in photo-plethysmography and NIRS (31). In laser-Doppler technique, monochromatic laser light is shown on an object; the change in its frequency and optical density is measured from the back scatter and used to calculate the quantitative estimate of the object (31, 179, 280). As the objects in question in the skin are RBCs, the moving cells produce a doppler-shift in the frequency of the back scattered light which is used to calculate their velocity (31, 179). Therefore the resultant RCF calculated by laser-Doppler is a product of RBC concentration and their velocity; represented in arbitrary perfusion units (PU) (280). The technique has been compared with others and is known to provide an accurate representation of perfusion that correlates well with volume flow (179).

The low powered red light laser-Doppler probes used in this study with the laser-Doppler monitor (DRT4, Moor Instruments Ltd, UK) allowed for tissue penetration of ~ 1 mm (280). Probes were attached to the iontophoresis wells; careful consideration was given to avoiding their placement directly over any superficial veins, abrasions, skin conditions, and/or hair.

### 8.2.3 Iontophoresis

Iontophoresis is a method of delivering ions (salts) to different tissues of the body in a non-invasive manner. The 260 year old technique involves passing small electrical-current through the ionized solution and the subject; allowing the “ions” (i.e., drug) to be delivered into the subject’s tissue (374). The technique is widely used in the dermatological practice to deliver

different drugs for the treatment of skin conditions (374). This study used this well established technique to deliver ACh into the skin (166, 258, 350, 382); its affects on cutaneous RCF were then studied as described below.

### 8.2.4 Protocol

Subjects visited the laboratory on three occasions; the initial visit was used to fill in the health and activity questionnaire, consent form, and for familiarization to the experimental protocol. Subjects in each age group were randomly assigned into one of the three sub-groups; each receiving air (21% $O_2$) and one of the 3 different $O_2$ concentrations during the experiment (40, 60, or 100%: hyperoxia responses of each sub-group were compared to their own placebo / Vitamin-C data). The two experiment visits were carried out in a single-blind cross-over manner; order of placebo and Vitamin-C was randomized. As the effect of high $O_2$ levels on cardiovascular variables can persist for an hour (426), hyperoxia always followed air breathing (Figure 8.1).

On experiment days, each subject was provided with a 200 ml orange-flavoured drink (placebo or Vitamin-C; 2 g). Vitamin-C was chosen as it acts as an effective anti-oxidant (13, 14, 54, 233); 2 g oral dose significantly increases plasma ascorbate concentrations to ~ 200 μmol/L; values that peak and plateau 2 hours after oral administration, remaining as such for at least further 3 hours (233, 310). Therefore, subjects were asked to visit the laboratory three hours after consuming the drink. Upon arrival they were asked to rest in a chair with their forearms uncovered for ~ 20 min to acclimatize to the room temperature (21 – 27 °C). The variation in the recorded room temperature was the result of weather conditions; i.e., young and older subject experiments were conducted at different times during the year. However, within these age groups, the skin and room temperature measured were reasonably consistent and there were no

significant changes in temperature during experiments. Recording equipment was set up while the subject acclimatized to the room. This included Finapres for MABP and HR as previously described in Chapter 2; right hand rested on the chest. In addition, after cleaning the ventral-side of the left forearm with isopropyl-alcohol, iontophoresis electrodes (anode; contained within a Perspex ring) and an indifferent electrode were placed on it and made secure with double-sided tape. Careful consideration was given to avoid hair or any visible veins directly underneath the electrode wells. The wells were then filled with 1% ACh solution prepared using ACh-chloride and sterile-water; ~ 0.6 ml solution was placed in each well. The laser-Doppler probes were inserted into the wells for RCF recording. A schematic of the arrangement of electrodes on the arm is shown in Figure 8.2 while Figure 8.3 shows the arrangement of equipment on one of the subjects. Initial pilot trials showed that ACh-induced increase in cutaneous circulation took at least 30 min to return to baseline, and as each visit included air and hyperoxia runs, two different electrode-containing wells were used in each experiment. The ventral-side of the left arm faced up throughout the experiment.

A facemask was put on the subject to deliver air and made secure with elastic-straps around the head; he/she were asked to breathe normally (See Section 2.4). Stable baseline recording were made for ~ 10 min and then the iontophoresis protocol was started; eight pulses, seven at 100 μA and eighth at 200 μA all at 60 s intervals (166). 5 min after the end of iontophoresis protocol, the gas flow was discretely switched to a designated concentration of hyperoxia (40, 60, or 100%). An appropriate Venturi-valve was used to deliver 40% and 60% $O_2$ while 100% $O_2$ was delivered via a modified valve (the openings for atmospheric air were sealed; See Section 2.4 for gas flow rates). The flow rate was kept similar to that achieved during air breathing with the aim of ensuring the subject was treatment blinded. After 10 min of hyperoxia, the iontophoresis protocol was repeated by using the second iontophoresis well. At the end of the protocol,

recordings were made for a further 5 min before the equipment was disconnected and the subject left the laboratory. Figure 8.4 shows the raw trace recorded during one of the experiments.

### 8.2.5 Data Extraction and Statistical analysis

HR, MABP, and RCF data were extracted over 60 s periods (averaged data; i.e., baseline and 60 s after each iontophoresis pulse). RCF was expressed in perfusion units where 1 V of Laser-Doppler meter represents 100 PU (166). Responses of each hyperoxia sub-group were compared to their own placebo and Vitamin-C runs; age comparison was also done to the corresponding hyperoxia sub-group. As placebo and Vitamin-C (air breathing) runs were performed by every subject, their comparison within each age group contained all participating individuals. Baseline HR, MABP, and RCF were analyzed using 2-way ANOVA for age, treatment, and their interaction effect. Within each age group, HR, MABP, and RCF data were analyzed using 2-way repeated-measures ANOVA for time, treatment, and their interaction effects. However, between age group comparison was done using Factorial ANOVA to identify time, treatment, age, and their interaction effects. The data for each treatment were also individually analyzed using 1-way repeated-measures ANOVA to detect within time effects. Upon detection of within and/or between significant effects, Tukey's HSD was used as a post-hoc. The area under curve (AUC) for the ACh-evoked change in RCF was computed to represent the full response to ACh-iontophoresis; and expressed as AU. This data were analyzed using 2-way ANOVA for age, treatment, and their interaction effects. Student's t-test was also used to where appropriate.

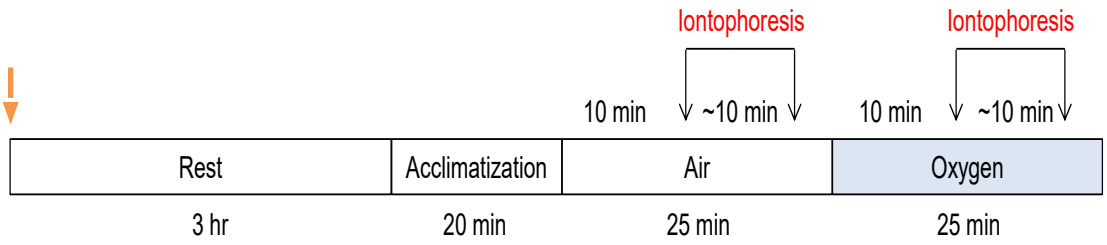



Figure 8.1 **Schematic of the protocol.** Each subject received air and one of the 3 different $O_2$ concentrations (40, 60, or 100%). The two experiment visits were carried out in a single-blind cross-over manner. Order of placebo and Vitamin-C was randomized; but hyperoxia followed air breathing during each experiment.

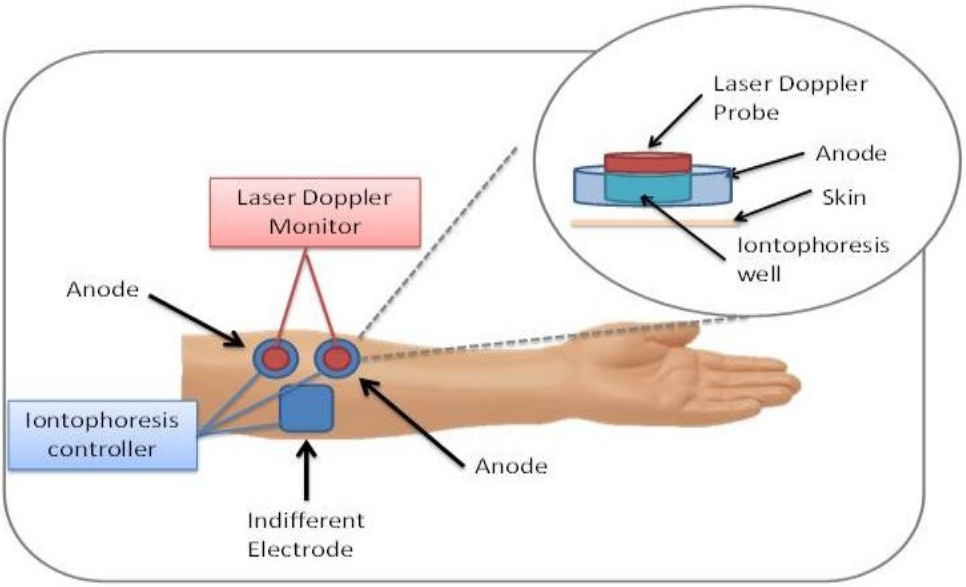



Figure 8.2 **Schematic showing the arrangement of iontophoresis electrodes and Laser-Doppler probes.** 2 anodes (1 each for different $O_2$ concentrations) were placed on the ventral surface of the left forearm, each well was filled with 1% ACh-solution for iontophoresis. Laser-Doppler probes were inserted into each well for measurement of cutaneous RCF.

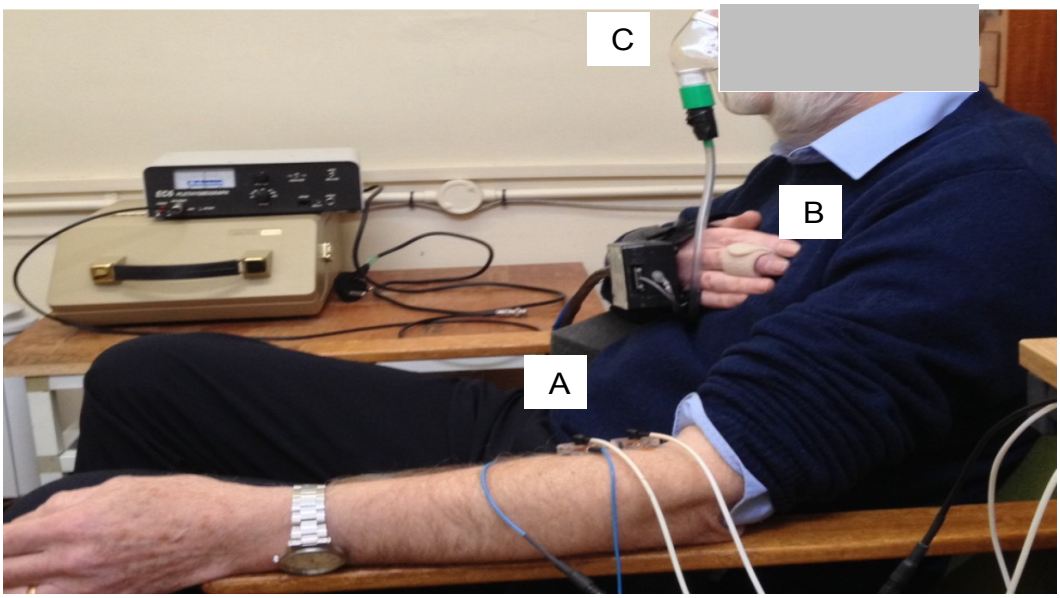



Figure 8.3 **Arrangement of equipment on the subject.** Iontophoresis electrodes were placed on the ventral surface of the left arm (A). Careful consideration was given to avoiding hair or any visible veins underneath the electrode wells. Finapres cuff was wrapped around the right middle finger for MABP measurement (B; the hand which rested on the chest). Different $O_2$ concentrations were delivered using a facemask connected to the venturi-valve (C). For subjects in 100% $O_2$ group, the holes in the valve were sealed (C).

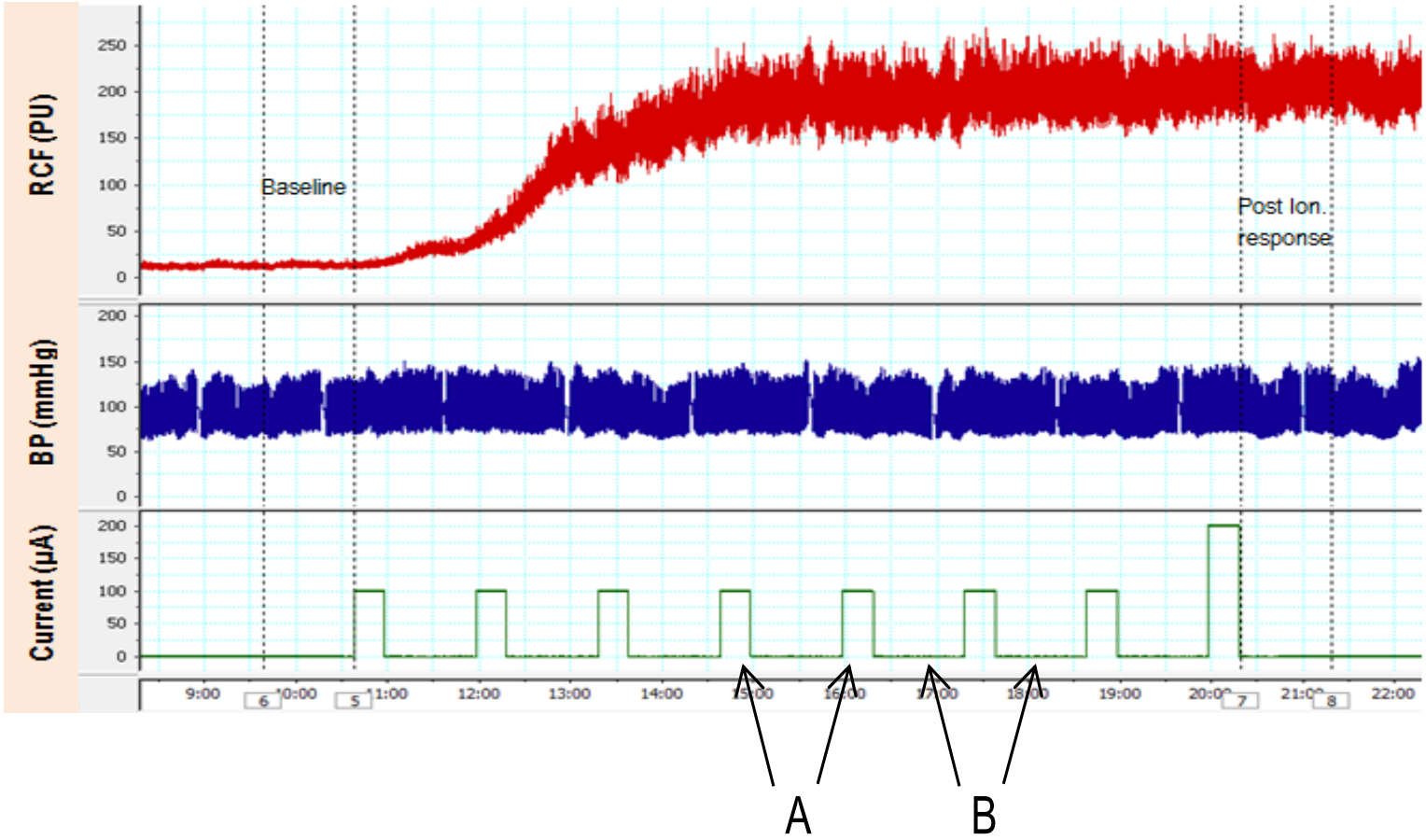



Figure 8.4 **Original trace recorded during one of the experiments.** Top (red) trace shows RCF recording, middle (blue) trace shows BP, while the bottom (green) trace shows iontophoresis current which was applied in eight 20 s pulses; seven at 100 µA (A) and eighth at 200 µA at 60 s intervals (B). Data were extracted for analysis over 60 s periods (Baseline, during iontophoresis, and post-iontophoresis; See trace). AUC was collected from the area under curve.

## 8.3 Results

Anthropometric data of the young and older subjects are shown in Table 8.1. The age and BMI of the subjects were not different between the three $O_2$ sub-groups of each age group.

### 8.3.1 Systemic cardiovascular responses

Baseline hemodynamic values were not affected by treatment condition in either age group (Table 8.2 and Table 8.3). Moreover there were no age-dependent differences in baselines (Table 8.3). HR was maintained close to baseline levels during the protocol and remained largely unaffected by the treatment conditions in either age groups (Table 8.4 and Table 8.5). Similarly, MABP values also remained largely unchanged both within and between treatments (Table 8.6 and Table 8.7). However, despite being within the normal physiological range, MABP were always higher in the older subjects (Table 8.7).

### 8.3.2 Cutaneous vascular responses

#### 8.3.2.1 Young subjects

As shown in Table 8.2, Baseline RCF was not different between treatments. Under placebo condition, cutaneous RCF increased significantly from baseline values of ~ 11 PU to 198 PU following ACh-iontophoresis. As shown in Figure 8.5, Vitamin-C did not affect the ACh-evoked response as RCF values increased from ~ 11 PU to 187 PU.

Figure 8.6 shows RCF and AUC recorded while breathing different $O_2$ concentrations. 40% $O_2$ had no effect on the ACh-induced increase in RCF but both 60 and 100% $O_2$ attenuated the peak increase in RCF to 180 and 160 PU, respectively. The AUC during placebo was ~ 14 AU which was attenuated to 10.24 and 8.89 AU with 60 and 100% $O_2$, respectively. By contrast as Figure 8.7 shows, after Vitamin-C the RCF and AUC values recorded during 60% $O_2$ (i.e.,

Vitamin-C + 60% $O_2$) were similar to those recorded during the placebo / Vitamin-C trials; RCF and AUC of 100% $O_2$ (i.e., Vitamin-C + 100% $O_2$) still remained significantly attenuated.

#### 8.3.2.2 Older subjects

Under placebo condition of air breathing and orange-flavoured drink, cutaneous RCF increased from baseline values of ~ 14 PU to 131 PU. As shown in Figure 8.8, the ACh-evoked increase in RCF after Vitamin-C was very similar, from ~ 13 PU to 136 PU. However, these ACh responses, whether considered as RCF or AUC, were much smaller than in young subjects (See Figure 8.5 and Figure 8.8).

As in young subjects, the ACh-induced increases in RCF responses recorded in older subjects were not attenuated by 40% $O_2$, but were attenuated with both 60 and 100% $O_2$ to 73 and 68 PU, respectively (Figure 8.9). Similarly, the AUC during placebo was ~ 9 AU and was attenuated to 6.6 and 4.8 AU with 60 and 100% $O_2$. After Vitamin-C the ACh-induced increases in RCF and AUC were augmented during both 60 and 100% $O_2$, but they were still significantly attenuated compared to placebo / Vitamin-C (i.e., air breathing responses; Figure 8.10).

| | Young Subjects | | | | | | Older Subjects | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Group 1 – 100% $O_2$ | | Group 2 – 60% $O_2$ | | Group 3 – 40% $O_2$ | | Group 1 – 100% $O_2$ | | Group 2 – 60% $O_2$ | | Group 3 – 40% $O_2$ | |
| | Age (yr) | BMI ($Kg/m^2$) | Age (yr) | BMI ($Kg/m^2$) | Age (yr) | BMI ($Kg/m^2$) | Age (yr) | BMI ($Kg/m^2$) | Age (yr) | BMI ($Kg/m^2$) | Age (yr) | BMI ($Kg/m^2$) |
| | 20♂ | 19.5 | 20♂ | 21.6 | 21♂ | 21.9 | 68♂ | 20.2 | 66♂ | 22.3 | 77♂ | 23.8 |
| | 21♂ | 21.6 | 20♂ | 24.3 | 21♂ | 18.1 | 68♂ | 25.7 | 78♂ | 21.3 | 65♂ | 24.8 |
| | 21♂ | 21.0 | 21♂ | 22.1 | 21♂ | 22.4 | 70♂ | 22.8 | 77♂ | 20.9 | 76♂ | 23.8 |
| | 21♂ | 20.6 | 21♂ | 21.0 | 22♂ | 22.0 | 77♂ | 24.2 | 76♂ | 24.2 | 72♂ | 24.1 |
| | 20♂ | 24.7 | 20♂ | 20.9 | 21♂ | 24.1 | 66♂ | 24.5 | 66♂ | 20.7 | 69♂ | 22.4 |
| | 20♂ | 20.7 | 21♂ | 24.8 | 21♂ | 24.4 | 62♂ | 24.4 | 65♂ | 19.0 | 67♀ | 23.1 |
| | 21♂ | 21.9 | 21♂ | 21.5 | 20♂ | 21.0 | 78♀ | 24.0 | 67♂ | 24.6 | 68♂ | 23.1 |
| | 22♂ | 22.1 | 22♂ | 23.9 | 20♂ | 19.0 | 68♀ | 17.8 | 66♀ | 25.1 | 68♀ | 21.6 |
| | | | | | | | 68♂ | 25.5 | 76♀ | 24.5 | 70♀ | 22.4 |
| Mean | 20.75 | 21.51 | 20.75 | 22.51 | 20.88 | 21.61 | 69.44 | 23.23 | 70.78 | 22.52 | 70.22 | 23.24 |
| SEM | 0.25 | 0.54 | 0.25 | 0.55 | 0.23 | 0.78 | 1.69 | 0.86 | 1.91 | 0.72 | 1.35 | 0.33 |

Table 8.1 **Anthropometric data of young and older subjects**. Female participants were included only in the older group.

| Age | n | Treatments | Baseline Responses | | |
|---|---|---|---|---|---|
| | | | HR | MABP | RCF |
| Young | 24 | Placebo | 65 ± 3 | 86 ± 2 | 11.37 ± 0.47 |
| | | Vitamin C | 65 ± 2 | 85 ± 2 | 11.35 ± 0.37 |
| | | P Value (Treatment) | *0.97* | *0.70* | *0.41* |
| | 8 | Placebo | 66 ± 5 | 88 ± 3 | 11.92 ± 3.86 |
| | | Vitamin C | 63 ± 4 | 85 ± 2 | 11.48 ± 4.06 |
| | | 40% $O_2$ | 63 ± 3 | 88 ± 1 | 10.98 ± 4.91 |
| | | 40% $O_2$ + Vitamin C | 63 ± 3 | 87 ± 3 | 13.59 ± 4.78 |
| | | P Value (Treatment) | *0.71* | *0.34* | *0.27* |
| | 8 | Placebo | 64 ± 8 | 84 ± 3 | 11.80 ± 3.81 |
| | | Vitamin C | 62 ± 3 | 83 ± 2 | 11.28 ± 3.99 |
| | | 60% $O_2$ | 59 ± 2 | 89 ± 4 | 11.53 ± 4.08 |
| | | 60% $O_2$ + Vitamin C | 58 ± 3 | 87 ± 2 | 11.62 ± 3.76 |
| | | P Value (Treatment) | *0.20* | *0.13* | *0.74* |
| | 8 | Placebo | 63 ± 5 | 88 ± 3 | 12.39 ± 3.38 |
| | | Vitamin C | 62 ± 4 | 86 ± 4 | 11.29 ± 3.99 |
| | | 100% $O_2$ | 61 ± 2 | 88 ± 2 | 13.64 ±3.82 |
| | | 100% $O_2$ + Vitamin C | 59 ± 2 | 80 ± 2 | 11.66 ± 3.12 |
| | | P Value (Treatment) | *0.96* | *0.35* | *0.79* |

Table 8.2 **Baseline haemodynamic values of young subjects.** Baseline values were recorded before iontophoresis and were not affected by treatment within each hyperoxia sub-group.

| Age | n | Treatments | | Baseline Responses | | |
|---|---|---|---|---|---|---|
| | | | | HR | MABP | RCF |
| Older | 27 | Placebo | | 65 ± 2 | 88 ± 3 | 14.04 ± 4.24 |
| | | Vitamin C | | 64 ± 2 | 88 ± 2 | 13.06 ± 3.92 |
| | | P Value | Treatment | *0.66* | *0.78* | *0.21* |
| | | | Age | *0.59* | *0.07* | *0.37* |
| | | | Interaction | *0.89* | *0.61* | *0.17* |
| | 9 | Placebo | | 66 ± 3 | 85 ± 3 | 14.77 ± 8.04 |
| | | Vitamin C | | 64 ± 2 | 88 ± 2 | 14.85 ± 6.20 |
| | | 40% $O_2$ | | 61 ± 3 | 88 ± 4 | 13.25 ± 2.48 |
| | | 40% $O_2$ + Vitamin C | | 62 ± 3 | 87 ± 2 | 10.67 ± 1.79 |
| | | P Value | Treatment | *0.52* | *0.56* | *0.14* |
| | | | Age | *0.83* | *0.07* | *0.13* |
| | | | Interaction | *0.91* | *0.62* | *0.26* |
| | 9 | Placebo | | 66 ±3 | 88 ± 6 | 12.90 ± 6.04 |
| | | Vitamin C | | 66 ± 2 | 88 ± 3 | 11.86 ± 6.93 |
| | | 60% $O_2$ | | 63 ± 3 | 92 ± 6 | 9.31 ± 1.30 |
| | | 60% $O_2$ + Vitamin C | | 63 ± 3 | 92 ± 3 | 11.41 ± 3.11 |
| | | P Value | Treatment | *0.71* | *0.84* | *0.84* |
| | | | Age | *0.16* | *0.08* | *0.36* |
| | | | Interaction | *0.83* | *0.99* | *0.63* |
| | 9 | Placebo | | 62 ± 2 | 92 ± 4 | 12.45 ± 6.83 |
| | | Vitamin C | | 63 ± 3 | 92 ± 3 | 12.14 ± 7.30 |
| | | 100% $O_2$ | | 60 ± 3 | 90 ± 5 | 9.65 ± 1.87 |
| | | 100% $O_2$ + Vitamin C | | 61 ± 3 | 91 ± 4 | 9.38 ± 1.15 |
| | | P Value | Treatment | *0.91* | *0.99* | *0.28* |
| | | | Age | *0.78* | *0.06* | *0.24* |
| | | | Interaction | *0.21* | *0.34* | *0.14* |

Table 8.3 **Baseline haemodynamic values of older subjects.** Baseline values were recorded before iontophoresis and were not affected by treatment within each hyperoxia sub-group. Moreover, an age effect was also not detected; although a trend for MABP was present.

| Young | | | Baseline | Iontophoresis | | | | | | | | P Value (within) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | Treatment | | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th | 8th | |
| HR (b.min$^{-1}$) | 24 | Placebo | 65 ± 3 | 65 ± 3 | 66 ± 3 | 65 ± 3 | 67 ± 3 | 65 ± 3 | 67 ± 3 | 67 ± 3 | 66 ± 3 | *1.00* |
| | | Vitamin C | 65 ± 2 | 64 ± 2 | 65 ± 2 | 65 ± 2 | 65 ± 2 | 64 ± 2 | 65 ± 2 | 65 ± 2 | 66 ± 2 | *1.00* |
| | | P Value | Time = *0.98* | | | Treatment = *0.12* | | | Time*Treatment = *1.00* | | | |
| | 8 | Placebo | 64 ± 5 | 64 ± 6 | 66 ± 6 | 65 ± 6 | 68 ± 7 | 65 ± 6 | 65 ± 7 | 64 ± 5 | 65 ± 6 | *1.00* |
| | | Vitamin C | 62 ± 4 | 62 ± 4 | 62 ± 4 | 63 ± 4 | 63 ± 4 | 62 ± 4 | 61 ± 4 | 62 ± 4 | 62 ± 3 | *1.00* |
| | | 40% $O_2$ | 63 ± 3 | 63 ± 4 | 65 ± 3 | 65 ± 4 | 64 ± 5 | 68 ± 4 | 66 ± 4 | 66 ± 4 | 65 ± 4 | *0.96* |
| | | 40% $O_2$ + Vitamin C | 62 ± 3 | 61 ± 3 | 62 ± 3 | 66 ± 4 | 63 ± 3 | 64 ± 3 | 63 ± 4 | 64 ± 4 | 63 ± 4 | *0.99* |
| | | P Value | Time = *1.00* | | | Treatment = *0.12* | | | Time*Treatment = *1.00* | | | |
| | 8 | Placebo | 65 ± 7 | 64 ± 7 | 67 ± 6 | 67 ± 6 | 63 ± 5 | 64 ± 7 | 64 ± 3 | 66 ± 6 | 63 ± 6 | *0.77* |
| | | Vitamin C | 62 ± 3 | 64 ± 4 | 66 ± 3 | 64 ± 4 | 64 ± 4 | 63 ± 4 | 65 ± 4 | 63 ± 5 | 66 ± 7 | *1.00* |
| | | 60% $O_2$ | 59 ± 2 | 61 ± 3 | 59 ±1 | 60 ± 2 | 58 ± 2 | 58 ± 2 | 64 ± 3 | 60 ± 3 | 62 ± 4 | *0.79* |
| | | 60% $O_2$ + Vitamin C | 58 ± 3 | 61 ± 2 | 58 ± 2 | 60 ± 2 | 60 ± 3 | 60 ± 2 | 62 ± 3 | 63 ± 4 | 63 ± 3 | *0.61* |
| | | P Value | Time = *0.20* | | | Treatment = *0.15* | | | Time*Treatment = *0.99* | | | |
| | 8 | Placebo | 63 ± 5 | 63 ± 4 | 63 ± 4 | 63 ± 4 | 63 ± 4 | 63 ± 4 | 62 ± 3 | 60 ± 2 | 61 ± 3 | *0.98* |
| | | Vitamin C | 62 ± 4 | 66 ± 3 | 66 ± 3 | 65 ± 4 | 65 ± 2 | 65 ± 1 | 66 ± 3 | 66 ± 3 | 66 ± 2 | *0.69* |
| | | 100% $O_2$ | 61 ± 2 | 62 ± 2 | 63 ±2 | 59 ± 2 | 64 ± 3 | 62 ± 1 | 61 ± 2 | 62 ± 1 | 59 ± 2 | *0.95* |
| | | 100% $O_2$ + Vitamin C | 59 ± 2 | 58 ± 2 | 61 ± 3 | 59 ± 2 | 60 ± 3 | 60 ± 3 | 59 ± 3 | 62 ± 3 | 58 ± 2 | *1.00* |
| | | P Value | Time = *0.97* | | | Treatment = *0.08* | | | Time*Treatment = *0.99* | | | |

Table 8.4 **Baseline and post ACh-iontophoresis HR of young men.** No significant affect was detected within and between treatments.

| Older | | | Baseline | Iontophoresis | | | | | | | | P Value (within) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | Treatment | | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th | 8th | |
| HR (b.min$^{-1}$) | 27 | Placebo | 65 ± 2 | 65 ± 2 | 64 ± 2 | 65 ± 2 | 65 ± 2 | 65 ± 2 | 64 ± 2 | 66 ± 2 | 65 ± 2 | *1.00* |
| | | Vitamin C | 64 ± 2 | 63 ± 2 | 64 ± 2 | 65 ± 2 | 64 ± 2 | 63 ± 2 | 64 ± 2 | 64 ± 2 | 65 ± 2 | *0.99* |
| | | P Value | Time = *0.99* Treatment = *0.51* Age = *0.47* Time*Treatment = *0.99* Age*Treatment = *0.81* | | | | | | | | | |
| | 9 | Placebo | 66 ± 4 | 64 ± 4 | 65 ± 3 | 65 ± 3 | 65 ± 3 | 66 ± 4 | 65 ± 3 | 67 ± 4 | 65 ± 4 | *1.00* |
| | | Vitamin C | 64 ± 2 | 64 ± 2 | 64 ± 3 | 66 ± 3 | 63 ± 3 | 61 ± 2 | 64 ± 3 | 63 ± 3 | 65 ± 3 | *0.95* |
| | | 40% $O_2$ | 61 ± 3 | 62 ± 3 | 62 ± 3 | 64 ± 3 | 64 ± 3 | 65 ± 3 | 62 ± 2 | 64 ± 3 | 63 ± 3 | *0.99* |
| | | 40% $O_2$ + Vitamin C | 62 ± 3 | 61 ± 2 | 64 ± 2 | 64 ± 3 | 63 ± 2 | 62 ± 2 | 64 ± 2 | 63 ± 2 | 63 ± 2 | *1.00* |
| | | P Value | Time = *1.00* Treatment = *0.07* Age = *0.88* Time*Treatment = *1.00* Age*Treatment = *0.14* | | | | | | | | | |
| | 9 | Placebo | 66 ± 2 | 66 ± 3 | 65 ± 3 | 66 ± 3 | 66 ± 2 | 66 ± 3 | 65 ± 3 | 66 ± 3 | 66 ± 2 | *0.99* |
| | | Vitamin C | 66 ± 3 | 65 ± 3 | 66 ± 3 | 67 ± 3 | 67 ± 4 | 66 ± 3 | 67 ± 3 | 65 ± 3 | 66 ± 4 | *1.00* |
| | | 60% $O_2$ | 63 ± 3 | 62 ± 3 | 63 ± 2 | 63 ± 3 | 64 ± 2 | 62 ± 3 | 64 ± 3 | 64 ± 3 | 63 ± 2 | *1.00* |
| | | 60% $O_2$ + Vitamin C | 63 ± 3 | 63 ± 3 | 63 ± 3 | 63 ± 3 | 63 ± 3 | 61 ± 3 | 62 ± 3 | 62 ± 3 | 63 ± 3 | *1.00* |
| | | P Value | Time = *0.99* Treatment = *0.05* Age = *0.14* Time*Treatment = *1.00* Age*Treatment = *0.33* | | | | | | | | | |
| | 9 | Placebo | 62 ± 2 | 63 ± 2 | 62 ± 2 | 62 ± 3 | 63 ± 3 | 63 ± 2 | 63 ± 3 | 65 ± 3 | 64 ± 3 | *1.00* |
| | | Vitamin C | 63 ± 3 | 59 ± 3 | 59 ± 2 | 63 ± 3 | 62 ± 3 | 63 ± 3 | 62 ± 3 | 62 ± 3 | 62 ± 3 | *0.98* |
| | | 100% $O_2$ | 60 ± 3 | 59 ± 2 | 60 ± 3 | 60 ± 3 | 61 ± 3 | 60 ± 3 | 62 ± 4 | 63 ± 3 | 60 ± 2 | *0.99* |
| | | 100% $O_2$ + Vitamin C | 61 ± 3 | 61 ± 3 | 62 ± 3 | 61 ± 3 | 61 ± 3 | 60 ± 3 | 61 ± 3 | 60 ± 3 | 61 ± 3 | *0.99* |
| | | P Value | Time = *0.99* Treatment = *0.30* Age = *0.52* Time*Treatment = *1.00* Age*Treatment = *0.06* | | | | | | | | | |

Table 8.5 **Baseline and post ACh-iontophoresis HR values of older subjects.** No significant affect was detected within and between treatments. Moreover, no age effect was detected.

| Young | | | Baseline | Iontophoresis | | | | | | | | P Value (within) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | Treatment | | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th | 8th | |
| MABP (mmHg) | 24 | Placebo | 86 ± 2 | 86 ± 2 | 86 ± 2 | 85 ± 2 | 86 ± 2 | 85 ± 2 | 86 ± 2 | 85 ± 2 | 85 ± 2 | *1.00* |
| | | Vitamin C | 85 ± 2 | 85 ± 2 | 85 ± 2 | 86 ± 2 | 85 ± 2 | 85 ± 1 | 86 ± 2 | 86 ± 1 | 86 ± 2 | *1.00* |
| | | P Value | Time = *1.00* | | | Treatment = *0.61* | | | Time*Treatment = *0.98* | | | |
| | 8 | Placebo | 88 ± 3 | 87 ± 3 | 87 ± 3 | 87 ± 3 | 87 ± 3 | 87 ± 3 | 87 ± 3 | 87 ± 3 | 87 ± 2 | *0.97* |
| | | Vitamin C | 85 ± 2 | 84 ± 2 | 85 ± 2 | 85 ± 2 | 84 ± 2 | 85 ± 2 | 85 ± 2 | 86 ± 2 | 86 ± 2 | *0.99* |
| | | 40% $O_2$ | 88 ± 1 | 86 ± 1 | 87 ± 1 | 89 ± 2 | 90 ± 2 | 90 ± 2 | 89 ± 2 | 89 ± 2 | 91 ± 2 | *0.69* |
| | | 40% $O_2$ + Vitamin C | 87 ± 3 | 87 ± 2 | 87 ± 2 | 86 ± 3 | 86 ± 3 | 88 ± 3 | 87 ± 3 | 89 ± 3 | 87 ± 4 | *0.09* |
| | | P Value | Time = *0.99* | | | Treatment = *0.11* | | | Time*Treatment = *1.00* | | | |
| | 8 | Placebo | 80 ± 2 | 82 ± 2 | 81 ± 2 | 81 ± 2 | 81 ± 3 | 80 ± 3 | 84 ± 4 | 82 ± 3 | 80 ± 2 | *0.28* |
| | | Vitamin C | 83 ± 2 | 84 ± 2 | 84 ± 2 | 84 ± 2 | 85 ± 2 | 85 ± 2 | 86 ± 2 | 85 ± 2 | 85 ± 2 | *1.00* |
| | | 60% $O_2$ | 89 ± 4 | 88 ± 4 | 88 ± 4 | 89 ± 4 | 89 ± 4 | 88 ± 4 | 88 ± 4 | 87 ± 3 | 88 ± 3 | *1.00* |
| | | 60% $O_2$ + Vitamin C | 87 ± 2 | 85 ± 3 | 85 ± 3 | 86 ± 3 | 84 ± 2 | 87 ± 2 | 87 ± 2 | 87 ± 2 | 86 ± 2 | *1.00* |
| | | P Value | Time = *1.00* | | | Treatment = *0.05* | | | Time*Treatment = *1.00* | | | |
| | 8 | Placebo | 85 ± 3 | 86 ± 3 | 87 ± 3 | 88 ± 3 | 86 ± 3 | 85 ± 3 | 87 ± 3 | 85 ± 3 | 84 ± 2 | *1.00* |
| | | Vitamin C | 86 ± 4 | 86 ± 5 | 86 ± 4 | 87 ± 3 | 88 ± 4 | 86 ± 3 | 86 ± 5 | 87 ± 3 | 87 ± 4 | *1.00* |
| | | 100% $O_2$ | 87 ± 1 | 89 ± 2 | 88 ± 2 | 89 ± 2 | 89 ± 2 | 88 ± 2 | 87 ± 1 | 86 ± 2 | 85 ± 2 | *0.65* |
| | | 100% $O_2$ + Vitamin C | 80 ± 2 | 81 ± 2 | 81 ± 2 | 82 ± 2 | 83 ± 2 | 83 ± 3 | 84 ± 3 | 85 ± 3 | 86 ± 3 | *1.00* |
| | | P Value | Time = *0.99* | | | Treatment = *0.06* | | | Time*Treatment = *0.59* | | | |

Table 8.6 **Baseline and post ACh-iontophoresis MABP values of young men.** No significant affect was detected within and between treatments.

| Older | | | Baseline | Iontophoresis | | | | | | | | P Value (within) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | Treatment | | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th | 8th | |
| MABP (mmHg) | 27 | Placebo | 88 ± 3 | 87 ± 3 | 87 ± 3 | 88 ± 2 | 88 ± 2 | 89 ± 3 | 90 ± 3 | 89 ± 3 | 89 ± 3 | *0.99* |
| | | Vitamin C | 88 ± 2 | 88 ± 2 | 90 ± 2 | 90 ± 2 | 91 ± 2 | 90 ± 2 | 90 ± 2 | 90 ± 2 | 90 ± 2 | *0.98* |
| | | P Value | Time = *0.96* Treatment = *0.29* Age = *0.001* Time*Treatment = *1.00* Age*Treatment = *0.96* | | | | | | | | | |
| | 9 | Placebo | 86 ± 3 | 84 ± 4 | 84 ± 4 | 83 ± 4 | 84 ± 4 | 86 ± 4 | 86 ± 4 | 85 ± 4 | 84 ± 4 | *1.00* |
| | | Vitamin C | 85 ± 3 | 84 ± 3 | 84 ± 3 | 86 ± 3 | 87 ± 3 | 84 ± 3 | 85 ± 3 | 85 ± 3 | 86 ± 4 | *0.99* |
| | | 40% $O_2$ | 88 ± 4 | 89 ± 4 | 87 ± 4 | 88 ± 4 | 88 ± 4 | 87 ± 4 | 88 ± 4 | 88 ± 4 | 89 ± 4 | *0.99* |
| | | 40% $O_2$ + Vitamin C | 87 ± 2 | 87 ± 3 | 89 ± 2 | 88 ± 3 | 90 ± 2 | 89 ± 3 | 91 ± 4 | 90 ± 3 | 92 ± 3 | *0.99* |
| | | P Value | Time = *0.94* Treatment = *0.12* Age = *< 0.001* Time*Treatment = *1.00* Age*Treatment = *0.01* | | | | | | | | | |
| | 9 | Placebo | 88 ± 6 | 88 ± 5 | 86 ± 5 | 88 ± 4 | 88 ± 4 | 91 ± 5 | 90 ± 6 | 88 ± 6 | 91 ± 6 | *1.00* |
| | | Vitamin C | 88 ± 3 | 90 ± 3 | 92 ± 4 | 91 ± 3 | 93 ± 3 | 94 ± 3 | 94 ± 3 | 93 ± 4 | 93 ± 3 | *0.94* |
| | | 60% $O_2$ | 92 ± 6 | 92 ± 5 | 91 ± 4 | 91 ± 4 | 92 ± 5 | 92 ± 4 | 92 ± 5 | 92 ± 5 | 92 ± 5 | *1.00* |
| | | 60% $O_2$ + Vitamin C | 92 ± 3 | 88 ± 3 | 90 ± 4 | 92 ± 4 | 91 ± 4 | 93 ± 4 | 93 ± 4 | 94 ± 3 | 94 ± 3 | *0.93* |
| | | P Value | Time = *0.86* Treatment = *0.07* Age = *< 0.001* Time*Treatment = *1.00* Age*Treatment = *0.001* | | | | | | | | | |
| | 9 | Placebo | 93 ± 4 | 91 ± 5 | 91 ± 5 | 94 ± 4 | 92 ± 4 | 92 ± 4 | 95 ± 4 | 95 ± 3 | 94 ± 3 | *1.00* |
| | | Vitamin C | 92 ± 3 | 92 ± 4 | 94 ± 4 | 94 ± 4 | 93 ± 4 | 93 ± 3 | 92 ± 4 | 93 ± 4 | 93 ± 4 | *1.00* |
| | | 100% $O_2$ | 90 ± 5 | 92 ± 4 | 93 ± 4 | 94 ± 3 | 93 ± 4 | 94 ± 4 | 94 ± 5 | 95 ± 4 | 94 ± 4 | *1.00* |
| | | 100% $O_2$ + Vitamin C | 91 ± 4 | 90 ± 4 | 90 ± 5 | 91 ± 4 | 91 ± 4 | 93 ± 4 | 91 ± 4 | 94 ± 5 | 94 ± 5 | *0.63* |
| | | P Value | Time = *0.97* Treatment = *0.17* Age = *< 0.001* Time*Treatment = *1.00* Age*Treatment = *< 0.001* | | | | | | | | | |

Table 8.7 **Baseline and post ACh-iontophoresis MABP values of older subjects.** No significant affect was detected within and between treatments. However, overall MABP values were higher in older men.

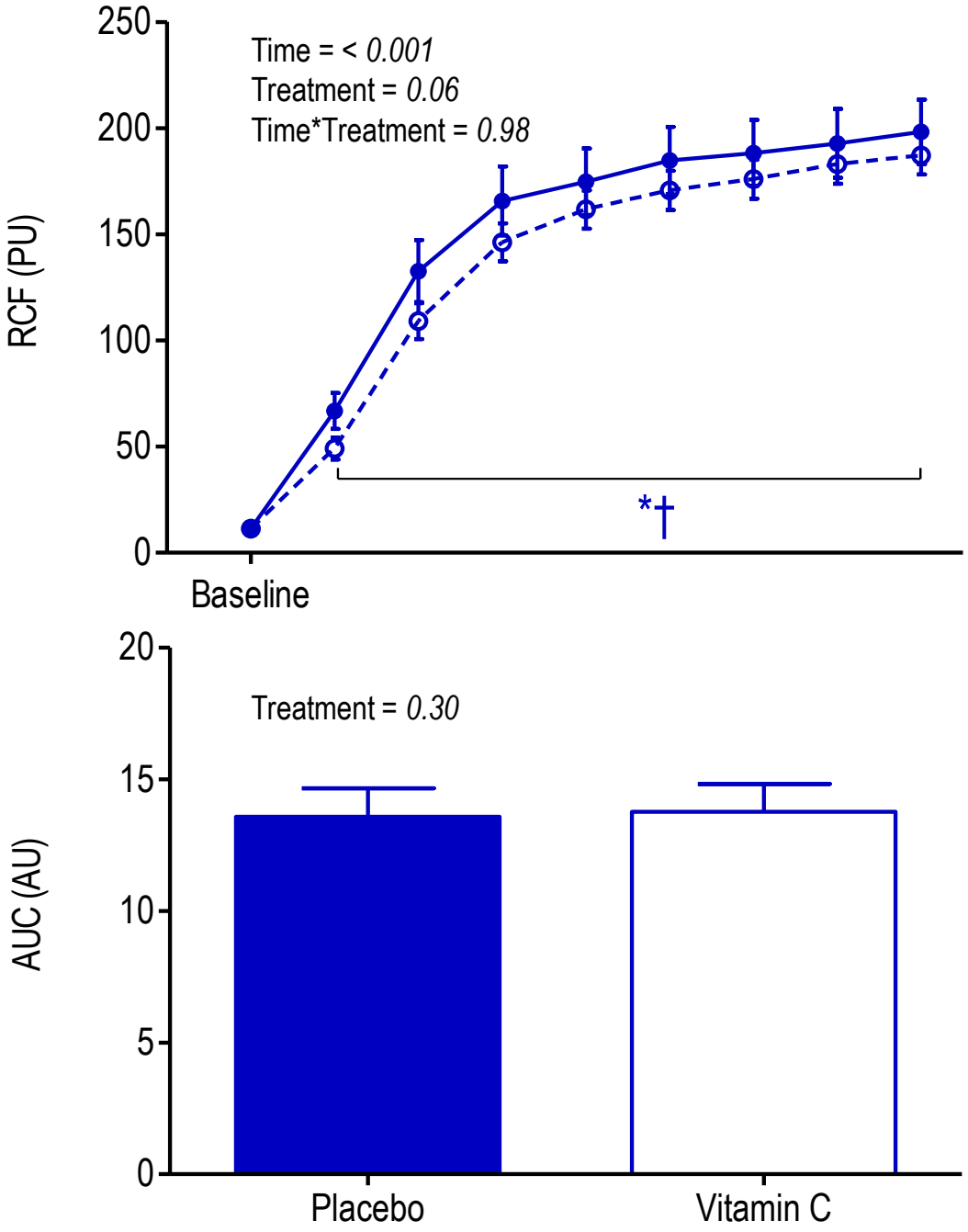



Figure 8.5 **Cutaneous vasodilator responses evoked by ACh-iontophoresis in young men (n = 24).** Upper panel shows RCF values while the lower panel shows AUC. Placebo is shown by solid line and column while Vitamin-C is shown by dashed line and open column. Cutaneous RCF increased significantly in response to ACh-iontophoresis, but the magnitude of this increase was not affected by Vitamin-C (AUC data). *,† represent placebo and Vitamin-C values that were significantly different from respective baselines (P < 0.001).

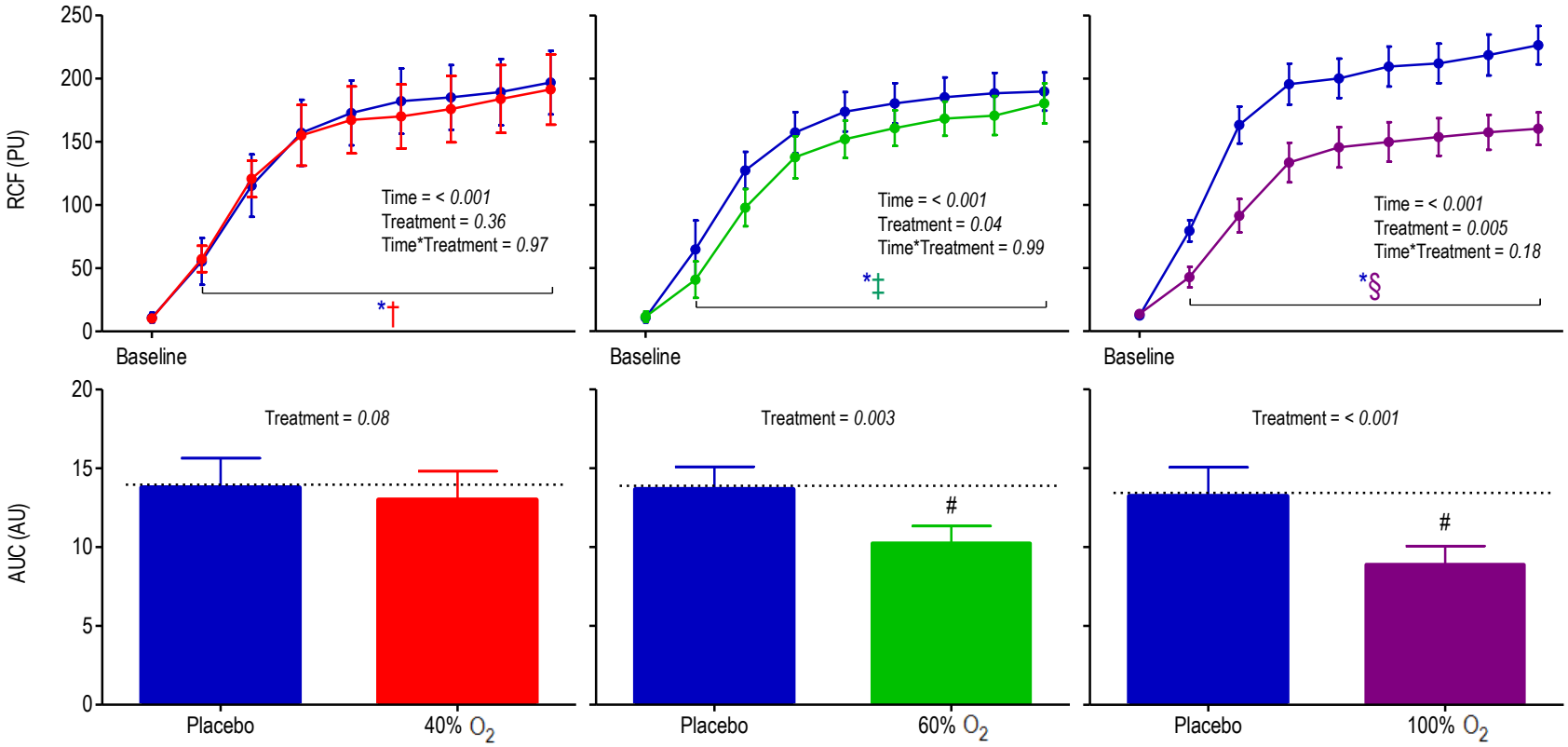



Figure 8.6 **Cutaneous vasodilator responses evoked by ACh-iontophoresis in young men during placebo, 40, 60, and 100% $O_2$.** Each hyperoxia sub-group's data are compared to their own placebo data; n = 8 in each sub-group. Cutaneous RCF increased significantly in response to ACh-iontophoresis but both 60 and 100% $O_2$ significantly attenuated this increase. */†/‡/§ represent placebo, 40, 60, and 100% $O_2$ values that were significantly different from respective baselines ($P < 0.001$). # represents treatment effect on the total ACh-induced vasodilatation ($P < 0.01$ vs. respective placebo response).

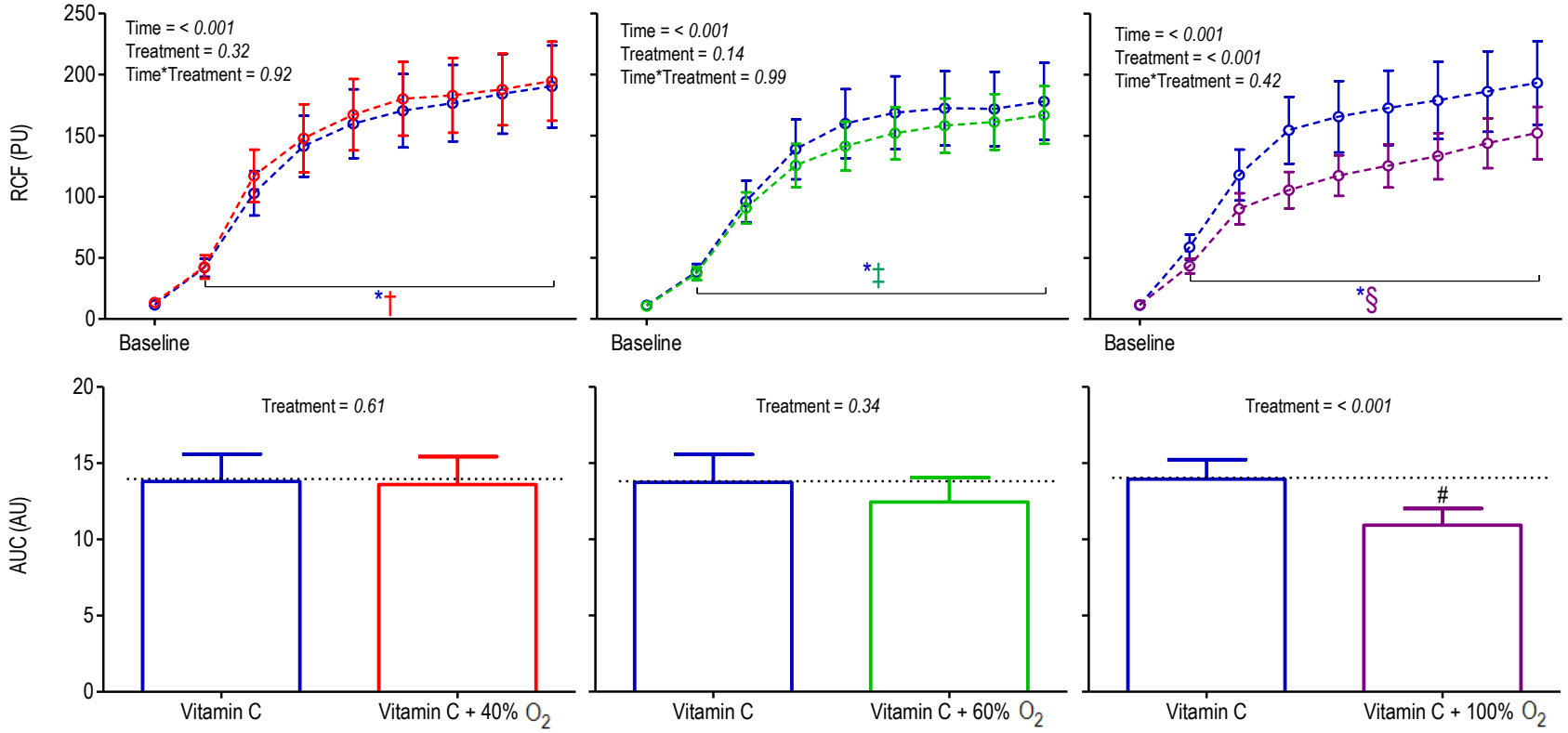



Figure 8.7 **Cutaneous vasodilator responses evoked by ACh-iontophoresis in young men during combination of Vitamin-C and different $O_2$ concentrations.** Each hyperoxia sub-group's data are compared to their own Vitamin-C data; n = 8 in each sub-group. Vitamin-C prevented significant decline in cutaneous RCF with 60% $O_2$ but responses of 100% $O_2$ still remained largely unaffected. */†/‡/§ represents Vitamin-C, Vitamin-C + 40%, Vitamin-C + 60%, and Vitamin-C + 100% $O_2$ values that were significantly different from respective baselines ($P < 0.001$). # represents significant treatment effect on the total ACh-induced vasodilatation response ($P < 0.01$ vs. respective Vitamin-C response).

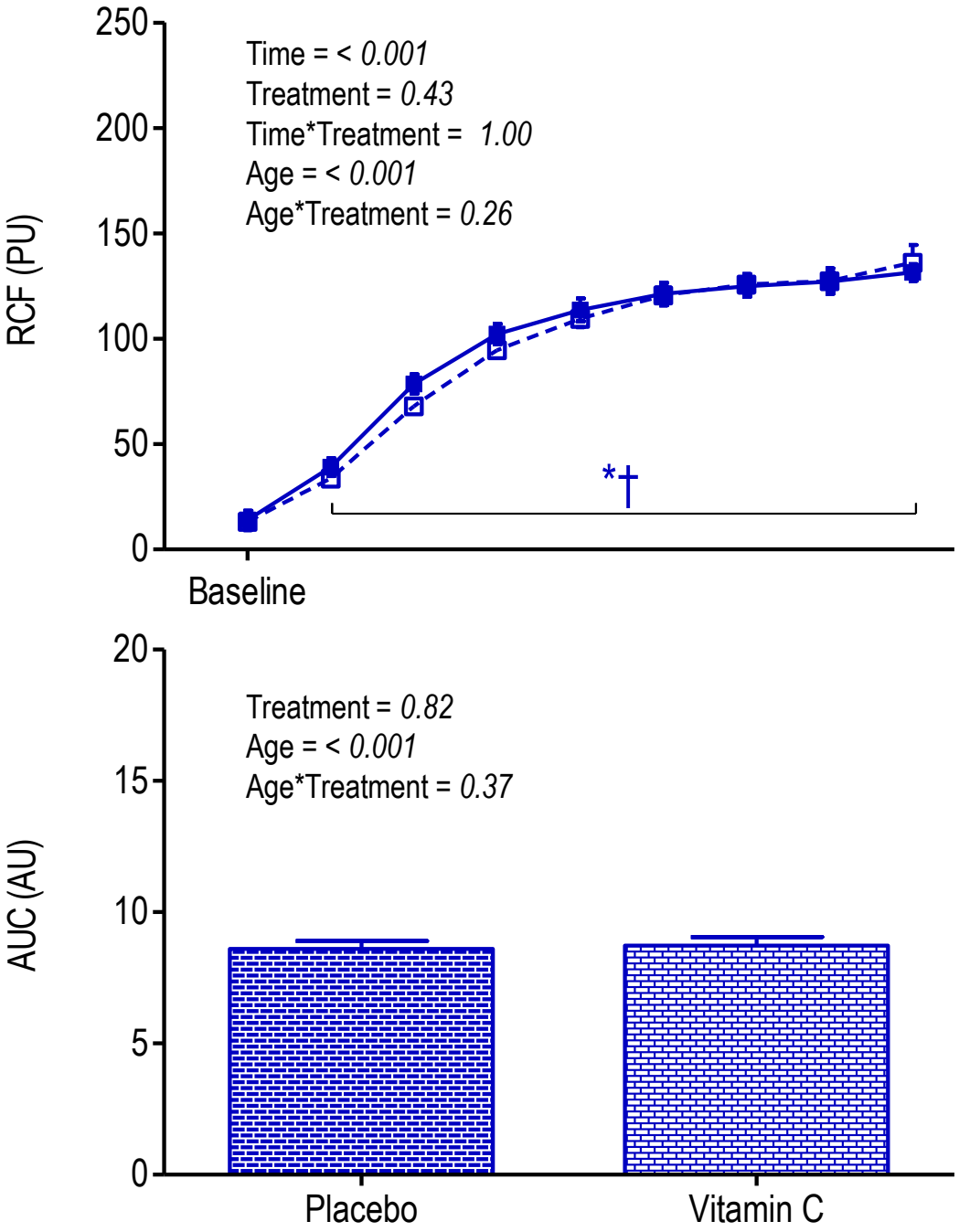



Figure 8.8 **Cutaneous vasodilator responses evoked by ACh-iontophoresis in older individuals (n = 27).** Upper panel shows RCF while the lower panel shows AUC during placebo (solid line and filled brick column) or Vitamin-C (dashed line and open brick column). Cutaneous RCF increased significantly in response to ACh-iontophoresis but the magnitude of this increase was unaffected by Vitamin-C. However, ACh's responses were significantly less in older participants during both placebo and Vitamin-C trials. *,† represent time effect of placebo and Vitamin-C, respectively ($P < 0.001$ vs. respective baseline).

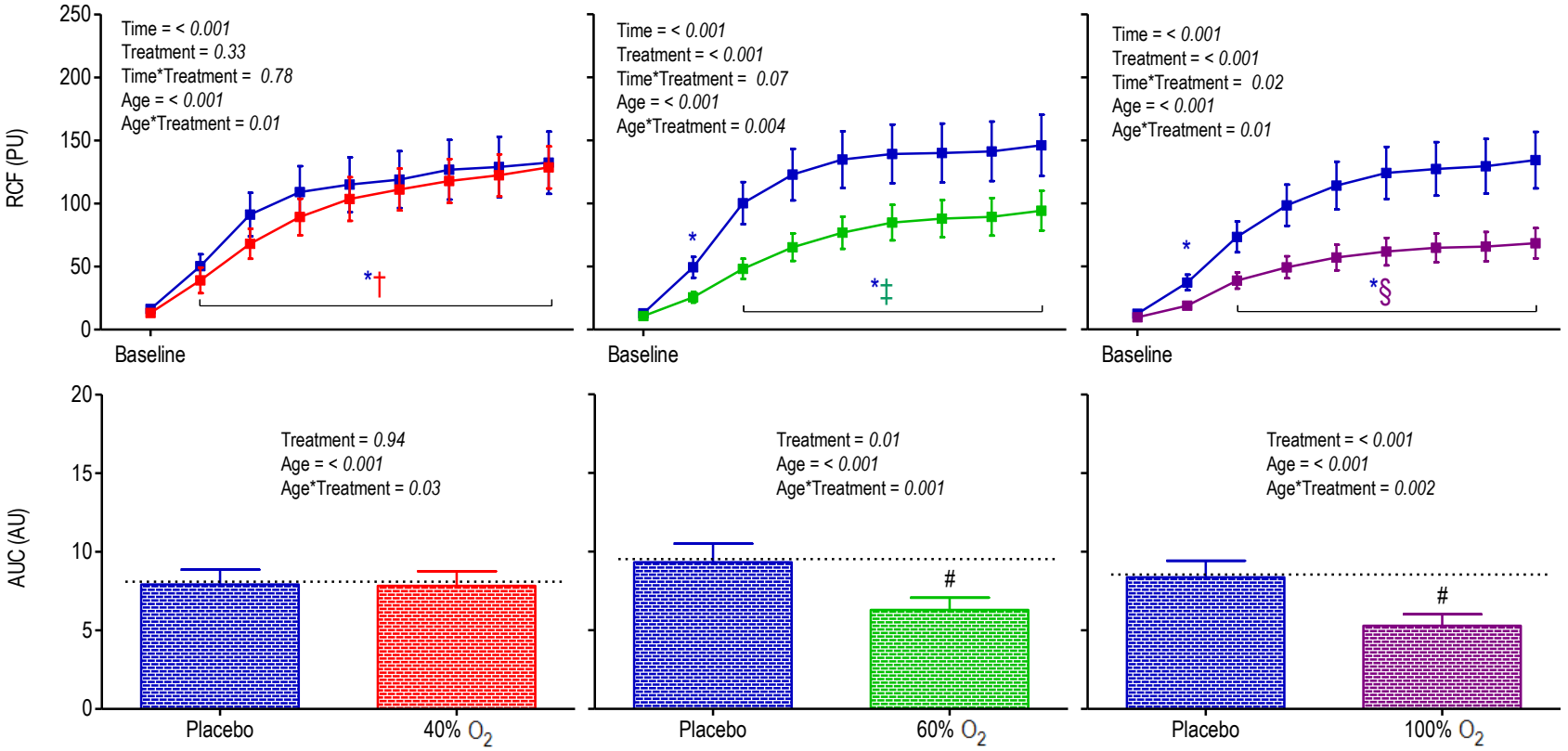



Figure 8.9 **Cutaneous vasodilator responses evoked by ACh-iontophoresis in older participants during placebo, 40, 60, and 100% $O_2$.** Each hyperoxia sub-group's data are compared to their own placebo data; n = 9 in each sub-group. Cutaneous RCF increased significantly in response to ACh-iontophoresis; 60 and 100% $O_2$ attenuated this increase. */†/‡/§ represents placebo, 40, 60, and 100% $O_2$ values that were significantly different from respective baselines. # represents significant treatment affect ($P < 0.001$ vs. respective placebo). The magnitude of ACh-induced increase in RCF was significantly less in older participants.

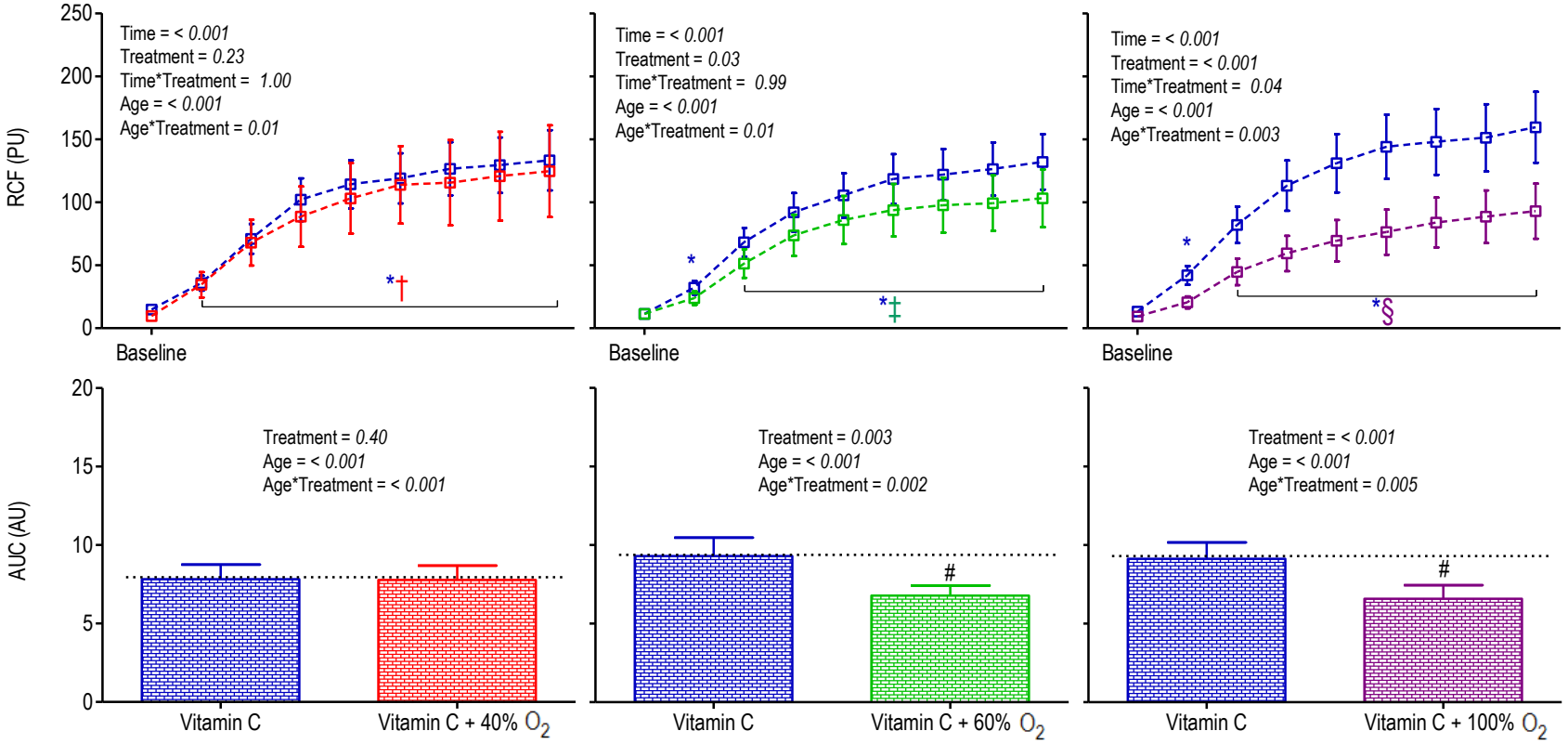



Figure 8.10 **Cutaneous vasodilator responses evoked by ACh-iontophoresis in older participants during combination of Vitamin-C and different $O_2$ concentrations.** Each hyperoxia sub-group's data are compared to their own Vitamin-C data; n = 9 in each sub-group. The attenuated increase in RCF during 60 and 100% $O_2$ was not completely reversed by Vitamin-C. */†/‡/§ represent Vitamin-C, Vitamin-C + 40%, Vitamin-C + 60%, and Vitamin-C + 100% $O_2$ values that were significantly different from respective baselines. # represents significant treatment affect ($P < 0.01$; i.e., vs. respective Vitamin-C data). The magnitude of ACh-induced increase in RCF was less in older participants.

## 8.4 Discussion

The principal novel finding of the present study is that breathing 40% $O_2$ did not affect ACh-mediated endothelium-dependent dilatation in forearm cutaneous circulation in either young or older individuals. Secondly, higher $O_2$ concentrations attenuated ACh-mediated dilatation in a dose-dependent manner in both age groups; and thirdly, Vitamin-C partially reversed this attenuation.

### 8.4.1 Systemic cardiovascular responses

HR and MABP values were not affected by treatment conditions within either age group. This is an interesting observation considering the ability of hyperoxia to attenuate endothelium-dependent vasodilatation and to raise ABP. However, Rousseau et al. who recorded a decrease in limb and cutaneous perfusion with 15 min of 100% $O_2$, also failed to record any change in the MABP in their studies (17, 348). It may be that any effect hyperoxia had in causing vasoconstriction was counteracted by a decrease in stroke volume (17). The exact reason for the disparity among studies regarding the systemic affects of hyperoxia is unclear, but it perhaps reflects between subject variability (17). Importantly, as MABP responses did not change significantly between treatments in the present study, it is reasonable to assume that the changes in RCF evoked by ACh were due to changes in vascular conductance within the cutaneous circulation.

### 8.4.2 Cutaneous responses of young subjects

Before ACh-induced changes to cutaneous blood flow can be discussed, it is important to remember that although baseline RCF values were similar between treatments, the recordings were made from two different cutaneous sites during each visit. Therefore, this study cannot

indicate whether hyperoxia or Vitamin-C affected baseline values in either age group, and therefore the primary focus is on the effect of different treatments on ACh-induced vasodilatation (i.e., AUC data).

As mentioned above, ACh-induced endothelium-dependent dilatation is mediated by NO, PGs, and non NO / PG relaxing factors (182, 206, 301, 350). The evidence available already indicates that hyperoxia can attenuate this dilatation by increasing the oxidative-stress which interferes with the production / action of these endothelial vasodilators (66, 256, 350, 353, 415). In the present study, during placebo ACh-iontophoresis induced an endothelium-dependent dilator response which significantly increased forearm cutaneous RCF in a progressive manner, before achieving a plateau. This is consistent with the results of others (166, 257, 283, 301). The finding that ACh-evoked increase in cutaneous blood flow with Vitamin-C was similar to that evoked under placebo suggests that during air breathing, ROS did not interfere with the dilatation; similar findings have been made by others (350). Although some *in-vitro* evidence suggests that Vitamin-C reacts with iron among other metals to produce radicals (27, 30, 269, 366), and its own oxidation also induces oxidative damage (366), no such effects were observed in the present study; ACh-evoked increase in cutaneous blood flow was similar between Vitamin-C and placebo.

The influence of hyperoxia and endothelium-dependent dilatation was investigated using different $O_2$ concentrations. Breathing 100% $O_2$ attenuated the ACh-evoked dilatation by ~ 35%. The magnitude of this attenuation was similar to that observed by Rousseau et al., (349, 350). Given their evidence that vasodilator responses of SNP iontophoresis were not affected by 100% $O_2$ (350), and that hyperoxia increases oxidative load (187, 256, 321, 348, 350, 415), the results of the present study are consistent with the proposal that attenuation of the endothelium-dependent dilatation was the result of oxidative-stress caused by 100% $O_2$. However, the

present study also yielded novel findings that ACh-evoked dilatation of cutaneous vessels was attenuated in a dose-dependent manner by $O_2$, in that the effect of 60% inspired $O_2$ was less than that of 100% $O_2$ (~ 25%) and 40% $O_2$ failed to attenuate ACh-evoked increase in cutaneous RCF. Previously, Rousseau et al. have shown that resting CBF declined progressively with increasing $PO_2$ concentrations (348). The results of this study are consistent with their findings, but add to them in showing a graded effect on endothelium-dependent ACh-mediated dilatation.

The influence of ROS was investigated with help of Vitamin-C; the dose used in this study has previously been used effectively to attenuate oxidative-stress (54, 233). Moreover, as its plasma concentrations peak 2 hours after oral dosage remaining stable for at least further 3 hours (233, 310), it should have acted as an effective antioxidant during the period of this study. Vitamin-C reduced the attenuation in ACh-evoked dilatation caused by 100% $O_2$ in agreement with the work of Rousseau et al. (350), and reversed the attenuating effect of 60% $O_2$. By contrast, responses evoked by ACh during 40% $O_2$ were similar without and with Vitamin-C. These results are therefore consistent with 60 and 100% $O_2$ attenuating the ACh-evoked response by inducing an increase in ROS that counteracted the endothelium-dependent dilatation. On the other hand, they indicate a lack of a significant effect of 40% $O_2$ on ROS generation.

The mechanisms by which ROS might interfere with endothelium-dependent dilatation have briefly been discussed (See this Chapter's Introduction). However, the present study does not elucidate on any of these mechanisms. Studies show that ROS react with NO and PGs to reduce their bioavailability. In fact, interaction between NO and $O_2^-$ occurs between the rates of $1.9 \times 10^9$ mol/L/s (215) to $6.7 \times 10^9$ mol/L/s (404) to produce peroxynitrite; at the higher end, the response is three times faster than $O_2^-$ reaction rate with superoxide-dismutase (48). Further, ROS are known to affect the COX pathway, such that their action can stimulate the production of vasoconstrictors $PGH_2$ / $TXA_2$ (69). Indeed, Rousseau et al. observed that the effect of 100% $O_2$

in attenuating ACh-induced increase in cutaneous blood flow was eliminated by COX inhibition (350). Thus, any and/or all these factors might be contributing to the attenuation of ACh-induced increase in cutaneous blood flow with 60% and 100% $O_2$. It is important to mention here that experiments performed by Furchgott and colleagues make it clear ACh-induced dilatation is endothelium-dependent in nature: removal of endothelium abolishes ACh-induced dilatation (131-134). However, given the effects of 40, 60, and 100% $O_2$ on responses evoked by SNP, an endothelium-independent dilator (217, 283), were not tested in the present study, we do not know whether 60 and 100% $O_2$ affected the ability of vascular smooth muscle to dilate in response to endothelium-independent or dependent substances. Nonetheless, the novel results of this study suggest that supplementary $O_2$ attenuates endothelium-dependent dilatation in a dose-dependent manner.

### 8.4.3 Cutaneous responses of older individuals

Despite similar baseline RCF values, the magnitude of the ACh-evoked increase in RCF was significantly less in older individuals than that observed in young (AUC ~ 8.6 vs. 13.6 AU). This age-related decline in ACh-evoked dilatation of the cutaneous vessels is consistent with the findings of others (6, 182, 401, 402). For example, Tew et al. measured changes to cutaneous circulation evoked by ACh iontophoresis in young, sedentary older, and exercise trained older subjects (402). Their results showed that peak ACh-induced hyperaemia in the cutaneous circulation was higher in young and unrelated to the $VO_2$-max status of their older participants. Moreover, before considering the effect of other treatments on ACh-induced endothelium-dependent dilatation, it is important to note that there was no difference between the ACh responses observed in older men and women in the present study. In agreement, Algotsson et al. observed that ACh-induced changes to cutaneous circulation were not different between post-menopausal women and elderly men (6). However as the responses to SNP were not compared

in the present study, it is unclear from our results whether the attenuated responses of older individuals reflected blunted endothelial-dependent dilatation or blunted relaxation of the vascular smooth muscle. However, as Holowatz et al. showed that the PG component of ACh-induced cutaneous dilatation was substantially attenuated in older individuals (182), it seems reasonable to assume that the blunted responses seen in older subjects of the present study at least partly reflect reduced endothelium-dependent dilatation mediated by vasodilator PGs.

Despite evidence that oxidative-stress increases with age (181, 214, 417), oral dose of 2 g of Vitamin-C failed to significantly increase the ACh-induced dilatation of cutaneous vessels. This observation differs from the observations of Holowatz et al. who noticed that ascorbate microdialysis augmented the reflex heating induced increase in cutaneous blood flow in older individuals; combination of ascorbate supplementation and arginase inhibition completely eliminated the age-associated differences (181). It is unclear why the results of the present study differ from those of Holowatz et al. (181). However, it may be that oral supplementation of Vitamin-C did not raise the levels of antioxidant high enough in the cutaneous tissue to reveal an effect of aging induced oxidative-stress on ACh-induced dilatation.

Nevertheless, the effects of different $O_2$ concentration on ACh-evoked responses in the cutaneous circulation of older individuals are completely novel. Importantly, similar to the observations made in young, ACh-evoked dilatation was attenuated by 100% $O_2$ in the older subjects and this attenuation was slightly greater as a percentage of the control response (~ 44%); consistent with the idea of an age-related increase in oxidative-stress (181, 214, 417). Moreover, the percentage attenuation in cutaneous RCF with 60% $O_2$ was also greater in older individuals (~ 25%), but again, importantly 40% $O_2$ did not cause any attenuation in their ACh-mediated increase in cutaneous RCF. Further, Vitamin-C supplementation attenuated the decline in endothelium-dependent dilatation observed with 60% and 100% $O_2$ but did not completely

reverse this decline. These results are in agreement with the findings of Holowatz et al. that oxidative-stress is increased in the cutaneous circulation of the older individuals. However, in order to test that the dose of Vitamin-C used in this study was effective as an antioxidant, markers of oxidative-stress should have been measured. This remains another limitation to this study.

### 8.4.4 Relevance to the results of previous Chapters

The observations made in the present study regarding the relationship between inhaled $O_2$ concentrations and endothelial-dilation in both young and older subjects are of profound importance to the results of the earlier Chapters. Holowatz et al. have reviewed substantial evidence to show that human cutaneous circulation can be used as a valid model to study systemic vascular responses (180). Moreover, it is relevant to this project that the contribution of PGs to vasodilatation in limbs (300), cutaneous circulation (182), and to exercise hyperaemia (358) decreases with age. Therefore, collectively these observations support the use of cutaneous circulation as a valid model for study in this project. Assuming the argument of Holowatz et al. (180) to be valid, the present results indicate that the attenuation in exercise hyperaemia observed in Chapters 4 and 6 with 40% $O_2$ are not a reflection of increased oxidative-stress blunting endothelium-dependent dilatation; but rather, genuinely reflect attenuation in the release / action of $O_2$-dependent vasodilators. Thus, the present results help to validate the findings of previous Chapters that 40% $O_2$ attenuates exercise hyperaemia by improving tissue oxygenation (See Chapters 4 and 6). Indeed, on the basis of the results of this Chapter, it could be argued that the use of a higher $O_2$ concentrations would not only have increased the oxidative-stress and resulted in vasoconstriction in the exercising muscles; but might have increased reflex sympathetic vasoconstrictor responses in parallel (See Chapters 5 and 6). This proposal is in agreement with findings of Caruana and Marshall who noticed that the

attenuation in *post*-exercise hyperaemia of isometric handgrip contraction with 40% $O_2$ was not different without or with acute Vitamin-C supplementation (54).

In conclusion, this novel study using different $O_2$ concentrations shows for the first time that endothelium-dependent dilatation is attenuated by supplementary $O_2$ at 60 and 100% in a dose-dependent manner: an effect that is consistent in both young and old. Moreover, this study also provides the important observation that 40% $O_2$ does not affect ACh-mediated endothelial dilatation; suggesting it does not increase the oxidative load significantly. Therefore in conclusion, the hypotheses of this study are accepted.

### 8.4.5 Limitations and future work

Some of the limitations of this study have already been discussed. However, for completeness, they are again briefly discussed here along with others.

Firstly, although the skin temperate did not vary between subjects, it would have been better if the variation in the room temperature been kept to a bare-minimum for all of the experiments. Unfortunately, available laboratory facilities precluded this as the air-conditioning at times was inadequate.

Secondly, as the results of this study indicate that the inhaled hyperoxia had a dose-dependent effect in increasing the oxidative load, it would be good in future studies to take blood samples to measure markers of oxidative-stress. A related limitation is the lack of Vitamin-C's effect on ACh-induced dilatation under air breathing conditions in the older subjects. This is important given Holowatz et al. have shown improved heat-induced cutaneous dilatation in older subjects with ascorbate microdialysis (181); for it implies that oral Vitamin-C did not completely attenuate oxidative-stress, or at least, age-related oxidative-stress. In future, it would be good to repeat the protocol of the present study with ascorbate microdialysis to see whether this achieves a higher

local concentration of antioxidant and acts more effectively to attenuate the effects of hyperoxia on ACh-induced dilatation in young and older individuals. Separately, as SNP responses were not tested, another limitation of this study remains our inability to be sure whether the attenuated older responses were the result of age-associated endothelial or vascular smooth muscle dysfunction. Future work should address this limitation.

It might be argued that an important limitation to this whole study is the use of iontophoresis – passing current into the skin in various vehicle solutions has been shown to cause cutaneous vasodilatation; a response that is abolished by local / topical anaesthesia (102, 103, 149, 283, 284). Özbebit et al. showed that ACh-induced cutaneous vasodilatation has two components; one of which is removed following topical anaesthesia (309).However interestingly, Durand et al. observed that the current-induced vasodilatation is primarily the result of COX-dependent vasodilators (102-104). As experiments with local anaesthesia and/or control experiments with de-ionized water were not performed in the present study, the ACh-evoked vasodilator response should be considered with this in mind. Nonetheless, iontophoresis of vasoactive agents without the use of these added protocols has been successfully used in many studies to elucidate endothelium-dependent dilatation (166, 258, 382, 402); even if dilatation induced by nerve stimulation is a persistent contributor to the response. Indeed, as 40% $O_2$ did not inhibit the observed cutaneous vasodilatation but higher $O_2$ concentrations did, it can be argued that 40% $O_2$ does not inhibit / interfere with COX-dependent vasodilatation by increasing oxidative-stress. Nevertheless, in future, it would be important to test the effects of hyperoxia on dilator responses of ACh-iontophoresis in presence and absence of local anaesthetic. This would provide robustness to the ideas / conclusions of this study. Although, the technique of iontophoresis may carry disadvantages, it does provide a non-invasive way of testing endothelium-dependent

dilatation as compared with the widely used alternatives of microdialysis into skin and/or intra-arterial infusion into a limb.

# Chapter 9

# General Discussion

## 9.1 Exercise and *post*-exercise hyperaemia

Physical activity requires harmony between physiological systems; an important part of this accord is the ability of blood vessels to change their diameter. It is now recognised that exercise increases muscle $VO_2$ (143) and leads to a profound decline in the intracellular $PO_2$ in both trained and un-trained individuals (47, 337-339, 376). The idea of metabolic regulation of vascular tone is not new. However $O_2$-linked regulation of the vascular tone (143, 184, 221, 226, 352), and the release of similar vasodilator PGs under hypoxic (45, 46, 266) and exercise (73, 74, 100, 209, 302, 303, 359, 390) conditions suggest that the vasodilatation of exercise hyperaemia might itself be $O_2$-dependent. Gorczynski and Duling while performing experiments on rhythmically contracting hamster cremaster muscles observed a decline in tissue $PO_2$ with a concomitant increase in arteriolar diameter (143). Superfusion of the exposed muscle with hyperoxic solution increased tissue $PO_2$, attenuated the increase in arteriolar diameter, and led to the proposal that approximately half of the observed exercise hyperaemia was the result of the decline in tissue $PO_2$. This study was supported by the observations of Lash and Bohlen who showed that perivenular $PO_2$ exhibited a maintained fall during muscle contraction; and therefore proposed that vasodilator metabolites released from perivenular tissues when local $PO_2$ falls make an important contribution to exercise hyperaemia (226).

Such observations were the foundation for the work performed by Thet Su Win (437) and Graham Fordy (122, 123) under the supervision of Janice Marshall; investigators who's work underpins the questions that are raised and addressed in this thesis. In brief, Win and Marshall showed that *post*-exercise hyperaemia of 60% MVC isometric handgrip contractions performed by young men was partly dependent on COX products (437): an observation that in itself was not novel (73, 74, 100, 209, 302, 303, 359, 390). However importantly, their results also indicated that COX vasodilator products were acting in an $O_2$-dependent manner because the blockade of

COX enzyme attenuated *post*-exercise hyperaemia to a similar extent as 40% $O_2$, with no added attenuation upon their combined use. This finding was later confirmed by Fordy while working on his doctorate (122). However, Fordy and Marshall also discovered that the attenuation in *post*-exercise hyperaemia only occurred if 40% $O_2$ was provided *during* the period of isometric contractions and not during the *post*-exercise recovery (122, 123). These observations therefore suggested that the $O_2$-dependent metabolites are generated *during* contraction and raised new questions that required further investigation.

Firstly, there is evidence that both isometric (122, 209, 390, 437) and dynamic exercise (73, 74, 100, 209, 302, 303, 359, 435) are accompanied by the release of vasodilator PGs ($PGE_2$ and $PGI_2$); and that COX blockade attenuates their hyperaemia (73, 74, 122, 209, 437). There is also evidence suggesting that the signal for the release of these vasodilating PGs originates somewhere within the skeletal muscle tissue (123, 198, 199) and that PGs are released in an intensity-dependent manner (29, 198, 199, 447). While our group has proposed that the exercise (*post*-exercise) hyperaemia associated with isometric handgrip contraction is regulated by $O_2$-dependent COX products (122, 437), it was not known whether this is also the case for the hyperaemia of rhythmic (dynamic) handgrip exercise. Secondly, the conclusions of our group were based on the FBF and FVC data. Blood samples were not taken to conclusively test whether there actually is an $O_2$-dependent release of vasodilator PGs during exercise.

The aim of Chapter 4 was to address these questions: the results clearly showed that the *post*-exercise hyperaemia of both isometric and rhythmic handgrip contractions performed at 60% MVC by young men is equally attenuated by COX inhibition, and 40% inspired $O_2$. Importantly, as this attenuation was accompanied by attenuated concentrations of vasodilating PGs ($PGE_2$ and $PGI_2$) in the venous efflux, the results of Chapter 4 provide new evidence that confirms the $O_2$-dependent nature of the COX pathway and its contribution to exercise (*post*-exercise)

hyperaemic response. In addition, the results of Chapter 4 shed some light on the site of hypoxic stimulus that leads to the release of these PGs during exercise. Although hypoxia leads to the release of $PGI_2$ from endothelium (45, 46), the primary vasodilator PG released from the skeletal muscles belongs to the $E_2$ series (400). Chapter 4's PG data shows that $PGE_2$ as well as $PGI_2$ are released in an $O_2$-dependent manner during either exercise. The mechanism by which $O_2$ could be affecting their release is discussed below.

Another aim of Chapter 4 was to investigate the contribution of PGs to exercise (*post*-exercise) hyperaemia in healthy older individuals; and to establish whether their release is still $O_2$-dependent? This question was of interest, particularly because of the work of Schrage et al. (358). In order to investigate the contribution of COX pathway to exercise hyperaemia, they infused ketorolac into the brachial artery of healthy older individuals who were performing rhythmic handgrip contractions at 10% MVC (1 s contraction: 2 s relaxation). In contrast to young subjects (359), PGs were found not to contribute to exercise hyperaemia in the older subjects. However, as the release of PGs is exercise / contraction intensity-dependent (29, 198, 199, 447), the lack of any observable contribution of PGs to exercise hyperaemia in the older individuals might have reflected the low intensity of exercise used in the protocol. Alternatively, studies have shown that lifelong activity prevents the age-related decline in the contribution of NO to exercise hyperaemia (285, 305, 391). The older subjects of Schrage et al. (358) only performed household tasks as part of their physical activity regimen. Moreover, recently Zoladz et al. have shown that the venous efflux of $PGI_2$ evoked by cycling exercise shows a net increase in young men who have gone through a five week endurance training regimen (448). Therefore, the results of Schrage et al., (358) might also have reflected a decreased generation of PGs associated with the sedentary life style of their healthy older subjects.

The data of Chapter 4 clearly shows that when recreationally active, healthy older men perform isometric and rhythmic handgrip contractions at 60% MVC (1:1 s duty-cycle), their *post*-exercise hyperaemia, and the contribution of PGs to it are both maintained. Moreover, as in young men, *post*-exercise hyperaemia and the contribution of PGs to it were found to be $O_2$-dependent in the older men. However, despite releasing very similar $PGE_2$ and $PGI_2$ concentrations into the venous efflux as young men; judging from the attenuating effect of COX inhibition, the magnitude of their contribution to exercise hyperaemia was smaller in older subjects (~ 30% vs. ~ 18% for isometric and ~ 25% vs. ~ 15% for rhythmic handgrip). This perhaps reflects an age-related increase in the production of vasoconstrictors (396), increased ROS production (114, 118, 214, 305, 358, 391, 393, 417), and/or an age-associated decline in the ability of the vasculature to respond to the vasodilators (114, 118, 259). Nonetheless, confirmation of any of these pathways requires further investigation.

The recordings of THI (an index of change in haemoglobin; i.e., blood flow) obtained in the NIRS study (Chapter 6) on the exercising arm of young and older men, confirms that COX products are involved in the hyperaemia that occurs *during* muscle contraction and that their effect is $O_2$-dependent, as shown for *post*-exercise hyperaemia. These results indicated that at least in the flexor digitorum-superficialis, blood flow is not only maintained, but increases *during* isometric handgrip contraction at 60% MVC. Moreover, not only did 40% $O_2$ attenuate the increase in THI, but it also reduced the fall in TOI, and the increase in HHb. Thus, these results provided direct evidence that breathing 40% $O_2$ ameliorates tissue / muscle oxygenation *during* exercise; and that COX inhibition and 40% $O_2$ have similar non-additive effects on tissue perfusion. This observation lends support to the conclusion that $O_2$-dependent COX products are generated *during* exercise, contribute to the hyperaemia that occurs *during* this time, as well as to *post*-contraction hyperaemia in both age groups. This also supports Fordy and Marshall's observation

(122, 123) that supplementary $O_2$ provided only *during* the period of isometric handgrip contractions (60 and 100% MVC) attenuates the *post*-contraction hyperaemia.

## 9.2 Exercise-evoked vasoconstriction

Vasodilatation is not the only influence on skeletal muscle vasculature during exercise; there is also a vasoconstrictor influence on exercising and non-exercising muscle. The novel findings of Chapter 4 raised questions regarding the $O_2$-dependence of reflex-vasoconstriction. This question is appropriate considering that not only is AA (347) implicated in the activation of group III and IV afferents but so too are its derivatives (202, 207, 262): inhibition of COX pathway abolishes the afferent activation (346, 347). Further, the pressor response evoked during both isometric and dynamic exercise is attenuated by COX inhibition (72, 79, 246, 267, 346), and the exercise-evoked vasoconstriction is exercise intensity-dependent (399). It has also been shown that systemic hypoxia caused greater stimulation of metabolic than mechanical afferents in resting muscles (174). Moreover during ischemic exercise, group IV afferents were stimulated more than group III afferents (4, 174, 203). Together, these results point to the local metabolic control of exercise-evoked reflex vasoconstriction.

Therefore, based on the results of Chapter 4, it was hypothesized that the pathway of exercise-evoked reflex vasoconstriction was also $O_2$ and PG-dependent. The studies of Chapters 5 and 6 aimed to shed light on this. In the study of Chapter 5, the finding that the increase in $\Delta$ CVR evoked by forearm exercise was similarly attenuated by COX inhibition, and 40% $O_2$, whether or not *post*-exercise circulatory occlusion (PECO) was applied was entirely consistent with contribution of COX products to reflex vasoconstriction in an $O_2$-dependent manner. The observations in Chapter 6 were made in the resting arm only *during* the period of contraction (i.e., without PECO application). Therefore, although they reflect the combined contribution of

central command and reflex afferent activated vasoconstriction, the additional observations of a similarly attenuated decrease in THI responses in the resting arm of young men under COX inhibition, 40% $O_2$, and their combination are consistent with the results of earlier Chapter 5. Metabolites not only activate metaboreceptors, but are also known to activate / sensitize mechanoreceptors (3, 79, 267, 347, 372). However their relative importance / input cannot be deduced from these results. Nonetheless, Chapter 5's finding that the reflex vasoconstriction was maintained with PECO and gradually declined with NO PECO indicates that the stimulus for the exercise-evoked reflex vasoconstriction lies within the exercising muscles; and persists when the circulation is occluded. An idea that is supported by the observation that $\Delta$ CVR responses of 40% $O_2$ increase near the end of PECO. This idea is also supported by the observations of Chapter 6; the decline in resting arm's THI increases over time during exercise, reflecting exercise-evoked vasoconstriction that is independent of central command. These results therefore show for the first time that the $O_2$-dependent attenuation of COX products in the exercising arm not only attenuates exercise hyperaemia in the same arm, but also makes a major contribution to the attenuation of exercise-evoked reflex vasoconstriction that occurs in the contralateral arm, and calf muscles, and presumably to the other components (276).

In view of the inconsistent findings in the literature of age-related changes in exercise-evoked vasoconstriction (204, 247, 298, 299, 342), the study of Chapter 6 recruited healthy, recreationally active older men with the aim of avoiding some of the variability. Although the data was collected during exercise only, and PECO was not applied, this study aimed to address the $O_2$-dependence of exercise-evoked / reflex vasoconstriction in older subjects. In previous studies, Ng et al. showed that resting MSNA in older individuals was higher than in young subjects (298); while Taylor et al. observed an intensity-dependent and age-related increase in the exercise-evoked vasoconstriction; a response that was linked to increased sympathetic

activity in this age group (399). By contrast, Markel et al. observed an attenuated increase in peroneal nerve MSNA in older individuals when they performed incrementally ischemic handgrip exercise (247); observations that were in agreement with those of other studies (204, 299). The results and conclusion of Chapter 6 lend support to the findings of age-related attenuation in the magnitude of the exercise-evoked reflex vasoconstriction; at least in healthy, recreationally active older men while they perform isometric handgrip exercise at 60% MVC. In addition, the results of Chapter 6 showed for the first time that age does not affect the $O_2$-dependent nature of this vasoconstriction. Thus, the change in THI recorded from the flexor digitorum-superficialis of the resting arm was similarly attenuated with COX inhibition, 40% $O_2$, and their combination.

It is not clear how, or why the magnitude of this vasoconstriction is attenuated in older individuals. However, a few possible reasons for this observation can be advanced. As indicated above, animal (202, 207, 262, 346, 347) and human studies (267, 276) indicate that both mechanical and metabolic pathways are involved in the pressor response; and that the contribution of metaboreceptors to the pressor reflex is significant above a certain threshold (158, 276, 421, 422). As the TTI of isometric exercise was significantly smaller in the older subjects, the smaller vasoconstriction might simply reflect a smaller stimulation of the mechano and/or metaboreceptors. This hypothesis is consistent with the evidence that the attenuated increase in THI in the exercising arm of the older individuals in Chapter 6 was also associated with attenuated TTI responses (unlike Chapter 4).

An alternative explanation is offered by comparing the data of Chapter 6 with the findings of Markel et al. (247). Their subjects performed rhythmic handgrip contractions (30% MVC; 0.5 Hz) while blood flow in the exercising arm was gradually restricted. The results showed that under resting and unrestricted blood flow, the increase in peroneal nerve MSNA was greater in the older group, but restriction of blood flow reversed this age-related effect. These data appear

completely counter-intuitive but are consistent with those of Kawano et al. (204): at both absolute and relative exercise intensities, the evoked increase in ABP was attenuated with increasing age. As explained in Chapter 6, although studies suggest an age-related shift towards the oxidative metabolism with aging (8, 225, 389, 408), which should bring about a relative increase in their $VO_2$, quite a few studies have shown an age-related decline in the $VO_2$ kinetics (16, 23, 81), particularly in the muscles that are not normally active (61). The observations in Chapter 6 that the increase in HHb in the exercising arm of older men was less than that of young men, suggests attenuated $VO_2$ kinetics in the flexor digitorum-superficialis of older subjects. This might be expected to lead to an attenuated increase in the concentrations of the metabolites that stimulate muscle afferents; and thereby mediate the reflex vasoconstriction. However, it might also reflect the observation that the afferent-sympathetic reflex responses from oxidative fibres are considerably less than those from the glycolytic type (436).

Another, but not a mutually exclusive, possibility is that the smaller vasoconstrictor responses reflected the activity status of the older participants; i.e., recreational rather than endurance trained. Nyberg et al. found that the preservation of NO bioavailability in their recreationally active older individuals was between that of the sedentary and endurance exercise trained individuals (305). Therefore, it might be that the $VO_2$ responses of our older individuals were not high enough to lead to a sufficient decline in the local tissue oxygenation to elicit enough release of metaboreceptor-stimulating $O_2$-dependent metabolites. Nevertheless as their release did elicit a substantial $O_2$-dependent exercise hyperaemia response in the exercising arm, it is clear that metabolite accumulation was not *insignificant*. Moreover, this interpretation would raise a question regarding the similar efflux of $PGE_2$ and $PGI_2$ between young and older men in study of Chapter 4. Thus, it may be that the muscle afferents become less sensitive to PGs with age.

## 9.3 $O_2$-induced generation of ROS

The important question that is fundamental to the studies described in this thesis is whether 40% $O_2$ actually has its effects by increasing $O_2$ delivery to exercising muscle as argued; or whether its influences are explained by other effects. ROS are quick acting, highly reactive molecules (215, 404) that are produced by enzymatic and non-enzymatic pathways (48, 69, 320, 398, 419, 420, 449); and work to attenuate the blood flow by scavenging endothelium-dependent vasodilators (66, 256, 350, 353, 415). Their relevance to this entire project is evident from the observation that increased superoxide-dismutase activity improves ACh-induced dilatation (353); elimination of the superoxide-dismutase gene significantly attenuates the vasorelaxation (66). Of further relevance to this project are the observations of McNulty et al. that the ACh-evoked increases in coronary blood flow and conductance are substantially decreased while nitrotyrosine efflux increases when subjects breathe 100% $O_2$ for only 15 min (256). Although the PG venous efflux data of Chapter 4 suggested it unlikely that the attenuation of exercise hyperaemia observed during Chapters 4 and 6 were the result of increased oxidative-stress, rather than any other route; this question still needed investigating. The novel results of Chapter 8 are important in this respect. ACh-evoked endothelium-dependent cutaneous vasodilatation was attenuated by breathing 60 and 100% $O_2$ in both young and older individuals; but 40% $O_2$ had no such effects. Moreover the antioxidant, Vitamin-C, did not affect the vasodilator response to ACh during air breathing or 40% $O_2$ conditions; but reversed the attenuating effects of 60 and 100% $O_2$. This indicates that higher $O_2$ concentrations do indeed generate enough ROS to effect endothelium-dependent dilatation whereas 40% $O_2$ does not. Based on these results, it can be concluded that the attenuated exercise and *post*-exercise hyperaemia responses of earlier Chapters were indeed a reflection of attenuated generation of $O_2$-dependent vasoactive metabolites (i.e., PGs); not just additional oxidative-stress inhibiting the vasodilatation by decreasing the bioavailability or

action of NO and $PGI_2$. This is supported by the observations of Caruana and Marshall, who noticed that the attenuation in *post*-exercise hyperaemia of isometric handgrip contraction with 40% $O_2$ was not different without or with acute Vitamin-C supplementation (54).

It is interesting to note that while the results of Chapters 5 and 6 showed the exercise-evoked reflex vasoconstriction is $O_2$-dependent, earlier studies that utilized 100% $O_2$ failed to observe any such attenuation (183, 361); i.e., the increase in leg MSNA was either maintained or increased when their subjects performed handgrip exercise with supplementary $O_2$. As discussed, 100% $O_2$ increases the generation of ROS (256, 348-350); and these molecules not only decrease the bioavailability of endothelial vasodilators (256), but they are also involved in directly evoking central and peripheral sympathetic responses (51).

## 9.4 Contribution of other factors to exercise hyperaemia

The findings of Chapter 4, 5, and 6 are internally consistent; and implicate COX products as $O_2$-dependent substances that contribute to the exercise hyperaemia and exercise-evoked reflex vasoconstriction of handgrip exercise. However, published studies show that adenosine and its nucleotides also contribute to exercise hyperaemia (18, 304, 333) and that they can influence the production of NO and PGs (286, 289, 304, 306). Thus, the contribution of PGs to hyperaemia may be at least partially dependent on other metabolites (289). As both localized and systemic hypoxia augment adenosine concentrations (70, 71, 274), a study was needed to investigate the relationship of adenosine, $O_2$, and $O_2$-dependent COX products in exercise hyperaemia. This study was carried out using rats as an effective, specific, adenosine-receptor antagonist is unavailable for use in humans (249). The results of Chapter 7 showed that 40% $O_2$, and the combination of 40% $O_2$ and adenosine $A_{2A}$-receptor inhibition caused similar attenuation of the exercise hyperaemia associated with electrically induced twitch contractions in the anaesthetised

rat. Further COX inhibition had no added attenuating effect. However, as the attenuation in exercise hyperaemia with 40% $O_2$ alone or in combination with $A_{2A}$-receptor inhibition was greater than that previously observed with lone $A_{2A}$-receptor inhibition (333), it suggested that some other factors were also contributing to exercise hyperaemia independent of adenosine pathway, particularly in the initial periods of exercise. Both Ballard et al. (18) and Ray and Marshall (333) observed that adenosine's contribution to exercise hyperaemia increased gradually over time while the results of Chapters 6 and 7 showed that COX inhibition and/or 40% $O_2$, attenuated exercise hyperaemia from the very start of exercise.

## 9.5 Mechanism of action of 40% $O_2$ on PG generation

The discussion at the end of Chapter 4 (See Section 4.4.3) proposed a tentative mechanism by which the release of $O_2$-dependent vasodilating PGs may be regulated; directly, or indirectly, by 40% $O_2$. Based on the results of Chapter 7, the following discussion presents multiple pathways that might be contributing to the $O_2$-dependent release of PGs during exercise; perhaps in unison.

Firstly, exercise is associated with a profound decline in the concentrations of intra-muscular / interstitial $PO_2$ concentrations (143, 337-339), and the major PG produced by the skeletal muscles is of PGE series (400). The attenuated venous efflux of $PGE_2$ with 40% $O_2$ in both isometric and rhythmic handgrip exercise conditions suggested that added $O_2$ ameliorates tissue oxygenation; and thereby directly inhibits its release. However, the observations made by Lash and Bohlen (226) showed that exercise was also accompanied by a significant decline in the peri-venular $PO_2$; and the decline in intravascular $PO_2$ is associated with an increase in $PGI_2$ concentrations (45, 46, 266). Thus, it is possible that the 40% acts on these peri-venular / capillary structures and inhibits the $PGI_2$ production; observation supported by the venous efflux

data of Chapter 4. It is not being suggested here that this added $O_2$, *only* attenuates $PGE_2$ production in the extra-vascular compartment, and $PGI_2$ efflux in the intravascular compartment. Rather, this proposal implies a direct action of 40% inspired $O_2$ in attenuating the $O_2$-dependent release of these vasodilators. The blue arrows in Figure 9.1 show this pathway.

The alternative explanation for the effect of 40% $O_2$ involves adenosine and/or ATP. Before proceeding, it is important to remember that the release of these compounds is linked to hypoxia (24, 70, 71, 88, 142, 186, 243, 331), and exercise-intensity (142, 253). Moreover, they also influence the production of NO and PGs (286, 289, 304, 306). Therefore, in theory adenosine in the intra (306) and extra-vascular (306) compartments might affect the release of vasodilating PGs. The results of Chapter 7 show that although the contribution of adenosine to exercise hyperaemia via $A_{2A}$-receptors is not added to by 40% $O_2$ and COX inhibition, the pattern of this attenuation in hyperaemia is not the same as observed with lone $A_{2A}$-receptor inhibition (333). This implies that during the initial period of exercise, adenosine release does not contribute to exercise hyperaemia, but that other $O_2$-dependent pathways do. The importance of testing the contribution of $A_1$-receptors to exercise hyperaemia is discussed in section 7.4.2. However, whether early release of adenosine contributes to the release of PGs or whether their release occurs independently is not clear at this time. Nonetheless, given the relationship between hypoxia and adenosine concentrations (24, 70, 71, 243, 331), its association with the release of NO and PGs (289, 306), and the fact that its interstitial concentrations reach near-maximum with relatively low-workload (165), it is possible that 40% $O_2$ ameliorates tissue oxygenation and prevents the release of adenosine; and thereby attenuates the release of $O_2$-dependent PGs during exercise.

It is also important to remember that not only does ATP release occur from RBCs as the haemoglobin structure changes from oxygenated to deoxygenated (88, 142, 186), but ATP

concentrations also increase in response to exercise (290). Moreover, an increase in the ATP concentration is linked to the release of NO and $PGI_2$ (306). The importance of this interdependent pathway is evident from the observation that the increase in blood flow induced by ATP infusion was attenuated by COX inhibition (286, 290). Therefore, as ATP can be acted on by 5'ectonucleotidase to release adenosine (164, 314, 315), its contribution to the $O_2$-dependent release of PGs during exercise could be direct (i.e., increased $PO_2$ attenuating the increase in the numbers of deoxygenated RBCs) or indirect (via adenosine). Although the contribution of indirect pathway cannot be denied, the observations in Chapter 6 of an attenuated increase in HHb with 40% $O_2$ lend support to the direct pathway. Purple, red, and green arrows in Figure 9.1 show this complicated relationship between adenosine, ATP, and $O_2$-dependent vasodilating PGs. These same mechanisms would also explain the PG and $O_2$-dependency of the exercise-evoked reflex vasoconstriction. The yellow arrows in Figure 9.1 show stimulation of group III and IV afferents by these $O_2$-dependent PGs.

In summary, it is clear from the data presented in this thesis that the exercise / *post*-exercise hyperaemia of isometric and rhythmic handgrip contractions at 60% MVC and the exercise-evoked reflex vasoconstriction of similar-intensity isometric handgrip contractions have an $O_2$-dependent component: vasoactive COX products. Moreover, the $O_2$-dependent nature of this pathway, and the contribution of COX products to exercise / *post*-exercise hyperaemia and exercise-evoked reflex vasoconstriction remains preserved in recreationally active healthy older men.

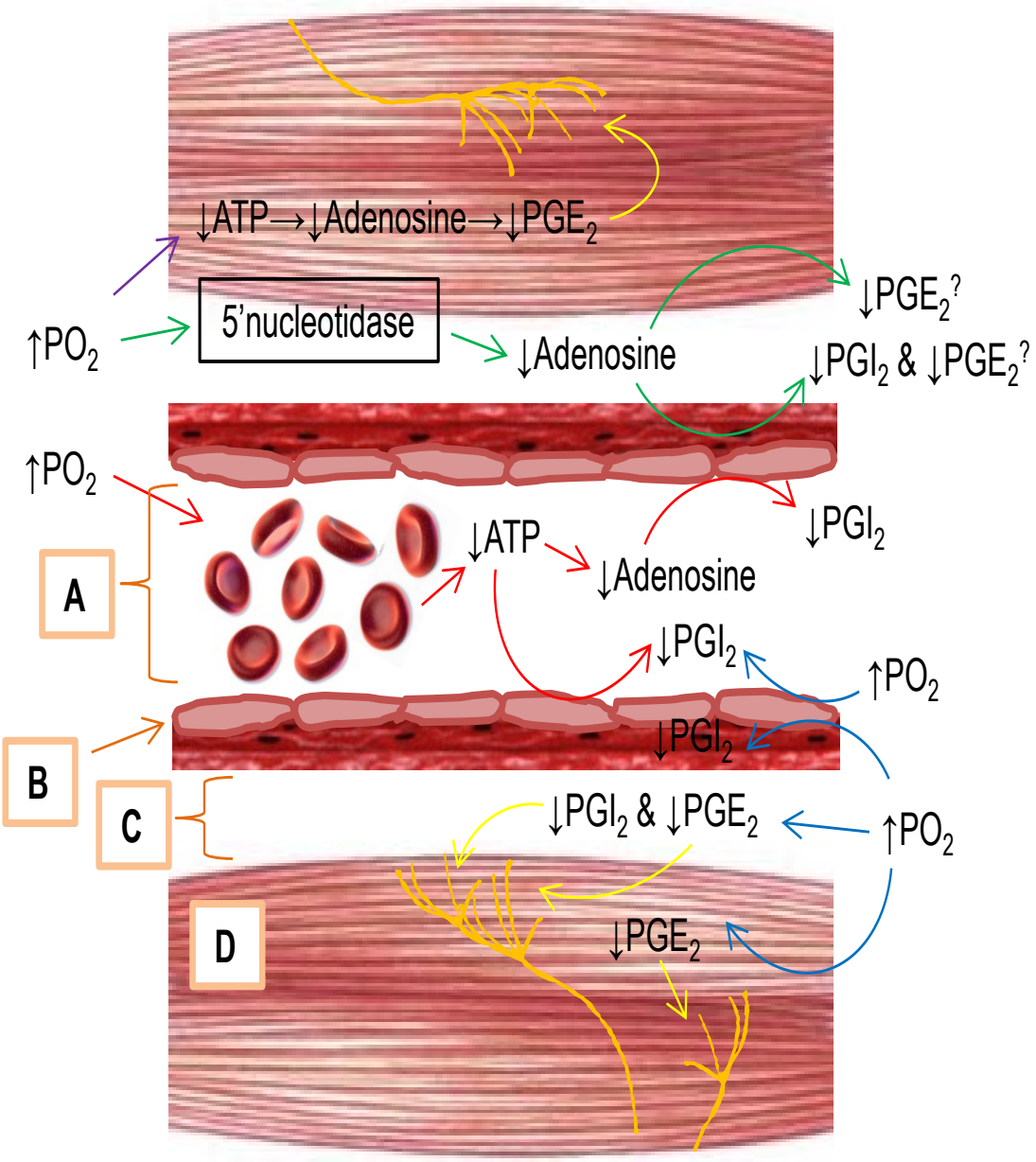



Figure 9.1 **Possible mechanisms by which 40% $O_2$ attenuates the release of vasodilator PGs**. Compartment A is intra-vascular compartment, B is endothelium, C represents interstitium while D represents skeletal muscle. Different coloured arrows represent four distinct, but not mutually exclusive pathways that could be affecting the release of vasoactive $PGE_2$ and $PGI_2$ during exercise in an $O_2$-dependent manner. The presence or absence of these COX products affects local and peripheral vascular diameter (i.e., exercise / *post*-exercise hyperaemia and exercise-evoked reflex vasoconstriction).

## 9.6 Future Work

The limitations and future work sections of previous Chapters raised questions that need addressing in future studies. Although, all of these issues are not raised here again, some that remain at the end of this project are briefly discussed below.

Firstly, there is evidence that the exercise-related release of PGs is not a universal finding (i.e., responders and non-responders) (448). Moreover, certain individuals do not respond to exogenous adenosine and their exercise hyperaemia is not attenuated by theophylline (251-253). Thus, the relationship between exercise, 40% $O_2$, and its dependent vasoactive metabolites needs investigation in such individuals. Based on the results of Chapter 4 and 6, questions are also raised regarding the responsiveness of older vasculature to vasoactive metabolites (particularly COX products). Series of experiments involving intra-arterial infusion of COX products *in-vivo* and/or myograph techniques on isolated vessels would shed more light on these issues. Moreover, as endothelium-dependent dilatation appears to be maintained with lifelong activity, a clear comparative study between sedentary and lifelong active older individuals with regards to $O_2$-dependent metabolites would shed more light on the effects of healthy aging on the contributions of $O_2$-dependent PGs to exercise hyperaemia / reflex vasoconstriction.

Separately, as de-oxygenation of RBCs leads to release of ATP (111, 112), and the nucleotides stimulate synthesis and release of NO and PG from endothelium (304), a future is study is needed to investigate the contribution and interdependent relationship between $O_2$, adenosine, ATP, and vasodilator COX products in exercise hyperaemia.

In addition, the results of Chapter 5 and 6 raise the obvious question of whether 40% $O_2$ attenuates MSNA during rest and exercise? Based on the observations that metaboreceptors are stimulated by derivates of the COX pathway (202, 207, 262, 347) and by ROS (51), the results of

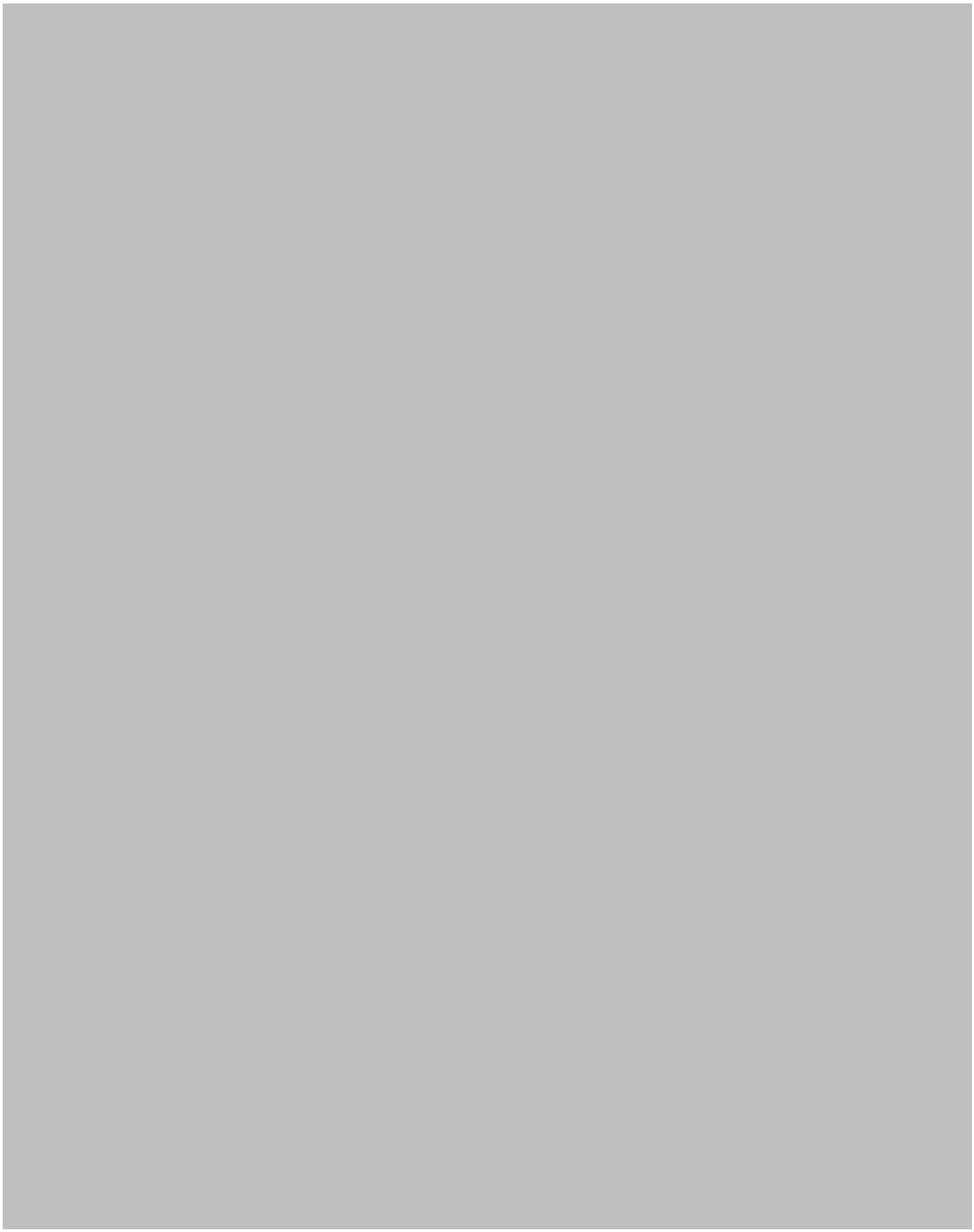
Chapters 5, 6, and 8 along with the venous efflux data of Chapter 4, would indeed suggest that the increase in MSNA is attenuated with 40% $O_2$. However, these experiments would not only confirm this hypothesis, but could form an integral part of the investigation into the neural and local control of cardiovascular system in CHF and COPD patients. They could also help address the question whether 40% $O_2$ would have beneficial exercise promoting and fatigue delaying affects in these patient groups.

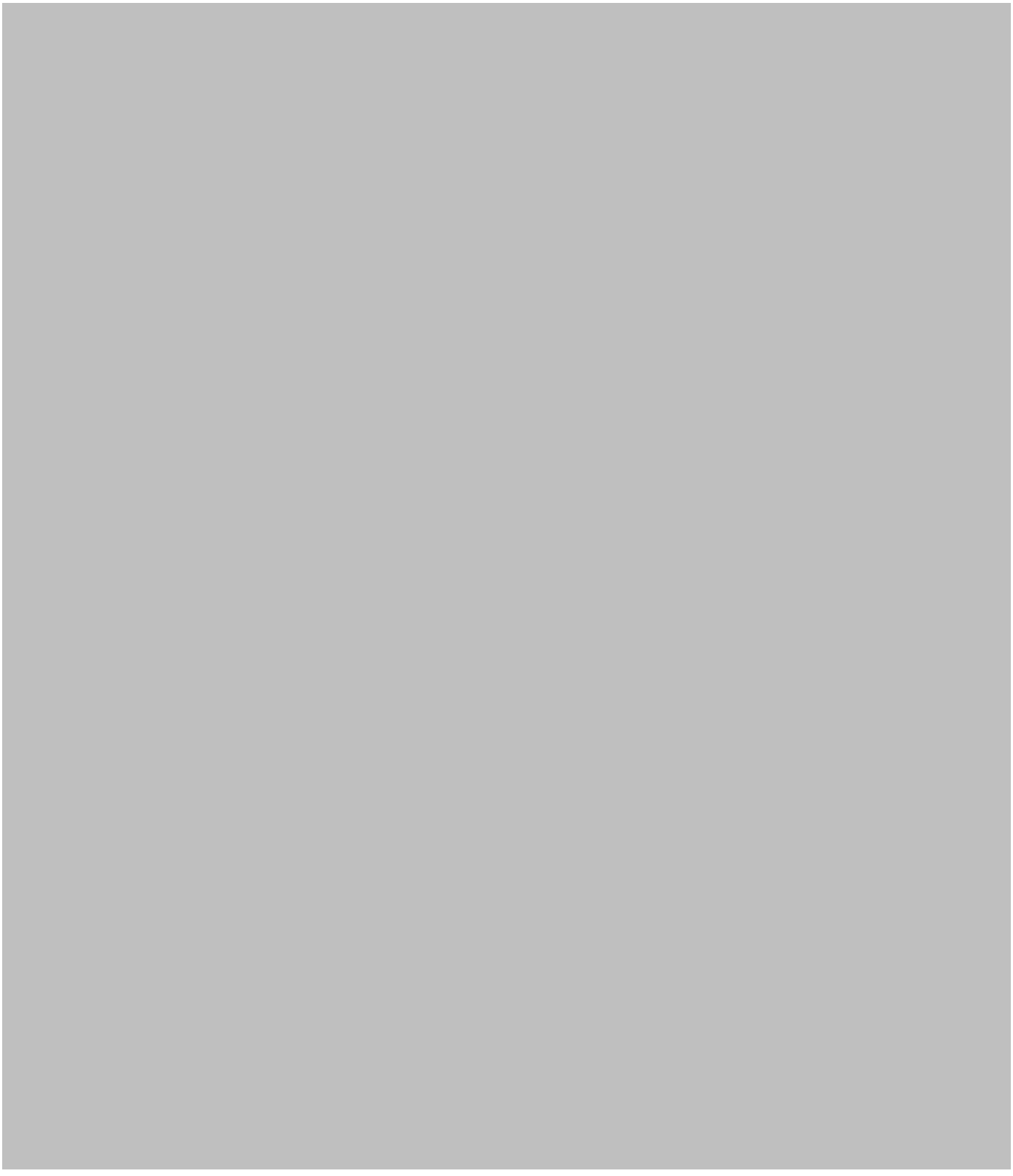
Finally, the $VO_2$ data from the resting arm of young and older men while they performed isometric handgrip exercise has revealed a completely novel phenomenon. Clearly, experiments involving the neural and local control of haemodynamics, and $VO_2$, in the resting muscles during exercise need to be performed. These experiments will help further elucidate / investigate this novel response.

Clearly, the results of this entire project have important implications: they not only help advance our understanding regarding the role of vasoactive COX products to exercise and *post*-exercise hyperaemia; but they also increase our understanding of their contribution to exercise-evoked reflex vasoconstriction, both in young and older individuals. Moreover, this project provides novel data that clearly shows that their relationship to exercise-linked vasoactive changes is $O_2$-dependent. This advancement in scientific understanding regarding the COX-linked control of vasculature will certainly help any future scientific work. In addition, the observations that breathing 40% $O_2$ can ameliorate tissue oxygenation in exercising muscle while not inhibiting endothelium-dependent dilatation may be of potential value to the medical profession; particularly in the rehabilitation and care of patients with cardiovascular and/or respiratory disease who have reduced exercise tolerance; for example, those with COPD or heart failure.

# Appendix 1

# Ethics Approval for the Project





# Appendix 2

# Screening Questionnaire 1 (Chapters 3 – 6)

**School of Clinical and Experimental Medicine**
**College of Medical and Dental Sciences**
**University of Birmingham**

## Healthy Volunteer Screening Questionnaire

*Please remember, all the information will be treated in the strictest of confidence.*

### VOLUNTEER INFORMATION

Subject No (for researcher only):_____

Name:_____

Date of Birth:_____

Address:_____

_____

_____

_____

Phone No:_____

Email Address:_____

Height:_____ Cm

Weight:_____ Kg

BMI:_____ $Kg/m^2$

Resting Heart Rate:_____ B/min

Resting Blood Pressure:_____ mm HG

Dominant Arm: _____ (R or L)

Arm Circumference (Dominant):_____ cm

Arm Circumference (Resting):_____ cm

Maximum Voluntary Contraction 1:_____ Kg

Maximum Voluntary Contraction 2:_____ Kg

Maximum Voluntary Contraction 3:_____ Kg

### ACTIVITY STATUS

How would you assess your present level of fitness? **POOR / AVERAGE / HIGH / TRAINED**

Leisure time questionnaire;

| Activity | Did you perform this activity? | | Month of activity | | | | Average time per week | |
|---|---|---|---|---|---|---|---|---|
| | No | Yes | Week 1 | Week 2 | Week 3 | Week 4 | hrs | min |
| Walking for pleasure | | | | | | | | |
| Walking to work | | | | | | | | |
| Walking during work breaks | | | | | | | | |
| Cycling | | | | | | | | |
| Using stairs when lift is available | | | | | | | | |
| Carrying – loading/unloading | | | | | | | | |
| Gardening | | | | | | | | |
| Household tasks (cooking / cleaning / Vacuuming, etc) | | | | | | | | |
| Home repairs | | | | | | | | |
| Occupational manual Labour (Building / Mining / Farming / Machine tooling, Nursing, etc) | | | | | | | | |
| Fishing/Hunting | | | | | | | | |

If yes, please provide the details of the activities._____

_____

Do you do any other exercise regularly? **Yes / No**

If yes, how regularly do you take part in physical activity? Please state the type of physical activity and how often? If you have multiple exercise schedules, please do not forget to list them all.

_____

_____

Approximately, how long do you exercise for on each occasion?

_____

How long have you been exercising regularly?

_____

On a Scale of 1 to 10, with 10 being highest, how strenuous would you say your each exercise routine is?

_____

Do you take part any sport activities? If yes, please provide the details to these activities and how often do you participate in them?

_____

On a Scale of 1 to 10, with 10 being highest, how strenuous would you say your sports regimen is?

_____

Do you take part in other activities? Please tick the appropriate one from the list provided. If the activity is not present, please specify.

- Rowing / Canoeing / Kayaking
- Corps training
- Cycling – for pleasure
- Dancing
- Martial arts
- Mountaineering
- Rock climbing
- Sailing
- Skiing / Snowboarding
- Swimming
- Other_____

Please specify the duration and regularity of your outdoor physical activity?

_____

**MEDICAL / DIET HISTORY**

Do you currently smoke? **Yes / No**

Have you ever smoked? **Yes / No**

If yes, Please provide details of when you quit?

_____

Do you or have you ever used ANY drugs for recreational purposes? **Yes / No**

Are you taking ANY medication? **Yes / No**

If yes, please give details:

_____

Are you allergic to non steroidal anti-inflammatory drugs (NSAIDs) e.g. Aspirin, Nurofen, Ibuprofen, Naproxen, etc?

**Yes / No**

If yes, please give details:

_____

Do you or have you ever suffered from any of the following?

- Cardiovascular Disease **Yes / No**
- Hemodynamic / Clotting Disorder **Yes / No**
- Metabolic Disease (e.g. Diabetes) **Yes / No**
- Respiratory Disease **Yes / No**
- Liver Disease **Yes / No**
- Kidney Disease **Yes / No**
- Ulcers (e.g. Stomach) **Yes / No**
- Any Other Chronic Medical Condition **Yes / No**

If yes to any of the above, please give details:

_____

Have you ever fainted (*e.g. on standing, following fasting, during exercise)*? **Yes / No**

If yes, please give details: _____

Do you consume alcohol? **Yes / No**

If yes, how many units of alcohol do you consume per week?

_____ Units

Note: *Half a pint of ordinary strength larger/beer/cider, or one small glass of vine, or one pub measure of spirits (e.g. a shot of tequila), or half a bottle of alcopop (e.g. Smirnoff Ice) is equal to* ***1 Unit.***

Roughly, how many cups of coffee, tea, cola or any other caffeinated / energy drink (e.g. Red Bull) do you drink in a day?

_____ Cups

Do you take dietary supplementation (Vitamins, Botanicals, Amino-acids, etc)? **Yes / No**

If yes, what do you take and how regularly?

_____

How often do you eat citrus fruits?

_____

How many portions of fruit and vegetable do you have daily?

_____

How often do you eat fish rich in oil (Omege 3) or consume Fish oil supplements?

_____

Have you taken part, or do you intend to take part, in any other trial within the three months prior to and following this particular study (e.g. other studies, blood donor)?

If yes, please give details? **Yes / No**

_____

**DECLARATION:**

I declare that all of the above information is correct to the best of my knowledge. I understand that this information will be treated in the strictest of confidence.

Signature:_____ Date:_____

# Appendix 3

# Screening Questionnaire 2 (Chapter 8)

**School of Clinical and Experimental Medicine**
**College of Medical and Dental Sciences**
**University of Birmingham**

## Healthy Volunteer Screening Questionnaire

*Please remember, all the information will be treated in the strictest of confidence.*

### VOLUNTEER INFORMATION

Subject No (for researcher only):_____

Name:_____

Date of Birth:_____

Address:_____

_____

_____

_____

Phone No:_____

Email Address:_____

Height:_____ Cm

Weight:_____ Kg

BMI:_____ $Kg/m^2$

Resting Heart Rate:_____ B/min

Resting Blood Pressure:_____ mm HG

Ethnicity; Please tick the most appropriate:

☐ Caucasian

☐ African / Afro-Caribbean

☐ South Asian

☐ East Asian

☐ Other (please specify): _____

### ACTIVITY STATUS

How would you assess your present level of fitness? **POOR / AVERAGE / HIGH / TRAINED**

Leisure time questionnaire;

| Activity | Did you perform this activity? | | Month of activity | | | | Average time per week | |
|---|---|---|---|---|---|---|---|---|
| | No | Yes | Week 1 | Week 2 | Week 3 | Week 4 | hrs | min |
| Walking for pleasure | | | | | | | | |
| Walking to work | | | | | | | | |
| Walking during work breaks | | | | | | | | |
| Cycling | | | | | | | | |
| Using stairs when lift is available | | | | | | | | |
| Carrying – loading/unloading | | | | | | | | |
| Gardening | | | | | | | | |
| Household tasks (cooking / cleaning / Vacuuming, etc) | | | | | | | | |
| Home repairs | | | | | | | | |
| Occupational manual Labour (Building / Mining / Farming / Machine tooling, Nursing, etc) | | | | | | | | |
| Fishing/Hunting | | | | | | | | |

If yes, please provide the details of the activities._____

_____

Do you do any other exercise regularly? **Yes / No**

If yes, how regularly do you take part in physical activity? Please state the type of physical activity and how often? If you have multiple exercise schedules, please do not forget to list them all.

_____

Approximately, how long do you exercise for on each occasion?

_____

How long have you been exercising regularly?

_____

On a Scale of 1 to 10, with 10 being highest, how strenuous would you say your each exercise routine is?

_____

Do you take part any sport activities? If yes, please provide the details to these activities and how often do you participate in them?

_____

_____

On a Scale of 1 to 10, with 10 being highest, how strenuous would you say your sports regimen is?

_____

Do you take part in other activities? Please tick the appropriate one from the list provided. If the activity is not present, please specify.

- Rowing / Canoeing / Kayaking
- Corps training
- Cycling – for pleasure
- Dancing
- Martial arts
- Mountaineering
- Rock climbing
- Sailing
- Skiing / Snowboarding
- Swimming
- Other_____

Please specify the duration and regularity of your outdoor physical activity?

---

**MEDICAL / FOOD INTAKE HISTORY**

Do you currently smoke? **Yes / No**

Have you ever smoked? **Yes / No**

If yes, Please provide details of when you quit?

---

Do you or have you ever used ANY drugs for recreational purposes? **Yes / No**

For female participants;

How long ago did you reach menopause?

---

Are you taking ANY medication (this includes hormone replacement therapy)? **Yes / No**

If yes, please give details:

---

Do you or have you ever suffered from any of the following?

- Cardiovascular Disease (e.g. Hypertension, Raynaud's Disease, etc) **Yes / No**
- Hemodynamic / Clotting Disorder **Yes / No**
- Metabolic Disease (e.g. Diabetes) **Yes / No**
- Respiratory Disease **Yes / No**
- Liver Disease **Yes / No**
- Kidney Disease **Yes / No**
- Skin Disorders (e.g. Eczema, etc) **Yes / No**
- Any Other Chronic Medical Condition **Yes / No**

If yes to any of the above, please give details:

---

Do you consume alcohol? **Yes / No**

If yes, how many units of alcohol do you consume per week?

Units

Note: *Half a pint of ordinary strength larger/beer/cider, or one small glass of vine, or one measure of spirits (e.g. a shot of tequila), or half a bottle of alcopop (e.g. Smirnoff Ice) is equal to* ***1 Unit.***

Roughly, how many cups of coffee, tea, cola or any other caffeinated / energy drink (e.g. Red Bull) do you drink in a day?

_____ Cups

Do you currently take any vitamin supplements ☐ Yes ☐ No

What is your average weekly intake of the following foods?

| | | | |
|---|---|---|---|
| Citrus Fruits and soft fruit (not tinned) | ☐ None | ☐ a few times a week | ☐ everyday |
| Fruit Juice (e.g orange, grapefruit, tomato, etc) | ☐ None | ☐ a few times a week | ☐ everyday |
| Vitamin C enriched cordials | ☐ None | ☐ a few times a week | ☐ everyday |
| Potatoes (incl. instant) | ☐ None | ☐ a few times a week | ☐ everyday |
| Green Vegetables | ☐ None | ☐ a few times a week | ☐ everyday |
| Other fruits (incl. tinned) | ☐ None | ☐ a few times a week | ☐ everyday |

Do you currently take fish oil supplements ☐ Yes ☐ No

Average Weekly Fish Intake ☐ None ☐ 1 or 2 times ☐ 3 or more times

**DECLARATION:**

I declare that all of the above information is correct to the best of my knowledge. I understand that this information will be treated in the strictest of confidence.

Signature:_____ Date:_____

# Appendix 4

# Consent Form

**School of Clinical and Experimental Medicine**
**College of Medical and Dental Sciences**
**University of Birmingham**

## Healthy Volunteer's Consent Form

Please read this form carefully and sign it after the aims and procedures of the study have been explained to you.

- I have voluntarily agreed to take part in this study.
- I confirm that the aims of the study have been explained and that I have read and understood the information sheet given to me.
- I have been given the opportunity to ask questions and discuss the study on all aspects of the study and have understood the advice and information given as a result.
- I agree to comply with the reasonable instructions of the investigators and will notify them immediately of any unexpected unusual symptoms.
- I understand that any data and/or samples collected will be strictly used for scientific research/study.
- I authorize the investigators to disclose the results of my participation in the study.
- I understand that the information about me recorded during the study will be kept secure.
- I understand that the data collected and/or results of the study may be published in a scientific journal and Thesis and that if published, my personal details will be protected.
- I authorize the investigators to disclose to me any abnormal test results.
- I understand that I can ask for further instructions and/or explanations at any time.
- I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing. I also understand that any data / samples collected during the course of my participation will not be discarded due to my withdrawal from the study.
- I confirm that I have disclosed all the relevant medical information before the study.

**Volunteer's Name:**_____

**Signature:**_____

**Date:**_____

**Investigator's Name:**_____

**Signature:**_____

**Date:**_____

Subject Number (for researcher only):_____

# Appendix 5

# Supplemental Data (Chapter 4)

| Young - Rhythmic Exercise | | Mean Baseline | Exercise | | | Post Exercise Responses | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | | | 1st min | 2nd min | 3rd min | 0 s | 30 s | 1 min | 2 min | 3 min | 4 min | 5 min | 6 min | 7 min |
| P | HR (b.min$^{-1}$) | 64.38±1.43 | 87.77±6.10 * | 89.61±6.55 * | 89.00±4.26 * | 70.98±4.32 | 65.30±3.23 | 62.50±1.55 | 64.15±2.27 | 61.38±2.28 | 62.87±2.31 | 61.21±2.33 | 66.55±2.80 | 62.11±2.24 |
| | MABP (mmHg) | 78.52±3.07 | 96.50±5.67 * | 108.37±6.15 * | 116.61±6.42 * | 101.72±6.04 * | 95.13±5.98 * | 86.51±5.25 | 86.24±4.81 | 84.88±4.48 | 85.89±4.20 | 83.92±4.77 | 83.26±4.74 | 82.97±4.56 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) | 4.45±0.43 | --- | | | 56.18±4.87 * | 48.80±3.43 * | 40.62±4.25 * | 33.51±4.80 * | 29.76±5.45 * | 26.34±4.58 * | 22.51±4.26 * | 22.58±3.26 * | 18.76±3.52 * |
| | FVC (CU) | 0.06±0.01 | --- | | | 0.55±0.08 * | 0.51±0.05 * | 0.47±0.07 * | 0.39±0.05 * | 0.35±0.05 * | 0.30±0.05 * | 0.27±0.04 * | 0.27±0.03 * | 0.23±0.03 * |
| 40% $O_2$ | HR (b.min$^{-1}$) | 62.29±1.53 | 80.93±2.62 * | 81.49±2.35 * | 85.24±2.51 * | 66.64±2.69 | 61.94±3.15 | 64.57±3.33 | 60.43±2.51 | 60.32±1.88 | 62.74±3.22 | 60.97±2.79 | 62.23±3.34 | 61.77±2.02 |
| | MABP (mmHg) | 76.17±3.13 | 91.87±4.90 * | 104.99±5.32 * | 112.06±5.16 * | 99.36±3.68 * | 90.90±5.05 * | 85.38±4.67 | 82.84±4.94 | 83.12±4.63 | 82.52±4.49 | 82.67±4.58 | 82.87±4.57 | 80.63±4.77 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 4.34±0.38 | --- | | | 40.63±3.97 *‡ | 35.93±2.41 *‡ | 34.18±1.88 *‡ | 25.58±2.14 *‡ | 20.51±1.87 *‡ | 19.72±1.93 *‡ | 15.86±1.57 *‡ | 16.39±1.77 *‡ | 12.68±1.34 *‡ |
| | FVC (CU) † | 0.06±0.01 | --- | | | 0.41±0.04 *‡ | 0.39±0.03 *‡ | 0.39±0.02 *‡ | 0.31±0.03 *‡ | 0.27±0.02 *‡ | 0.24±0.02 *‡ | 0.19±0.02 *‡ | 0.20±-0.02 *‡ | 0.16±0.02 *‡ |
| A | HR (b.min$^{-1}$) | 67.20±1.93 | 86.12±4.19 * | 86.25±3.76 * | 88.96±4.52 * | 70.24±3.14 | 64.27±2.03 | 63.69±1.90 | 63.12±2.51 | 63.58±2.94 | 65.92±2.64 | 65.65±2.61 | 64.70±2.19 | 65.29±2.70 |
| | MABP (mmHg) | 77.53±2.75 | 96.34±4.58 * | 107.26±4.68 * | 112.47±4.65 * | 95.56±4.66 * | 92.07±5.57 * | 85.28±5.19 | 81.63±4.48 | 81.64±4.07 | 82.58±5.13 | 81.32±4.39 | 82.31±5.02 | 81.97±5.02 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 4.78±0.45 | --- | | | 41.95±3.63 *‡ | 37.61±3.54 *‡ | 31.86±3.45 *‡ | 25.92±2.86 *‡ | 19.92±2.46 *‡ | 19.18±2.78 *‡ | 16.39±1.69 *‡ | 15.91±1.71 *‡ | 12.84±1.30 *‡ |
| | FVC (CU) † | 0.06±0.01 | --- | | | 0.44±0.04 *‡ | 0.40±0.02 *‡ | 0.37±0.04 *‡ | 0.32±0.02 *‡ | 0.25±0.02 *‡ | 0.23±0.02 *‡ | 0.20±0.02 *‡ | 0.19±0.02 *‡ | 0.16±0.02 *‡ |
| A + $O_2$ | HR (b.min$^{-1}$) | 62.04±1.61 | 79.90±3.29 * | 81.01±2.90 * | 84.37±3.94 * | 65.99±2.05 | 59.53±2.57 | 59.99±3.00 | 61.57±2.33 | 60.61±2.61 | 60.49±2.04 | 60.65±2.01 | 62.46±3.68 | 60.14±2.63 |
| | MABP (mmHg) | 73.32±2.20 | 83.10±3.87 * | 100.22±5.04 * | 107.31±5.50 * | 92.99±4.72 * | 91.08±4.94 * | 81.47±4.74 | 81.89±4.27 | 80.53±4.57 | 80.17±5.80 | 80.34±5.14 | 78.13±4.84 | 81.48±5.24 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 4.59±0.41 | --- | | | 41.85±2.35 *‡ | 34.28±4.44 *‡ | 28.03±2.66 *‡ | 23.67±1.74 *‡ | 20.95±2.74 *‡ | 18.94±2.25 *‡ | 16.56±2.35 *‡ | 16.62±1.56 *‡ | 14.73±1.88 *‡ |
| | FVC (CU) † | 0.06±0.01 | --- | | | 0.45±0.03 *‡ | 0.38±0.04 *‡ | 0.34±0.03 *‡ | 0.29±0.02 *‡ | 0.26±0.04 *‡ | 0.24±0.03 *‡ | 0.21±0.02 *‡ | 0.21±0.02 *‡ | 0.18±0.02 *‡ |

Figure 1 **HR, MABP, FBF and FVC responses of rhythmic handgrip contractions performed by young men.** * represent values significantly different from respective baselines while † represents values different from placebo (P) condition (P < 0.05). Significant time x treatment interaction is denoted by ‡ (vs. Placebo)

| Young – Isometric Exercise | | Mean Baseline | Exercise | | | Post Exercise Responses | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | | | 1st min | 2nd min | 3rd min | 0 s | 30 s | 1 min | 2 min | 3 min | 4 min | 5 min | 6 min | 7 min |
| P | HR (b.min$^{-1}$) | 66.72±3.31 | 84.67±3.97 * | 87.12±4.05 * | 93.04±4.55 * | 75.51±3.91 * | 62.52±2.71 | 62.61±3.17 | 62.26±1.77 | 62.13±1.70 | 61.12±2.13 | 61.93±1.73 | 62.94±1.97 | 60.98±2.43 |
| | MABP (mmHg) | 80.27±2.72 | 94.38±4.69 * | 108.77±5.73 * | 122.89±7.12 * | 104.68±5.56 * | 90.85±5.32 | 90.18±4.47 | 84.08±4.65 | 83.17±4.62 | 79.58±4.62 | 76.31±5.57 | 78.48±4.91 | 76.07±5.64 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) | 5.92±0.55 | --- | | | 63.32±6.09 * | 48.47±4.50 * | 43.14±5.43 * | 40.36±4.72 * | 32.93±3.68 * | 29.97±3.84 * | 24.92±3.89 * | 22.82±2.78 * | 19.86±3.05 * |
| | FVC (CU) | 0.08±0.01 | --- | | | 0.61±0.06 * | 0.54±0.06 * | 0.48±0.07 * | 0.48±0.07 * | 0.40±0.05 * | 0.38±0.06 * | 0.33±0.05 * | 0.29±0.04 * | 0.26±0.04 * |
| 40% $O_2$ | HR (b.min$^{-1}$) | 63.67±2.00 | 85.99±5.62 * | 87.72±4.59 * | 91.29±3.50 * | 75.35±2.48 * | 60.23±2.12 | 58.72±2.17 | 58.05±2.35 | 60.00±2.74 | 61.98±3.42 | 58.71±3.30 | 57.04±1.34 | 58.11±2.08 |
| | MABP (mmHg) | 79.73±2.90 | 97.54±4.45 * | 116.18±6.62 * | 123.19±8.85 * | 100.93±4.42 * | 91.55±3.97 | 84.83±5.48 | 87.24±4.12 | 87.71±4.23 | 87.61±3.80 | 86.33±4.56 | 86.01±4.64 | 85.29±4.57 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 5.98±0.42 | --- | | | 42.36±3.75 *‡ | 36.86±3.54 *‡ | 30.65±2.24 *‡ | 30.00±1.54 *‡ | 24.40±2.10 *‡ | 23.60±2.31 *‡ | 18.90±1.96 *‡ | 16.76±1.24 *‡ | 14.24±0.97 *‡ |
| | FVC (CU) † | 0.08±0.01 | --- | | | 0.42±0.05 *‡ | 0.40±0.03 *‡ | 0.36±0.05 *‡ | 0.34±0.02 *‡ | 0.27±0.02 *‡ | 0.27±0.03 *‡ | 0.22±0.02 *‡ | 0.19±0.01 *‡ | 0.17±0.01 *‡ |
| A | HR (b.min$^{-1}$) | 68.69±1.80 | 87.78±4.81 * | 90.23±5.01 * | 94.19±7.17 * | 83.75±5.50 * | 69.09±4.28 | 66.58±2.63 | 61.90±2.09 | 64.57±2.68 | 65.58±3.61 | 63.92±3.44 | 66.03±3.04 | 66.24±3.07 |
| | MABP (mmHg) | 78.72±2.14 | 95.91±3.50 * | 111.60±4.95 * | 120.12±6.88 * | 99.55±6.22 * | 96.38±6.03 | 90.73±3.77 | 87.51±4.26 | 86.18±4.02 | 82.74±3.54 | 79.34±3.08 | 82.05±2.84 | 82.47±3.29 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 6.22±0.46 | --- | | | 44.85±5.42 *‡ | 36.45±4.17 *‡ | 33.65±2.94 *‡ | 28.88±3.01 *‡ | 25.13±3.00 *‡ | 20.55±2.01 *‡ | 17.94±1.70 *‡ | 17.35±1.97 *‡ | 13.33±1.27 *‡ |
| | FVC (CU) † | 0.09±0.01 | --- | | | 0.44±0.07 *‡ | 0.38±0.06 *‡ | 0.37±0.04 *‡ | 0.33±0.03 *‡ | 0.29±0.04 *‡ | 0.25±0.03 *‡ | 0.23±0.02 *‡ | 0.21±0.02 *‡ | 0.16±0.02 *‡ |
| A + $O_2$ | HR (b.min$^{-1}$) | 62.47±1.13 | 79.70±4.18 * | 82.47±4.05 * | 93.09±6.21 * | 77.59±4.63 * | 63.45±1.98 | 62.04±1.85 | 60.74±1.61 | 57.63±1.63 | 58.59±1.42 | 57.29±1.57 | 59.28±2.60 | 60.62±2.53 |
| | MABP (mmHg) | 75.50±2.69 | 96.41±5.12 * | 118.89±8.47 * | 124.33±8.64 * | 103.42±6.54 * | 93.59±5.37 | 82.51±5.97 | 84.37±6.27 | 86.03±4.53 | 82.87±4.80 | 80.98±3.87 | 82.62±3.84 | 81.29±4.58 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 7.23±0.81 | --- | | | 44.82±5.57 *‡ | 35.59±4.00 *‡ | 33.04±2.47 *‡ | 29.84±3.81 *‡ | 22.03±3.22 *‡ | 21.91±2.62 *‡ | 16.90±1.92 *‡ | 16.67±1.53 *‡ | 14.00±1.88 *‡ |
| | FVC (CU) † | 0.09±0.01 | --- | | | 0.43±0.04 *‡ | 0.38±0.04 *‡ | 0.35±0.03 *‡ | 0.35±0.05 *‡ | 0.26±0.04 *‡ | 0.26±0.04 *‡ | 0.21±0.02 *‡ | 0.20±0.01 *‡ | 0.17±0.01 *‡ |

Figure 2 **HR, MABP, FBF and FVC responses of isometric handgrip contractions performed by young men.** * represent values significantly different from respective baselines while † represents values different from placebo (P) condition ($P < 0.05$). Significant time x treatment interaction is denoted by ‡ (vs. Placebo).

| Older - Rhythmic Exercise | | Mean Baseline | Exercise | | | Post Exercise Responses | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | | | 1st min | 2nd min | 3rd min | 0 s | 30 s | 1 min | 2 min | 3 min | 4 min | 5 min | 6 min | 7 min |
| P | HR (b.min$^{-1}$) | 64.93±1.83 | 73.46±3.21 * | 74.67±3.44 * | 77.91±3.51 * | 68.78±3.85 | 62.72±3.16 | 62.29±3.25 | 63.40±3.30 | 63.79±3.33 | 63.08±3.26 | 65.48±3.50 | 64.82±3.23 | 65.51±3.18 |
| | MABP (mmHg) | 83.75±1.70 | 98.84±4.14 * | 106.81±4.40 * | 112.47±4.92 * | 100.52±4.19 * | 88.35±4.07 | 86.09±4.13 | 84.35±3.23 | 84.82±3.24 | 83.50±3.49 | 83.23±3.38 | 84.10±3.38 | 84.93±4.25 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) | 6.59±0.77 | --- | | | 64.26±5.81 * | 47.28±4.45 * | 37.65±3.75 * | 32.64±3.54 * | 24.92±2.13 * | 22.87±1.55 * | 20.57±1.64 * | 17.15±1.71 * | 12.58±1.05 |
| | FVC (CU) | 0.08±0.01 | --- | | | 0.64±0.06 * | 0.54±0.06 * | 0.44±0.05 * | 0.39±0.05 * | 0.29±0.03 * | 0.27±0.03 * | 0.25±0.03 * | 0.20±0.02 * | 0.15±0.01 * |
| 40% $O_2$ | HR (b.min$^{-1}$) | 62.61±1.69 | 71.95±3.54 * | 73.53±3.52 * | 73.78±3.43 * | 66.30±3.98 | 63.84±3.20 | 62.29±3.08 | 62.34±3.19 | 62.41±2.83 | 64.39±3.64 | 62.73±3.43 | 62.27±2.92 | 62.12±3.37 |
| | MABP (mmHg) | 86.25±2.64 | 102.55±4.38 * | 112.20±5.55 * | 117.20±6.56 * | 107.95±7.64 * | 92.46±5.06 | 89.36±4.70 | 87.26±4.22 | 86.47±4.24 | 88.61±4.60 | 88.85±5.14 | 87.02±4.40 | 86.98±4.47 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 6.04±0.53 | --- | | | 48.46±4.33 *‡ | 38.91±3.51 *‡ | 29.70±2.90 *‡ | 23.40±2.17 *‡ | 18.50±2.24 *‡ | 17.17±2.15 *‡ | 16.45±1.91 *‡ | 13.60±1.50 * | 11.02±0.78 |
| | FVC (CU) † | 0.07±0.01 | --- | | | 0.49±0.06 * ‡ | 0.42±0.05 *‡ | 0.33±0.05 *‡ | 0.26±0.03 *‡ | 0.21±0.03 *‡ | 0.19±0.03 *‡ | 0.18±0.03 *‡ | 0.16±0.02 * | 0.13±0.01 |
| A | HR (b.min$^{-1}$) | 62.01±1.55 | 73.94±3.52 * | 74.51±3.62 * | 75.06±3.49 * | 67.50±4.23 | 62.65±3.09 | 62.22±3.18 | 62.90±3.21 | 62.33±2.82 | 61.87±2.99 | 61.63±3.07 | 62.62±3.25 | 62.51±2.72 |
| | MABP (mmHg) | 88.37±2.13 | 106.98±4.33 * | 115.38±4.42 * | 121.82±5.22 * | 117.32±3.77 * | 96.91±3.64 | 96.92±4.27 | 92.00±4.77 | 92.73±4.66 | 91.15±4.23 | 91.52±4.19 | 90.18±3.55 | 87.91±3.70 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 5.12±0.50 | --- | | | 52.79±5.48 *‡ | 38..93±3.56 *‡ | 30.85±3.49 *‡ | 24.30±2.58 *‡ | 22.07±2.09 *‡ | 20.14±2.22 * | 17.58±2.42 * | 15.25±2.05 * | 13.83±1.74 |
| | FVC (CU) † | 0.06±0.01 | --- | | | 0.45±0.05 * ‡ | 0.40±0.04 *‡ | 0.31±0.05 *‡ | 0.26±0.03 *‡ | 0.24±0.03 *‡ | 0.22±0.03 * | 0.19±0.03 * | 0.17±0.02 * | 0.16±0.02 * |
| A + $O_2$ | HR (b.min$^{-1}$) | 61.57±1.72 | 72.06±4.03 * | 72.73±3.87 * | 72.65±3.71 * | 64.87±3.94 | 61.04±3.30 | 59.60±3.22 | 60.50±3.38 | 60.37±3.42 | 61.35±3.41 | 61.10±3.40 | 60.47±3.20 | 62.05±3.25 |
| | MABP (mmHg) | 84.57±2.22 | 103.59±4.98 * | 114.14±5.31 * | 118.23±5.16 * | 112.20±5.39 * | 93.93±4.18 | 91.94±4.36 | 87.31±3.33 | 86.80±3.47 | 86.38±3.54 | 86.12±3.54 | 84.24±4.50 | 86.12±4.75 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 5.02±0.30 | --- | | | 55.26±5.74 *‡ | 38.52±3.86 *‡ | 30.36±3.42 *‡ | 23.75±2.42 *‡ | 18.70±2.17 *‡ | 16.43±1.80 *‡ | 15.37±1.56 *‡ | 14.81±1.49 * | 12.40±1.00 * |
| | FVC (CU) † | 0.06±0.00 | --- | | | 0.49±0.06 * ‡ | 0.41±0.06 *‡ | 0.33±0.05 *‡ | 0.27±0.04 *‡ | 0.22±0.04 *‡ | 0.19±0.03 *‡ | 0.18±0.02 *‡ | 0.18±0.02 * | 0.14±0.01 |

Figure 3 **HR, MABP, FBF and FVC responses of rhythmic handgrip contractions performed by older men.** * represent values significantly different from respective baselines while † represents values different from placebo (P) condition ($P < 0.05$). Significant time x treatment interaction is denoted by ‡ (vs. Placebo).

| Older – Isometric Exercise | | Mean Baseline | Exercise | | | Post Exercise Responses | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | | | 1st min | 2nd min | 3rd min | 0 s | 30 s | 1 min | 2 min | 3 min | 4 min | 5 min | 6 min | 7 min |
| P | HR (b.min$^{-1}$) | 61.82±1.37 | 70.33±3.66 * | 74.35±4.45 * | 75.89±4.10 * | 67.00±3.31 * | 62.11±3.01 | 60.93±3.62 | 61.36±2.37 | 61.41±3.63 | 61.44±2.98 | 62.34±3.21 | 62.14±3.26 | 61.25±2.88 |
| | MABP (mmHg) | 81.53±1.92 | 98.60±4.78 * | 109.41±5.45 * | 117.89±5.99 * | 108.26±5.08 * | 97.48±4.39 * | 87.79±4.01 | 88.39±4.61 | 87.70±4.88 | 85.48±4.09 | 85.36±4.64 | 82.97±4.39 | 85.73±4.67 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) | 6.67±0.67 | --- | | | 71.45±6.52 * | 56.54±5.14 * | 47.42±3.70 * | 38.04±4.41 * | 28.56±2.81 * | 22.01±1.63 * | 18.22±1.94 * | 15.72±1.86 | 13.55±1.65 |
| | FVC (CU) | 0.08±0.01 | --- | | | 0.66±0.06 * | 0.58±0.08 * | 0.54±0.05 * | 0.43±0.06 * | 0.32±0.05 * | 0.26±0.03 * | 0.21±0.03 * | 0.19±0.03 * | 0.16±0.02 |
| 40% $O_2$ | HR (b.min$^{-1}$) | 59.84±1.56 | 70.39±4.15 * | 72.04±3.88 * | 74.53±3.48 * | 66.18±3.11 * | 59.64±2.79 | 59.37±3.14 | 59.30±3.13 | 58.72±3.13 | 59.38±3.06 | 58.79±2.74 | 59.60±3.09 | 59.87±2.84 |
| | MABP (mmHg) | 85.42±2.14 | 101.25±5.07 * | 113.46±6.44 * | 121.14±7.03 * | 105.51±5.57 * | 94.08±5.91 * | 89.76±4.69 | 89.07±3.60 | 88.78±3.92 | 88.26±2.82 | 90.13±3.76 | 89.49±3.68 | 88.77±3.82 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 6.09±0.37 | --- | | | 51.54±4.03 *‡ | 41.65±4.24 *‡ | 33.41±4.13 *‡ | 24.35±3.55 *‡ | 18.90±3.05 *‡ | 15.53±1.83 *‡ | 11.62±1.62 ‡ | 11.65±1.29 | 10.32±1.30 |
| | FVC (CU) † | 0.07±0.01 | --- | | | 0.48±0.05 *‡ | 0.44±0.06 *‡ | 0.37±0.05 *‡ | 0.27±0.05 *‡ | 0.21±0.04 *‡ | 0.18±0.03 *‡ | 0.14±0.02 ‡ | 0.13±0.2 | 0.12±0.02 |
| A | HR (b.min$^{-1}$) | 60.77±1.45 | 69.91±3.82 * | 72.31±4.05 * | 75.72±4.27 * | 68.42±3.12 * | 60.90±2.88 | 57.87±2.88 | 59.33±2.70 | 58.94±2.96 | 58.95±2.85 | 59.00±2.97 | 59.91±3.02 | 59.44±2.89 |
| | MABP (mmHg) | 85.26±2.22 | 102.30±5.21 * | 117.01±6.35 * | 124.50±5.20 * | 113.31±4.90 * | 95.26±4.28 * | 91.18±4.06 | 87.62±3.13 | 89.63±4.20 | 88.19±4.16 | 88.45±4.61 | 89.82±4.66 | 88.79±4.90 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 6.91±0.33 | --- | | | 52.98±7.53 *‡ | 38.59±5.43 *‡ | 33.80±5.49 *‡ | 28.59±4.29 *‡ | 22.96±3.17 *‡ | 19.03±2.28 * | 16.74±2.20 | 13.88±1.79 | 11.93±1.34 |
| | FVC (CU) † | 0.08±0.01 | --- | | | 0.47±0.07 *‡ | 0.41±0.06 *‡ | 0.37±0.07 *‡ | 0.33±0.05 *‡ | 0.26±0.04 *‡ | 0.22±0.03 * | 0.19±0.03 * | 0.16±0.02 * | 0.13±0.02 |
| A + $O_2$ | HR (b.min$^{-1}$) | 60.59±1.64 | 69.27±3.89 * | 73.65±3.73 * | 75.74±3.59 * | 65.22±3.27 * | 58.17±3.17 | 59.50±3.27 | 57.37±3.17 | 59.16±3.81 | 59.28±3.33 | 59.77±3.19 | 60.56±3.39 | 59.51±3.35 |
| | MABP (mmHg) | 82.76±1.94 | 100.98±4.20 * | 113.59±4.86 * | 118.98±4.66 * | 103.20±3.20 * | 93.86±4.15 * | 89.59±3.88 | 86.52±3.36 | 86.98±3.29 | 84.82±3.45 | 84.96±4.21 | 85.29±3.88 | 84.63±3.56 |
| | FBF (ml.dl$^{-1}$.min$^{-1}$) † | 6.85±0.46 | --- | | | 55.13±5.03 *‡ | 45.93±6.04 *‡ | 37.60±5.93 *‡ | 29.69±2.70 *‡ | 22.11±2.22 *‡ | 18.61±1.94 * | 15.08±1.47 * | 14.55±1.85 | 13.36±1.69 |
| | FVC (CU) † | 0.08±0.01 | --- | | | 0.53±0.05 *‡ | 0.48±0.08 *‡ | 0.42±0.07 *‡ | 0.34±0.04 *‡ | 0.25±0.03 *‡ | 0.22±0.03 * | 0.17±0.03 * | 0.17±0.03 * | 0.16±0.03 |

Figure 4 **HR, MABP, FBF and FVC responses of isometric handgrip contractions performed by older men.** * represent values significantly different from respective baselines while † represents values different from placebo (P) condition ($P < 0.05$). Significant time x treatment interaction is denoted by ‡ (vs. Placebo).

# Appendix 6

# Published Abstracts

1. **Junejo, R.T**., Ray, C.J., Lucas, S.J. and Marshall, J.M., 2016. Investigation into effects of supplementary $O_2$ on tissue oxygenation during isometric handgrip in the ipsilateral and contralateral limb in young (Y) and older (O) men using Near Infra Red Spectroscopy (NIRS). *The FASEB Journal*, *30*(1 Supplement), pp.761-9.

2. **Junejo, R.T.**, Ray, C.J. and Marshall, J.M., 2015. Role of oxygen ($O_2$) and cyclooxygenase (COX) products in muscle fatigue in healthy young (Y) and older (O) men. In *Proceedings of The Physiological Society*. The Physiological Society.

3. **Junejo, R.**, Ray, C. and Marshall, J., 2015. Prostaglandins (PGs) Contribute in an $O_2$ Dependent Manner to Reflex Vasoconstriction in Exercise. *The FASEB Journal*, *29*(1 Supplement), pp.994-20.

4. **Junejo, R.**, Ray, C. and Marshall, J., 2015. $O_2$ Dependent Contributions of Prostaglandins ($PGI_2$ $PGE_2$) to Exercise Hyperaemia in Young (Y) and Older (O) Men. *The FASEB Journal*, *29*(1 Supplement), pp.994-19.

5. Ray, C., **Junejo, R.** and Marshall, J., 2015. Does Adenosine make an $O_2$ Dependent Contribution to Exercise Hyperaemia? *The FASEB Journal*, *29*(1 Supplement), pp.994-14.

6. Marshall, J., **Junejo, R.**, D'Souza, F. and Ray, C., 2015. Effects of Breathing $O_2$ at different Concentrations on Reactive Oxygen Species (ROS) and Endothelium Dependent Dilatation. *The FASEB Journal*, *29*(1 Supplement), pp.787-4.

7. **Junejo, R.**, Ray, C. and Marshall, J., 2014. Prostaglandins contribute in an $O_2$-dependent manner to exercise hyperaemia following rhythmic and isometric handgrip exercise in young and older healthy subjects (1106.4). *The FASEB Journal*, *28*(1 Supplement), pp.1106-4.

8. **Junejo, R.**, Pavliv, F., Ray, C.J. and Marshall, J.M., 2013. Comparison between automated inflation and manual inflation for forearm blood flow measurements made with venous occlusion plethysmography in humans. In *Proceedings of The Physiological Society*. The Physiological Society.

9. **Junejo, R.**, Ray, C. and Marshall, J., 2013. Evidence that (COX) products contribute in an $O_2$-dependent way to exercise hyperaemia associated with both static and rhythmic handgrip contractions. In *proceedings of International Early Career Symposium - Clinical and Translational Physiology*. The Physiological Society.

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