

The effects of obesity and different modes of training on the skeletal muscle microvasculature

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A thesis submitted to the
University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

School of Sport and Exercise Sciences
College of Life and Environmental Sciences
University of Birmingham
September 2012

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General Abstract

The principle aims of this thesis involved the development of methods to measure enzymes that determine nitric oxide (NO) production (eNOS) and quenching (NAD(P)Hoxidase) within the microvasculature of skeletal muscle, and the use of these techniques to investigate metabolic syndrome and various training modes. Chapter 2 describes the development of reliable methods to analyse enzymes responsible for NO bioavailability within the endothelium of skeletal muscle microvessels. Chapter 3 suggests that impaired eNOS ser¹¹⁷⁶ phosphorylation is instrumental to the reductions in insulin sensitivity of obese Zucker rats. Chapter 4 reveals that 6 weeks endurance training (ET) and sprint interval training (SIT) in sedentary males induce similar increases in capillary density, and that SIT is more effective than ET at increasing eNOS content. Chapter 5 fails to observe a change in any measure of microvascular structure or function following 6 weeks resistance training in sedentary males. Chapter 6 suggests that 4 weeks of constant-load SIT is an effective intervention to improve the content of endothelial enzymes controlling NO bioavailability in obesity. In conclusion this thesis describes novel techniques which will be valuable tools for future research into microvascular function, and suggests that SIT may be an effective time efficient strategy to improve microvascular function.

Acknowledgments

There are many people I need to thank for making the completion of this PhD possible, whether for their academic input or their personal help.

Firstly, I need to thank my supervisors, Professor Anton Wagenmakers and Dr Chris Shaw for giving me the opportunity to work within the group and for their support and guidance over the years. Your guidance and support has been essential to completing this thesis and providing me with great opportunities for the future. I would particularly like to thank Chris for his support even when not my supervisor in the first years of my PhD.

I would also like to thank Professor Kevin Tipton and Dr James Fisher for their guidance and advice on many of the chapters within this thesis.

I also need to thank the members of the exercise metabolism research group and Histology lab; Annie, Jules, Helen, Oliver, Stuart, and Dan for their various help in the lab. In particular I need to thank Sam for his day to day support and role in all the studies within this thesis. I would also like to thank the various medics whom without we could not have completed much of the work. In particular Dr Aaron Ranasinghe, Dr Thomas Barker and Dr Andrew Mcleans for their contributions. Finally I need to thank all the participants who gave their time; without them, this research would not be possible.

However it is not only members of the laboratory and those involved in the research that have made this PhD possible. I would like to thank all my friends within SportEx who have made the last 4 years so enjoyable, especially all the members of lunch club and those I have shared an office with.

Finally, to my mum and Dad, and Beth for your encouragement and always being there on the dark days, and putting up with listening to signalling pathways and looking at blood vessels on the good days.

Thank you all.

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During the period of postgraduate study at The University of Birmingham, work from the current thesis resulted in the following abstract:

Cocks M, Shepherd SO, Tipton KD, Shaw CS, Wagenmakers AJM. Macro- and Microvascular adaptations in response to Resistance training. 17th Proceedings of the European College of Sport Science, 2012.

Bradley H, Shepherd SO, **Cocks M**, Shaw CS, Wagenmakers AJM. GLUT4 protein expression and localisation in human skeletal muscle following endurance and high intensity interval training. 17th Proceedings of the European College of Sport Science, 2012.

Cocks M, Shepherd SO, Tipton KD, Ranasinghe AM, Barker TA, Shaw CS, Wagenmakers, AJM. Microvascular adaptations in response to high intensity interval training in skeletal muscle. 16th Proceedings of the European College of Sport Science, 2011.

Shepherd SO, **Cocks M**, Tipton KD, Ranasinghe AM, Barker TA, Wagenmakers AJM, Shaw CS. Enhanced intramuscular triglyceride metabolism and improved insulin sensitivity in response to traditional endurance and high intensity interval training. 16th Proceedings of the European College of Sport Science, 2011.

Cocks M, Shepherd SO, Ranasinghe AM, Barker TA, Shaw CS, Wagenmakers AJM. Effect of exercise on skeletal muscle microvascular and sarcolemma endothelial nitric oxide synthase phosphorylation. Proceedings of the Physiology Society 23, C24, 2011.

Shepherd SO, **Cocks M**, Tipton KD, Ranasinghe AM, Barker TA, Wagenmakers AJM, Shaw CS. Endurance exercise decreases the lipid droplet association with perilipin 2 in human skeletal muscle. Proceedings of the Physiology Society 23, PC98, 2011.

Cocks M, Shaw CS, Wagenmakers AJM. Quantification of skeletal muscle microvascular endothelial nitric oxide synthase (eNOS) content using immunofluorescence microscopy. Proceedings of the Physiology Society 19, C61, 2010.

Cocks M, Shepherd SO, Tipton KD, Wagenmakers AJM, Shaw CS. High-intensity interval training improves microvascular and macrovascular function and insulin sensitivity. *British Journal of Sports Medicine*, doi:10.1136/bjsm.2010.078972.27.

Shepherd SO, **Cocks M**, Tipton KD, Wagenmakers AJM, Shaw CS. Improvements in insulin sensitivity and whole-body fat oxidation after a period of high-intensity interval training. *British Journal of Sports Medicine*, doi:10.1136/bjsm.2010.078972.32.

During the period of postgraduate study, the data in this thesis also resulted in the following conference communications:

Cocks M, Shepherd SO, Tipton KD, Shaw CS, Wagenmakers AJM. Macro- and Microvascular adaptations in response to Resistance training. 17th Congress of the European College of Sport Science, Bruges, 2012.

Cocks M, Shepherd SO, Tipton KD, Ranasinghe AM, Barker TA, Shaw CS, Wagenmakers, AJM. Microvascular adaptations in response to high intensity interval training in skeletal muscle. 16th Congress of the European College of Sport Science, Liverpool, 2011.

Cocks M, Shepherd SO, Ranasinghe AM, Barker TA, Shaw CS, Wagenmakers AJM. Effect of exercise on skeletal muscle microvascular and sarcolemma endothelial nitric oxide synthase phosphorylation. Physiology, Oxford, 2011.

Cocks M, Shaw CS, Wagenmakers AJM. Quantification of skeletal muscle microvascular endothelial nitric oxide synthase (eNOS) content using immunofluorescence microscopy. Physiology, Manchester, 2010.

Cocks M, Shepherd SO, Tipton KD, Wagenmakers AJM, Shaw CS. High-intensity interval training improves microvascular and macrovascular function and insulin sensitivity. International sports science and sports medicine, Newcastle, 2010.

The following papers were also published during the period of postgraduate study:

Cocks M, Shaw CS, Shepherd SO, Fisher J, Ranasinghe AM, Barker TA, Tipton KD, Wagenmakers AJM. High intensity interval and endurance training are equally effective in increasing muscle microvascular density and eNOS content in sedentary males. The Journal of Physiology DOI: 10.1113/jphysiol.2012.239566.

Cocks M, Shepherd SO, Shaw CS, Achten J, Costa ML, and Wagenmakers AJM. Immunofluorescence microscopy to assess enzymes controlling NO availability and microvascular blood flow in muscle. *Microcirculation* DOI: 10.1111/j.1549-8719.2012.00199.x, 2012.

Shepherd SO, **Cocks M**, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJM, Shaw CS. Preferential utilization of perilipin 2-associated intramuscular triglycerides during 1 h of moderate-intensity endurance-type exercise. The Journal of Physiology DOI: 10.1113/expphysiol.2012.064592

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Chapter 1

Introduction

1.1 Defining a control

The genetic makeup of Homo sapiens has changed little during the past 10,000 years (53). Therefore, modern humans are genetically adapted to a pre-agricultural hunter-gatherer life style (58), entailing the obligatory and integral performance of regular physical activity (42). As a consequence our ancestor's physical activity patterns likely resembled what today would be classified as a combination of aerobic, sprint and strength training (52). We therefore believe that the sedentary, food abundant state of modern living is creating an abnormal phenotype, as our current genes evolved from physically active humans. As a result, and as suggested by Booth et al. (23) the following introduction will present a physically active trained phenotype as the control or optimal state for humans.

1.1.1 Aerobic capacity and insulin sensitivity

Two key adaptations to this physically active trained state are a high aerobic capacity and insulin sensitivity, leading to an optimal healthy phenotype. The importance of aerobic capacity is demonstrated by its effectiveness as a predictor of mortality, having been shown to be a more powerful predictor than clinical variables or established risk factors such as hypertension, smoking, and diabetes, as well as other exercise-test variables, including ST-segment depression, the peak heart rate, or the development of arrhythmias during exercise (133). The importance of insulin sensitivity for maintenance of an optimal healthy phenotype during the lifespan is also demonstrated by its association with age related clinical events (56).

Both aerobic capacity and insulin sensitivity are positively correlated to each other, with trained individuals participating in high levels of physical activity showing high aerobic

capacity and high insulin sensitivity (75). As a result a number of the mechanisms by which regular physical activity leads to a high aerobic capacity and insulin sensitivity are common. Of these, elevations in skeletal muscle mitochondrial density and improvements in lipid metabolism seem to be part of the key shared mechanisms (for a previous review see (84). Another adaptation responsible for optimal aerobic capacity and insulin sensitivity in the physically active state is an ability to effectively match perfusion of tissues with metabolic demand. Of particular importance is the perfusion of skeletal muscle as it constitutes 40% of body mass and is a principle determinant of aerobic exercise capacity (94) and peripheral insulin sensitivity (30).

1.2 Insulin's hemodynamic effects

The following sections will discuss the role of insulin on hemodynamic changes affecting delivery of insulin and glucose to the muscle and the mechanisms that lead to an increased delivery in the optimal trained state.

1.2.1 Effect of insulin on limb blood flow

The earliest interest in the hemodynamic actions of insulin was provoked by the work of Baron and colleagues (12, 109), showing that insulin could regulate its own delivery to the muscle by increasing total leg blood flow, measured using a thermal dilution method. In this work they showed a strong positive correlation between increase in leg blood flow and whole body insulin mediated glucose uptake (12, 109) measured during hyperinsulinemic-euglycemic clamps. Following this a number of other laboratories investigated the regulatory role of insulin on its own delivery, with the majority of studies supporting the role of elevated limb blood flow in response to insulin mediating an increase in glucose uptake (145, 146, 177,

191). The most direct evidence for the role of total limb blood flow in regulation of insulin stimulated muscle glucose uptake comes from studies where the vasodilatory action of insulin, a Nitric Oxide (NO) dependent action (discussed subsequently), are blocked using the NO synthase inhibitor L-N^G-monomethyl arginine (L-NMMA). Such studies show that inhibiting insulin dependent increases in total limb blood flow result in diminished glucose uptake.

However, physiological levels of insulin have not uniformly been shown to lead to increases in limb blood flow in humans (20, 36, 134, 145, 146, 191), leading to controversy over the significance of insulin's action on limb blood flow, when physiologically relevant conditions are used. Adding to this controversy are the findings that pharmacological elevation of limb blood flow through infusion of nitropusside or bradykinin did not elevate glucose uptake in insulin resistant individuals during a hyperinsulinemic-euglycemic clamp (110, 135, 139). Finally the time course of insulin mediated increases in limb blood flow do not reconcile with a significant metabolic effect in regulating delivery of insulin to the muscle. Studies using physiological insulin report approximately 2 hours or more are required before insulin's effect on limb blood flow are observed, which significantly lags behind the insulin mediated increase in skeletal muscle glucose uptake (208). Consequently, it is difficult to assess the role that limb blood flow plays in insulin mediated glucose uptake and therefore insulin sensitivity.

1.2.2 Insulin's actions on the microvasculature

The discrepancy between the time course of the insulin induced dilation of feed arteries and increases in muscle glucose uptake (from A-V measurements across leg and/or forearm) has

led to further work on the role of the skeletal muscle microvasculature in regulating insulin induced muscle glucose uptake. This work has led to the suggestion that low physiological increases in insulin lead to increases in microvascular blood volume, thus increasing the capillary surface area available for the transport of insulin and glucose, while larger increases in insulin as occur during hyperinsulinemic-euglycemic clamps also increase microvascular blood flow. The latter could be the consequence of the late increase in feeding artery blood flow seen with pharmacological doses of insulin. Barrett and collaborators specifically proposed a mechanism in which dilation of terminal arterioles leading to the recruitment of previously underperfused capillaries may actually be the key regulatory site of insulin's action on skeletal muscle glucose uptake (13, 14, 150). The importance of capillary recruitment for glucose uptake in man has also been shown under physiologically relevant conditions through the direct measurement of the capillary permeability surface area product. In this study Gudbjornsdottir et al. (77) showed that an increase in capillary permeability surface area product was essential for increases in skeletal muscle glucose uptake during an oral glucose tolerance test.

Progression in evaluating the role of insulin mediated skeletal muscle microvascular recruitment as the key step in the control of muscle glucose uptake has been limited by the lack of methods available to directly quantify skeletal muscle microvascular perfusion (34). Two methods have been developed to assess the effect of stimuli on skeletal muscle microvascular blood flow. These are 1-methylxanthine (1-MX) extraction (an index of endothelial surface area available), and contrast-enhanced ultrasound (CEU, a method to measure muscle microvascular blood volume and muscle microvascular blood flow velocity). Details of both methods can be found in the review of Clark, 2008 (34).

Use of these methods has generated data in rats and humans which suggest that insulin recruits previously underperfused skeletal muscle capillaries through dilation of terminal arterioles increasing the delivery of insulin, glucose and other nutrients in healthy active individuals (40, 151, 188, 189, 210). Using these techniques it has been shown that the time course of insulin induced increases in muscle microvascular blood volume is much faster than of the dilation of feeding arteries and total leg blood flow, with the earliest measurable increases being apparent within 5-10 minutes. The increases in microvascular blood volume also precede the activation of key intermediates of the insulin signalling cascade and glucose uptake in skeletal muscle (186, 188). Finally it has been demonstrated that recruitment of the skeletal microvasculature occurs at considerably lower insulin infusion rates ($1 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) than required to enhance total limb blood flow (210). Therefore studies conducted using 1-MX and CEU give compelling evidence that insulin mediated dilation of terminal arterioles is important in insulin sensitivity within healthy active individuals.

1.3 Molecular mechanisms of insulin's action on the vasculature

1.3.1 Lessons from primary cell cultures

As a result of the interest in the vasodilatory actions of insulin several studies have attempted to find the mechanisms leading to vasodilatation following insulin stimulation. As a result an endothelial biochemical insulin signalling cascade has been identified, in first instance in endothelial cell cultures, which as end product generates NO, a powerful vasodilator of vascular smooth muscle cells.

The rate limiting enzyme in the synthesis of NO is endothelial nitric oxide synthase (eNOS) (131). eNOS converts the substrate L-arginine to the products NO and L-citrulline. Insulin

activates a signalling cascade similar to that within myocytes resulting in phosphorylation and activation of Akt, which directly phosphorylates eNOS at the serine¹¹⁷⁷ residue leading to activation of eNOS and subsequent production of NO (131)(Fig. 1).

1.3.2 In vivo insulin signalling

Although as discussed above insulin's vasodilatory effect on the macrovasculature only plays a role in the maintenance of the arterial glucose supply to the skeletal muscle in the >120 min period after the start of an hyperinsulinemic euglycemic clamp, the interest in the vasodilatory role of eNOS within the macrovasculature has led to much of our current knowledge on the mechanisms leading to vasodilatation. An important early *in vivo* observation was that infusion of the nitric oxide synthase (NOS) inhibitor L-NMMA into the femoral artery of human volunteers 3 h after the start of a hyperinsulinemic euglycemic clamp acutely reduced the late increase in limb blood flow and the accompanying increase in glucose uptake (171). This observation clearly illustrates the NO dependency of insulin induced arterial dilation. Other studies have shown that the total content of eNOS and insulin-induced phosphorylation of eNOS at ser¹¹⁷⁷ collectively determine endothelial NO production and the late vasodilatory response of the macrovasculature to insulin in animals (156, 174). Studies using cultured endothelial cells have also shown that incubation with insulin leads to ser¹¹⁷⁷ phosphorylation of eNOS, and that ser¹¹⁷⁷ is the main activation site (126, 154). Vincent *et al.* (187) have pre-treated rats with the NOS inhibitor L-NAME (*N*-nitro-L-arginine-methyl ester) and observed that it both prevented the insulin induced increase in microvascular blood volume and increase in skeletal muscle glucose uptake which normally occur 5-10 min after the start of a hyperinsulinemic euglycemic clamp (187). To date no studies have investigated the specific role of eNOS content and insulin induced ser¹¹⁷⁷ phosphorylation of eNOS in the mechanism

leading to the early insulin induced increase in microvascular dilation in skeletal muscle of rat and man.

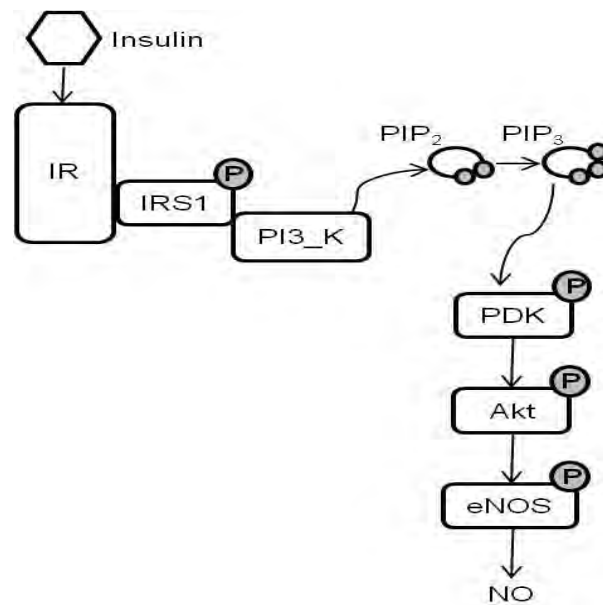


Figure 1.1. Scheme of the insulin signalling cascade within the vascular endothelium leading to NO production.

Abbreviations: IR, Insulin receptor; IRS, insulin receptor substrate; PI3-K, phosphatidylinositol-3-kinase; PIP₂, phosphatidylinositol-3,4-biphosphate; PIP₃, phosphatidylinositol-3,4,5-triphosphate; PDK, phosphoinositide-dependent kinase 1 ;eNOS, endothelial nitric oxide synthase.

1.4 Exercise-induced changes in perfusion

A key determinant of the aerobic exercise capacity is adequate perfusion of skeletal muscle, so that the fuel and oxygen needs of the exercising muscle are covered, while metabolites accumulating during contraction and playing a role in fatigue development are efficiently removed from skeletal muscle.

The initiation of exercise provokes an increase in blood flow to the active skeletal muscle, which is maintained during sustained exercise (160). As observed during insulin stimulation, exercise also recruits previously underperfused muscle capillaries leading to an increase in the capillary endothelial surface area available for exchange of blood borne fuels and oxygen (96, 189). This capillary recruitment observed with exercise has been shown to be very sensitive, with workloads that do not affect total limb blood flow increasing microvascular volume by 2-3 fold (189). Therefore it appears that delivery of nutrients to the active skeletal muscle can be exquisitely controlled by this staged vascular response allowing oxygen and nutrient delivery to match the metabolic needs of the exercising tissue.

1.4.1 Mechanisms of exercise hyperemia

A number of mechanisms have been reported to increase blood flow to skeletal muscle during exercise (48). A comprehensive review of all these mechanisms is beyond the scope of this introduction. Therefore, we will focus on the potential role of NO and eNOS activation in the regulation of the arterial and muscle microvascular blood flow during exercise.

The role of NO in exercise hyperaemia is controversial. Studies using eNOS inhibitors have produced equivocal results with some suggesting a role for NO (25, 89, 100, 129, 161) and

others excluding a role in exercise hyperaemia (59, 144, 164). However simultaneous inhibition of NO and prostaglandins produce a ~ 30% reduction in blood flow to exercising limbs (25, 89, 100, 129, 161). A matter which makes it difficult to draw hard conclusions from these studies is the number of complementary mechanisms, with other mechanisms potentially taking over when NO production is reduced with eNOS inhibitors (48). We, therefore, believe that today it is impossible to conclude whether the production of endothelial NO plays a role in exercise hyperaemia both at the arterial and skeletal muscle microvascular level.

Evidence in support for a potential role of NO in exercise hyperaemia comes from the finding that treadmill exercise increases eNOS ser¹¹⁷⁷ phosphorylation in arteries of active mice (211). It is proposed that this phosphorylation of eNOS is the result of shear stress mediated activation of AMPK and Akt (latter via the same signalling cascade as insulin) (211).

Hester et al. (87) have shown that inhibition of NO using the NOS inhibitor L-NAME reduces functional dilation in response to electrical stimulation of both first and second order arterioles within the superfused hamster cremaster muscle, but had no effect on third order arterioles. However studies investigating microvascular blood flow in humans and rats have produced conflicting results regarding the role of NO in exercise hyperaemia. Inhibition of NOS does not appear to reduce microvascular blood volume or flow measured using CEU (86, 158), however combined NOS and cyclooxygenase inhibition reduces microvascular blood flow (86). Also in the case of the muscle microvasculature, it is therefore not clear today whether increases in eNOS activation and microvascular endothelial NO production play a role in the increased perfusion of the muscle capillary bed during exercise.

It has been observed in several studies that there are substantial reductions in exercise hyperemia and muscle perfusion in sedentary, obese and elderly individuals with and without the metabolic syndrome, while it has also been reported that the vascular eNOS content is reduced in these conditions (118). It, therefore, is important that future studies investigating the potential role of eNOS and NO in the mechanisms leading to exercise hyperemia will also include these groups with known exercise limitations.

1.5 Microvascular density

In addition to the ability of terminal arterioles to dilate in response to insulin and exercise, microvascular density also plays a role in determining insulin sensitivity and aerobic capacity as it determines the maximal capillary surface area that is available for exchange of insulin, oxygen and nutrients (61). In active individuals capillary density has been shown to be much higher than in sedentary individuals (19) and there is strong positive correlation of muscle capillary density with both aerobic capacity (19) and insulin sensitivity (114).

1.6 Physical inactivity and obesity

1.6.1 Epidemiology: inactivity

As discussed earlier, throughout history daily physical activity has been a necessity for survival of *homo sapiens* (33). However, in the second half of the 20th century substantial changes have occurred in the need to be physically active, the most important changes being a reduction in professional workload requirements (more office jobs) and the introduction of automobiles, television and computer games. This has resulted in rapid increases in the proportion of adults and children leading a sedentary lifestyle. In the US 70% of the adult population is classed as sedentary (<30 min exercise 5 days/week) (33), while in the UK only

40% of males and 28% of females attain these guidelines (3). These trends for reduced physical activity are not only apparent in developed nations. Recent studies from India show high rapidly increasing levels of physical inactivity in men and women (169) and physical activity is documented to already dramatically decline during adolescence (105).

The landmark study of Morris et al. (128) was the first to show detrimental effects of physical inactivity on health, through the observation that the more active bus conductors had reduced risk of myocardial infarction than their more sedentary bus driver colleagues. Subsequently physical inactivity has been shown to affect at least 20 chronic health conditions (23), with reduced exercise capacity and energy expenditure from weekly activity more powerful predictors of all-cause mortality than clinical variables (132, 133). In addition to the direct health implication of sedentary lifestyles the economic cost of the prevention and treatment of chronic diseases resulting from physical inactivity in the UK has been estimated at £8.2 billion a year, involving both direct (treatment of lifestyle related disease) and indirect (sick absence) costs (1). This estimate excludes the contribution that physical inactivity makes to the direct and indirect costs of obesity (see next subsection).

1.6.2 Epidemiology: obesity

The latter half of the 20th century has also seen a massive increase in the availability (abundance) of food, variety of food products and marketing of food products via modern communication means. Although difficult to quantify, the combination of a life-style related reduction in energy expenditure with an increased energy intake or unhealthy nutritional choices and habits, has led to the dramatic increase in the prevalence of obesity in recent decades. The World Health Organisation (WHO) defines obesity as the excess accumulation

of adipose tissue to an extent which presents a health risk to the individual (4) and it is crudely defined using body mass index (BMI), with a BMI >30 defining an individual as obese.

Obesity has become a global epidemic with 396 million adults worldwide classified as obese (BMI > 30kg.m⁻²) (103). If recent trends continue this figure is expected to grow to 1.12 billion by 2030 (103). In the UK ~24% of adults are obese with an additional ~44% of men and 35% of women classified as overweight (BMI > 25kg.m⁻²) (3). As with physical inactivity obesity is not confined to developed nations with obesity rates increasing at dramatic rates in developing countries (205). Of particular concern is the increase in childhood obesity, which has increased in all countries for which data is available (195), as the negative health consequences of obesity are expected to appear at earlier ages and lead to more severe obesity related pathologies in future generations of the rapidly growing elderly population.

Obesity has been shown to double the risk of all-cause mortality (1), with the development of obesity being associated with the progression of a number of additional pathologies including cardiovascular disease, type II diabetes, and hypertension (170). When a number of these risk factors appear in conjunction with each other, in addition to obesity, the combination leads to “metabolic syndrome”, a condition which can dramatically increase the risk of negative health outcomes (64). The economic costs of obesity are also staggering with an estimated £2 billion spent on the treatment of obesity and its consequences by the NHS annually and the impact on employment predicted to be as much as £10 billion per annum (2).

Where possible in the subsequent discussion of vascular impairment, the negative consequences of inactivity and obesity will be separated, however in many cases there is a lack of experimental data on the role of inactivity alone.

1.7 Critical role of endothelial derived nitric oxide bioavailability

In the first part of this chapter the crucial role of increases in endothelial NO production in achieving insulin- and exercise-induced increases in skeletal muscle perfusion were explained. In the following subsections the mechanisms that impair NO production in sedentary and obese individuals will be reviewed and the functional consequences will be explained for insulin- and exercise-induced increases in arterial dilation and blood flow and muscle microvascular blood volume and blood flow in sedentary and obese individuals.

1.7.1 Impaired nitric oxide bioavailability in inactivity and obesity; lessons from the macrovasculature

NO dependent dilation following intra-arterial infusion of Acetylcholine (ACh) is reduced in obese individuals in comparison to lean controls (141). Further evidence of impaired NO production or bioavailability in obesity comes from studies measuring flow mediated dilation (FMD). FMD is measured as described by Thijssen et al. (178), and is the increase in vessel diameter (dilation) observed in an artery in response to a predefined period of 5 minutes occlusion and has been shown to be an NO-dependent process. FMD is the gold standard test used to measure whether NO production or bioavailability is reduced in certain individuals or populations. A reduced FMD has been observed in the brachial artery of individuals with obesity and the metabolic syndrome (51) and this has led to the suggestion that reduced NO

bioavailability leading to endothelial dysfunction may play a key role in a number of the developing pathologies in these groups (29).

The evidence that supports a reduction in NO bioavailability leads to endothelial dysfunction and impairments in vasodilatation of arteries has been supported by a number of studies in animal models of obesity, among others in studies of obese Zucker rats (OZR), which is the best known rat model of the metabolic syndrome (64).

The FMD response of conduit arteries of sedentary individuals has been observed to be larger than in trained individuals (181). This counter-intuitive result was explained by the fact that structural remodelling occurs in response to training which leads to a larger lumen size in trained individuals and which, therefore, will reduce the FMD in trained individuals in comparison to sedentary individuals (142). Periods of inactivity lead to reductions in the size of the lumen of conduit arteries, (17, 18, 45, 172) and are therefore expected to increase the shear stress response to FMD (181). Another mechanism which may contribute to the increase in FMD in sedentary individuals is that thickening of the artery wall in response to inactivity may increase responsiveness to vasoactive substances resulting in a positive correlation between wall thickness and FMD (143, 165, 166, 179). Finally an increased sensitivity to NO of the smooth muscle in arteries of sedentary individuals has been reported to be responsible for the elevated FMD (181). Although many of the studies show this increase in FMD in sedentary individuals, a short term bed rest study found reduced FMD which was not accompanied by a change in baseline lumen diameter (21), suggesting a possible role of reduced NO bioavailability.

Suvorava et al. (173) have shown that dilation in response to ACh is impaired in aortic rings of sedentary mice, suggesting that insulin induced activation of eNOS and increases in NO production may well be reduced in the sedentary state, similar to the observation made in obese individuals.

1.7.2 Impaired nitric oxide bioavailability within the microvasculature of sedentary and obese individuals

Obese individuals display a marked reduction in the dilation of the cutaneous microvasculature in response to iontophoresis of ACh, suggesting an impaired endothelial mediated response to this classical vasodilator (121). However use of ACh to demonstrate NO mediated vasodilatation in the cutaneous microcirculation has been questioned as inhibition of NOS does not or only partially inhibits ACh mediated cutaneous dilation (26, 92).

Studies in animal models of obesity suggest that NO production or bioavailability is reduced within the muscle microvasculature. Frisbee & Stepp (67) have shown that the NO mediated dilation in response to ACh and elevated shear stress is severely impaired in situ in arterioles of the cremaster muscle of OZR in comparison to LZR. In addition studies using isolated skeletal muscle 1a arterioles have also shown an impaired vasodilator effect to various NO mediated dilators in arterioles obtained from OZR in comparison to arterioles from LZR (64). However to the author's knowledge the effect of obesity on the ability of terminal arterioles in skeletal muscle to dilate in response to ACh has not been investigated in rat or man in vivo.

The effect of inactivity has also only been investigated in the cutaneous microvasculature in humans and the results obtained are not necessarily the same as in the skeletal muscle

microvasculature. In the cutaneous microvasculature ACh induced vasodilation was impaired following bed rest (50, 136). The cutaneous response to heating (which also is NO mediated) was also reduced following bed rest (44).

Evidence that inactivity leads to reduced NO production or NO bioavailability in skeletal muscle comes from a hind limb unloading study in rats. In these animals endothelium dependent dilation in response to ACh is impaired in both the soleus feed artery and 1a arterioles (47), suggesting that the NO bioavailability is reduced in both the macrovasculature and the skeletal muscle microvasculature.

1.7.3 Insulin mediated vasodilation

Hyperinsulinemic-euglycemic clamps in obese individuals have shown that late (>120 min) insulin mediated increases in leg blood flow are attenuated suggesting an impaired response of the leg macrovasculature to insulin (12, 109). However, as was discussed earlier the role of this late increase may be less important under more physiological conditions, such as in the period after ingestion of a mixed meal.

Insulin mediated microvascular recruitment in skeletal muscle has been studied in obese individuals in comparison to lean humans using the CEU method and was shown to be substantially impaired both during a hyperinsulinemic euglycemic clamp (38) and following mixed meal ingestion (37, 104). Insulin mediated microvascular recruitment, assessed with the 1-methylxanthine method, and skeletal muscle glucose uptake were also severely impaired in OZR in comparison to LZR (193). This impairment in insulin mediated microvascular recruitment in skeletal muscle is likely to contribute to the reduced insulin

mediated glucose disposal in obesity, as the impaired microvascular recruitment will significantly reduce the muscle capillary surface area available for diffusion of insulin and glucose, thereby severely limiting delivery of insulin and glucose to the muscle fibres (13, 14, 150).

To the author's knowledge there are no studies regarding the effect of inactivity on insulin mediated vasodilation in either feeding or resistance arteries or the muscle microvasculature. However physical inactivity through bed rest is associated with the development of insulin resistance and vascular dysfunction suggesting a possible role of impaired vasodilation in the insulin resistance developed through inactivity (79). Inactivity also has been reported to lead to decreases in eNOS gene expression and protein content in the macrovasculature of humans and animal models (118).

1.7.4 Functional hyperaemia

In comparison to lean individuals vascular resistance is increased and exercise-induced increases in forearm blood flow in obese humans are impaired (137, 153). Unfortunately no data is available on differences in the exposed skeletal muscle capillary surface area in obese humans compared to lean healthy humans during exercise. However it has been shown that exercise induced microvascular recruitment is impaired in patients with type II diabetes displaying microvascular complications (198). In animal models of obesity muscle blood flow during contraction has been shown to be impaired in the OZR, due to attenuated functional hyperaemia in skeletal muscle arterioles in OZR compared to lean controls (60, 203, 204).

To the authors knowledge no studies have investigated the effect of bed rest on the microvascular blood flow of skeletal muscle during exercise in humans. Therefore evidence of impaired functional hyperaemia following inactivity must come from animal models particularly hind limb unloading models, in which a reduced limb blood flow has been observed during exercise (201).

It appears that obesity and inactivity reduce the maximal microvascular blood flow of skeletal muscle during exercise. However as discussed above mechanisms of functional hyperaemia are complex and currently not completely understood, therefore the possible role of NO in the attenuated perfusion exhibited in obesity and inactivity currently is not known. However, there is evidence that NO production and bioavailability are reduced by inactivity and obesity and that this, therefore, is one of the candidate mechanisms for the reduction in maximal microvascular blood flow in skeletal muscle in response to exercise in these groups (90, 192). Of support for this theory is data showing that improved endothelial function following the removal of oxidative stress and vascular sources of prostaglandinH2/thromboxane A2 (combined treatment with Tempol and SQ-29548, respectively) is likely responsible for the increase in hemodynamics in response to electrical stimulation in the distal microcirculation of OZR to the level seen in LZR (65).

1.8 Potential mechanisms of impaired NO bioavailability

NO bioavailability is determined by the balance between NO production and NO removal. NO production is dependent on the activity of the rate limiting enzyme eNOS (156, 157, 174), which depends both on its protein content and phosphorylation state (156, 157, 174). In contrast NO removal is partly dependent on scavenging by ROS. The following sections will

focus on the effect of obesity and inactivity on eNOS gene expression, protein content and phosphorylation state and the main reactions that lead to ROS production in the vasculature.

1.8.1 Obesity: eNOS content

Hickner et al. (88) have shown that skeletal muscle eNOS content measured using the quantitative sandwich enzyme immunoassay technique in whole muscle homogenates from the *m. vastus lateralis* is negatively associated with body fat percentage, and that overweight women have lower eNOS content than lean women.

A number of animal models have investigated the effect of obesity on eNOS content. OZR and rats fed a high fat diet display reduced eNOS protein content in the aorta (24, 155, 156). In resistance arteries isolated from rat *cremaster m.* eNOS content is also markedly reduced in obese compared to LZR (55). Although the findings of reduced eNOS content are relatively consistent in the macrovasculature, analysis of eNOS content in skeletal muscle homogenates in models of obesity are less clear. eNOS content in *gastrocnemius muscle* homogenates of high fat fed rats is reduced in comparison to lean controls (24). OZR have the same eNOS content as their lean littermates in hind limb skeletal muscle homogenates (69). It should be noted though that the interpretation of these results is confounded by the fact that eNOS is not only present in the muscle microvascular endothelium, but also in the muscle fibres (39).

1.8.2 Obesity: eNOS phosphorylation

Phosphorylation of eNOS has been shown to acutely regulate its activity in cultured endothelial cells (130). eNOS is phosphorylated on multiple phosphorylation sites with ser¹¹⁷⁷

regarded as the main activation site (154). However eNOS ser¹¹⁷⁷ phosphorylation has not been assessed in arteries and the skeletal muscle microvasculature of obese humans.

In aorta of high fat fed rats basal eNOS ser¹¹⁷⁷ phosphorylation has been shown to be reduced (174). In addition the increase in ser¹¹⁷⁷ phosphorylation of eNOS induced by insulin infusion was also reduced in rats on the high fat compared to the control diet (174) and was, therefore, proposed to explain the reduced arterial vasodilation observed in the rats on the high fat diet.

The effect of obesity on basal and insulin stimulated eNOS ser¹¹⁷⁷ phosphorylation has not been investigated within skeletal muscle of rats and man. The only available information is that eNOS ser¹¹⁷⁷ phosphorylation was increased in cardiac muscle homogenates of obese compared to LZR (69), but the meaning of this observation is not clear, and could also involve a compensation mechanism required to protect the heart in metabolic syndrome from fatal microvascular underperfusion.

1.8.3 Obesity: ROS

In obese humans elevated levels of oxidative stress have been demonstrated by increased nitrotyrosine levels in endothelial cells harvested via a scraping technique from the antecubital vein (167). This observation indicates that the endothelial cells in obese individuals not only generate NO, but also superoxide anions (O_2^-). As both of these molecules are free radicals they react with each other when they meet under the formation of peroxynitrite, which itself is a free radical and therefore remains a highly reactive molecule. Peroxynitrite among others can lead to nitration of the tyrosine residues in nearby endothelial proteins. Nitration of endothelial proteins, therefore, is a marker of local O_2^- production,

which in turn will lead to formation of peroxynitrite and many other secondary free radical molecules, collectively named Reactive Oxygen Species (ROS). Indications of increases in oxidative stress have also been found in the aorta and femoral artery of OZR, a model of the metabolic syndrome. In comparison to their lean littermates there was an increase in the aorta and femoral artery of OZR in dihydroethidium fluorescence, which is a histochemical staining technique to identify sites of $\cdot\text{O}_2^-$ production (67, 202).

Elevations in $\cdot\text{O}_2^-$ production within the vasculature as explained above will reduce NO bioavailability through the scavenging of NO and subsequent formation of peroxynitrite. $\cdot\text{O}_2^-$ is generated within the vasculature by redox enzymes. Two main oxidant systems exist within the human vascular system, NAD(P)Hoxidase (NAD(P)Hox) and xanthine oxidase. Studies in human obesity suggest that endothelial protein expression of NAD(P)Hox is up regulated in venous endothelial cells (167) while no change was seen in xanthine oxidase, suggesting that elevated NAD(P)Hox is primarily responsible for the elevated oxidative stress associated with obesity. This finding of elevated NAD(P)Hox is supported by high fat feeding studies in rats which also show elevation in NAD(P)Hox content within the aorta (157). In addition to elevated NAD(P)Hox protein content its activity has also been shown to be elevated in the aorta of OZR (202).

NAD(P)Hox is a multi-enzyme complex, requiring the assembly of at least 6 components for its activation. Firstly NOX2 (Also known as gp91phox) is a transmembrane protein which transports electrons across cell membranes to reduce oxygen to $\cdot\text{O}_2^-$ (15). NOX2 is activated by a complex series of protein/protein interactions. P22phox is a membrane bound protein constitutively associated with NOX2 which helps stabilise the NOX2 protein and dock

cytosolic proteins. For NOX2 to be activated the cytosolic subunits P47phox, p67phox, Rac and p40phox need to translocate to the plasma membrane, thus allowing complex formation and activation of the NOX2 enzyme.

NAD(P)Hox protein expression has not been directly measured in the skeletal muscle microvasculature or in muscle homogenates of obese individuals. However treatment of *cremaster muscle* arterioles isolated from OZR with the $\cdot\text{O}_2^-$ scavengers polyethylene glycol-superoxide dismutase and catalase has been shown to improve ACh-induced dilation of arterioles (67). This suggests a potential role for elevated $\cdot\text{O}_2^-$ production in the impaired vasodilatory responses to insulin and exercise observed in obesity and the metabolic syndrome in the skeletal muscle microcirculation.

In addition to NAD(P)Hox, the enzyme eNOS itself may be a potential source of $\cdot\text{O}_2^-$ production through eNOS uncoupling, a phenomenon that is observed in obesity, metabolic syndrome and hypertension. Production of NO by eNOS requires several cofactors, the most important one is tetrahydrobiopterin (BH_4) which stabilises the eNOS dimer (11). Oxidation of BH_4 leads to loss of the interaction between BH_4 and eNOS and results in the production of $\cdot\text{O}_2^-$ rather than NO as eNOS transfers its electron to oxygen rather than to L-arginine (98, 138). Although eNOS uncoupling remains largely uninvestigated in obesity it has been shown that peroxynitrite is a potent oxidiser of BH_4 (111, 131, 140) and secondly peroxynitrite may oxidise the zinc thiolate core of eNOS causing enzymatic uncoupling (140, 212, 213).

1.8.3 Inactivity: eNOS content

To the authors knowledge there are no studies investigating bed rest or inactivity on eNOS protein expression in arteries and the skeletal muscle microvasculature in humans. Hindlimb unloading studies in rats have generated a wealth of relevant data though. Hindlimb unloading in rats reduced the eNOS mRNA and protein content both in feed arteries and class 1a arterioles of the *soleus m.*, which experienced an impaired vasodilator response to exercise (162, 200). Of interest eNOS expression and protein content did not change in the feed artery and class 1a arterioles of the *gastrocnemius m.*, which did not experience impairment in the endothelium dependent vasodilatory ability. These data collectively suggest that a reduction in total eNOS content in response to a reduction in physical activity may reduce both resting muscle blood flow and insulin and exercise-induced vasodilation both at the level of the muscle feed arteries and arterioles. The discrepancy between muscle groups also suggests a possible role for chronic reductions in blood flow in the reduced eNOS expression, as resting muscle blood flow was reduced in the *soleus m.* during hind limb unloading and maintained in the *gastrocnemius m.* As it has been shown that eNOS expression is regulated by fluid flow in monolayer's of cultured endothelial cells, this supports the role attributed to shear stress as one of the main signals controlling eNOS expression (22).

1.8.4 Inactivity: eNOS activation

There is no published data regarding the effects of inactivity on eNOS ser¹¹⁷⁷ phosphorylation in the basal state or in response to stimulation by insulin and exercise.

1.8.5 Inactivity: ROS

In inactive rats vascular lipid peroxidation has been shown to be elevated in addition to elevated $\cdot\text{O}_2^-$ release assessed by L-012 chemiluminescence and by the superoxide dismutase-inhibitable cytochrome C reduction assay (113). NAD(P)Hox activity and protein expression were both elevated in homogenates of the aorta (113). This suggests a possible role for NAD(P)Hox in the impaired NO-dependent vasodilatory ability of feed arteries. However studies investigating whether physical activity or bed rest increase biochemical markers of oxidative stress and NAD(P)Hox protein expression or activity have not been conducted at the level of the skeletal muscle microvasculature.

1.9 Microvascular rarefaction

Alterations in microvascular structure and density may also play a role in obesity and inactivity induced reductions in skeletal muscle blood perfusion. Obesity has been consistently shown to cause capillary rarefaction in both humans and animal models (63, 71). Although inactivity has not been directly studied, detraining causes a significant reduction in skeletal muscle capillary and microvascular density (106). This will significantly reduce the capillary surface area available for transport of insulin, glucose and other nutrients thus limiting their delivery and uptake by the muscle fibres (61).

1.9.1 Mechanisms of angiogenesis and microvascular rarefaction

Vascular endothelial growth factor (VEGF) has previously been shown to stimulate endothelial cell division and migration (209) and increases in VEGF mRNA and protein content and VEGF receptors (VEGFR-1 and VEGFR-2) have been shown to be an essential prerequisite for exercise induced angiogenesis (6, 70, 72). However, increased capillary

proliferation through the up regulation of VEGF and VEGFR-2 in the early stages of electrical stimulation has been shown to be NO dependent (122), as it did not occur following NOS inhibition with L-NMMA. In addition the effects of longer term electrical stimulation appear to be fully NO dependent as inhibition of NOS suppresses capillary growth without a concomitant reduction in VEGF or VEGFR-2 (122). As a result of the critical role of NO in exercise induced angiogenesis, much of the work on mechanisms of microvascular rarefaction in obesity has focused on the role played by the observed reduction in NO bioavailability (97). The clearest illustration that a reduced NO bioavailability leads to capillary rarefaction has been shown by Frisbee et al. (62) in OZR. In this study treatment of OZR with the antioxidant TEMPOL reduces microvascular rarefaction (62). The importance of reduced NO bioavailability to this process was further illustrated when these animals were treated with L-NAME and TEMPOL, in this situation the lower oxidative stress had no effect on microvascular rarefaction suggesting that it is the reduced NO bioavailability resulting from NO quenching by ROS and not the oxidative stress per se which caused the microvascular rarefaction. The mechanism by which the sedentary state (inactivity) leads to capillary rarefaction has not been investigated in great detail, however, it would appear likely that the lack of regular exercise bouts leading to regular increases in blood shear stress takes away the stimulus that is needed for VEGF production and angiogenesis, while the total eNOS content will also drop in prolonged periods of inactivity, thus leading to a new reduced set point for capillary density.

1.10 Endurance training to regain optimal function

As discussed earlier we believe that inactivity and obesity create an abnormal phenotype which increases the risk of ill health, in particular reduced aerobic capacity and insulin

sensitivity. However it has regularly been shown that endurance training (ET) is an effective way of improving health and regaining the optimal phenotype which occurs in trained individuals. In particular ET is shown to increase aerobic capacity (106) and insulin sensitivity (84) in previously sedentary or obese individuals. As a result of the beneficial effects of exercise on health the guidelines of the American College of Sports Medicine and the American Heart Association suggest that individuals between 18 and 65 should participate in moderate-intensity aerobic (endurance) physical activity for a minimum of 30 min five days a week or vigorous-intensity aerobic physical activity for a minimum of 20 min three days a week (82). Part of the beneficial effects of ET on aerobic capacity and insulin sensitivity in obesity are likely to occur via insulin and exercise-induced increases in the local perfusion (microvascular blood volume and blood flow) of skeletal muscle. In the next subsection of the introduction the vascular adaptations and mechanisms leading to this increase in local muscle perfusion will be discussed.

1.10.1 Endurance training (ET) to improve nitric oxide bioavailability in inactivity and obesity; lessons from the macrovasculature

Only one study has investigated the effects of traditional endurance-type exercise training in obese adults (190). Another study (163) has investigated the effect of a combined diet and physical activity intervention in obese adults, while other studies have been performed in obese adolescents and/or children following circuit training (combined aerobic training and resistance training). All of these studies show that aerobic exercise training is an effective method to increase FMD (120, 196, 199), suggesting that aerobic training increases NO bioavailability in the macrovasculature of obese individuals. One study has also shown that aortic stiffness measured by pulse wave velocity is reduced (8). A reduction in aortic stiffness

is the result of structural and functional improvements, which most likely will involve an improved vasodilatory function as a result of an improved NO bioavailability.

Animal studies also consistently show that ET is an effective method for improving NO bioavailability. Studies in a number of rat models of obesity have shown that ACh mediated dilation of the macrovasculature is improved by training in obese rats (9, 184). The improvements in endothelium dependent vasodilatory function observed in these studies were abolished following administration of the eNOS inhibitor L-NAME further suggesting that an increase in NO bioavailability is responsible for the improvement in the vascular dilatory function following training.

The effect of ET on macrovascular NO dependent dilation in previously sedentary individuals has produced contrasting results making it difficult to draw firm conclusions on the role of ET increasing NO bioavailability in this group. In conduit arteries several studies have shown an increased FMD response in previously sedentary individuals (35, 149). However not all studies investigating FMD have found this increase (127, 180). It is possible that the disparate results are due to the different durations of the interventions, with only a single functional assessment being made at the end of the studies. Recent evidence suggests that functional adaptations (such as improved FMD) in conduit arteries may precede arterial remodelling leading to increases in lumen diameter. Therefore, multiple time point assessments in future studies are required to gain a more comprehensive understanding of the time course of conduit artery adaptations to training in previously sedentary individuals (182). As in conduit arteries, studies in resistance vessels have also produced conflicting results (182).

Animal studies have also produced mixed findings into the role of ET in previously sedentary animals with results dependent on the species, vascular bed and duration of the intervention. As in the human studies initial increases in endothelium dependent vasodilation are abolished after long term training (99). However studies in aortic rings of sedentary rats have shown an increased response to ACh following training and that this response was abolished following L-NAME suggesting that improvements are primarily mediated by an increased NO dependent vasodilatory function (49).

1.10.2 Endurance training to improve nitric oxide bioavailability within the microvasculature in inactivity and obesity

There is no data on the effect of ET on muscle microvascular vasodilatory capacity in humans. However 2 studies in animals have suggested that ACh induced vasodilation of skeletal muscle arterioles is improved following ET in OZR (204). Frisbee et al. (66) have shown that 10wk of ET improved reactivity of arterioles to ACh and that this improvement was due to an elevated NO bioavailability in response to stimuli.

One study has investigated the effect of ET on the cutaneous microvasculature and observed that ET increased ACh mediated microvascular perfusion in previously sedentary humans (194). More compelling evidence for a role of elevated cutaneous microvascular NO bioavailability following training in previously sedentary older adults comes from Black et al. (16). In this study cutaneous heating was used as the stimulus and the contribution of NO to the improved cutaneous perfusion following ET was directly assessed by blocking the NO production with L-NAME. The results suggest that ET reversed the impaired microvascular NO bioavailability in the previously sedentary older adults. Future research should confirm

that this effect of ET can be extrapolated to insulin and exercise induced dilation in the muscle microvasculature.

It is difficult to draw a firm conclusion on the effect of ET in previously sedentary animals due to a large variation in responses of different order arterioles and different vascular beds. However, most of the studies investigating skeletal muscle arterioles have shown that the vasodilatory response to ACh and FMD is increased, suggesting that endothelial function can be improved within the microvasculature of previously sedentary animals (99). Spier et al (168) have suggested that the enhanced response of *gastrocnemius m.* arterioles to FMD following ET in rats is mediated exclusively through upregulation of NO mediated dilation, as the increased response to FMD was abolished by L-NAME administration (168).

1.10.3 Insulin mediated vasodilation

To the authors knowledge there is only one study investigating the effect of training on insulin mediated vasodilation. This study investigated the effect of 14 d of ET on limb blood flow and skeletal muscle microvascular recruitment during a hyperinsulinemic euglycemic clamp in previously sedentary rats (152). This study found that, although total limb blood flow in response to insulin was unchanged following training, hindleg 1-MX disappearance was increased indicating that there was an increase in skeletal muscle microvascular recruitment. These observations imply that the so-called 'nutrient blood' flow to skeletal muscle fibres was increased and that the insulin-induced dilation of terminal arterioles is improved following training, likely through increased NO bioavailability as the insulin induced microvascular recruitment is NO dependent (188). This increase in skeletal muscle microvascular recruitment was accompanied by an increase in whole body insulin sensitivity

suggesting an increased uptake of the glucose infused during the clamps by the skeletal muscle fibres.

1.10.4 Exercise induced hyperemia and arteriolar vasodilation

Information regarding the effects of ET on exercise induced hyperaemia in humans is unavailable for both obesity and inactivity. However two studies using OZR have investigated the effect of ET on femoral artery blood flow and the vasodilatory response of skeletal muscle arterioles to electrically induced muscle contractions (66, 204). Both studies observed an increased femoral artery blood flow and an increased vasodilatory response of the arterioles after training in the OZR, suggesting that both the response of feeding arteries and the skeletal muscle microvasculature to exercise-induced increases in shear stress is elevated following ET in the OZR model of the metabolic syndrome.

In sedentary rats exercise induced vasodilation was increased in the femoral artery and class 1a and 2a arterioles following 8 weeks of ET. This effect, however, was lost following 16 weeks of ET in all but the femoral artery, suggesting a time and order effect of training in previously sedentary animals, with increases in microvascular structure and density eventually leading to a reduction in the shear stress experienced by individual arterioles during exercise (112).

1.11 Mechanisms of improved vasodilation following training of sedentary and obese individuals

The increased availability of NO appears to play a key role in the augmented vascular vasodilatory function observed following ET. Therefore the following subsection will focus

on the potential mechanisms that lead to an increase in NO bioavailability. These theoretically could involve increases in eNOS content and ser¹¹⁷⁷ phosphorylation leading to eNOS activation and increased endothelial NO production. A reduced production of $\cdot\text{O}_2^-$ and other ROS could also lead to a reduction in NO scavenging.

1.11.1 Human studies

Hoeir et al (91) is the only study to investigate eNOS content in humans following ET. In this study skeletal muscle eNOS content measured in whole muscle homogenates was increased following 4 weeks of training in previously sedentary men. However, no human studies are available investigating the effects of exercise training of sedentary or obese individuals on eNOS ser¹¹⁷⁷ phosphorylation, NAD(P)Hox protein expression, or local vascular ROS production, and therefore the following subsections will focus on animal work alone. The only human studies to investigate the effect of ET on eNOS content and phosphorylation or NAD(P)Hox content in humans were conducted in coronary artery disease patients and showed that both eNOS content and ser¹¹⁷⁷ phosphorylation were increased (78), while NOX2 content (5) was reduced in the left internal mammary artery following ET.

1.11.2 Animal studies

1.11.2.1 eNOS content and phosphorylation

Only 2 studies have investigated the effect of ET on eNOS content and phosphorylation in obesity and both of these investigated the effect in the aorta. One study investigated eNOS content and phosphorylation in aortic homogenates of high fat fed rats (184) and the other aortic endothelial cells of Otsuka Long-Evans Tokushima fatty rats (displaying obesity and TIID) (27) before and after ET. In both studies there was an increase in eNOS ser¹¹⁷⁷

phosphorylation under basal (unstimulated) conditions and no change in eNOS content following ET. This may suggest that ET in obese individuals increases the bioavailability of NO via an increase in ser¹¹⁷⁷ phosphorylation and therefore via activation of eNOS rather than via an increase in eNOS protein content. However no studies have investigated the effect of ET on insulin or exercise induced eNOS phosphorylation, which would be the most important variable to understand the mechanism behind improvements in the vasodilatory response to insulin and exercise.

A study in previously sedentary rats has shown that aortic endothelial cell eNOS protein content and mRNA are increased following training (49, 206). This suggests that elevated eNOS content may play a role in the improved macrovascular vasodilatory function following ET. In rats eNOS content is elevated in isolated arterioles assessed by Western blotting of the red portion of the gastrocnemius in response to ET (117). Although eNOS content appears to increase in both the skeletal muscle microvasculature and in feed arteries following ET, vascular eNOS phosphorylation following ET has never been assessed.

1.11.2.2 ROS

One of the most important effects of ET is an upregulation of antioxidant enzymes. These systems convert $\cdot\text{O}_2^-$ and ROS into other molecules that are not free radicals. They therefore protect against quenching of NO by $\cdot\text{O}_2^-$ and ROS and thus help to improve the vasodilatory response to insulin and exercise. The predominant antioxidant systems within the vasculature are superoxide dismutase (SOD), catalase and glutathione peroxidase. Of these systems SOD has received most attention following exercise training, as total SOD activity is shown to be elevated in skeletal muscle following training (176). SOD rapidly converts $\cdot\text{O}_2^-$ to hydrogen

peroxide. SOD has three different isoforms, Cu/Zn dependent isoforms SOD-1 and 3 and Mn dependent SOD-2. SOD-1 is the primary isoform of SOD within blood vessels and is primarily localised within the cytosol of endothelial cells and vascular smooth muscle cells (57). SOD-2 and 3 are also present in blood vessels but less abundantly, and are localised to the mitochondria (57) and cell membrane (68), respectively of endothelial cells and vascular smooth muscle cells.

The effect of ET on antioxidant systems in obesity is understudied, as to our knowledge only one study has investigated the effect of training. In this study, high calorie fed rats had higher levels of the antioxidant SOD-1 in aorta and mesenteric arteries following training than the sedentary litter mates, which was associated with elevated endothelial function (46). No information is available on the effect of ET on the expression of antioxidant enzymes in the microvasculature of skeletal muscle in obese individuals.

In previously sedentary pigs long term ET increase aortic SOD-1 protein content and activity (159). However there was no increase in protein content of other antioxidant enzymes (Mn-SOD and catalase). These adaptations to oxidant enzymes were accompanied by a reduction in lipid peroxidation a marker of oxidative stress. Unlike this study in porcine aorta McAllister et al. (117) showed no difference in SOD-1 protein content from a number of conductance vessels following ET in previously sedentary rats(117). In addition SOD-1 was not up regulated in most orders of arterioles (1a-5a) in both red and white portions of the gastrocnemius. Although the gastrocnemius feed artery SOD-1 content was increased. The differences in these studies may be due to differences in species (rat vs. pigs) used and training duration (8-12 wks Rush et al. (159), vs. 16-18wk McAlister et al.(117)). The results do seem to suggest that reduction in oxidative stress through upregulation of antioxidant

enzymes may contribute to elevated endothelial function, but more studies should investigate its role, particularly in humans.

In addition to the up regulation of antioxidant defence proteins, the content of NAD(P)Hox subunit p67phox has been shown to be reduced following training in previously sedentary pigs (159). Suggesting that in addition to the upregulation of antioxidant defences production of O_2^- is reduced following ET training. This observation is waiting for confirmation in rat and man.

1.11.3 Angiogenesis

Whether ET can reverse the negative impact of obesity on skeletal muscle angiogenesis has not been investigated in human obesity and only one study has investigated the effects in animal models of obesity. In this study Frisbee et al. (66) demonstrated that ET reversed the microvascular rarefaction observed in OZR. They suggested that the increase in microvessel density was a result of the ability of ET to increase the low NO bioavailability in the OZR at the start of the training.

Both studies in humans and rodents have shown that ET leads to substantial increases in muscle capillarization in previously sedentary groups (7). The angiogenic response of skeletal muscle to exercise training is mediated by a combination of signals which include contractile activity, increased shear stress and metabolic activity (54). Of these stimuli, increased shear stress through elevated blood flow is the most studied. As with functional vasodilation NO is proposed to have a significant influence on the angiogenic response to exercise, likely through a synergistic/ complementary association with VEGF. It has been shown that the initial effect of elevated blood shear stress by electrical stimulation causes an

increase in the release of NO which is followed by an increase in capillary proliferation through up regulation of VEGF and VEGF receptor2 proteins (122). The exact roles of NO and VEGF in capillary growth are not clear, but as long term inhibition of NOS during electrical stimulation had no effect on the increase in the VEGF protein but still reduced capillary growth it is likely that NO is instrumental for VEGF to increase capillary proliferation.

1.12 HIT a time efficient training strategy to mirror the effects of ET

Recently various forms of high intensity interval training (HIT) have received much attention as alternative training strategies due to their ability to mirror or even surpass the effects of traditional ET (73). As a result of this interest a number of different protocols have been developed varying greatly in mode, intensity and time commitment of training. The main protocols are aerobic interval training (AIT) developed by Wisloff and colleagues (197) and low volume, sprint interval training (SIT) and constant load high intensity interval training (HIT) developed by Gibala and colleagues (107). The typical training session using AIT involves 4x4 minute intervals of treadmill exercise at 90-95% heart rate max interspersed by rest periods of 4 minutes at 50-70% heart rate max. SIT sessions have been developed using the Wingate test, which involves 30s of “all out” cycling against a high resistance. The typical training protocol involves 4-6 repeated Wingate tests separated by 4 or 4.5 minutes of recovery. The SIT method of training therefore provides a very time efficient training stimulus as exercise is limited to 2-3 minutes per 30 min session and total time commitment is ~90 minutes per week for 3 sessions. The SIT protocol has subsequently been translated in constant workload HIT protocols which involve 1 min cycling at a constant workload of approximately 100% of maximal workload predefined following a maximal exercise test to

exhaustion and lead to reductions in training intensity and total time commitment making it a more practical training method (116).

Despite the overwhelming evidence for the beneficial effects of ET on health and prevention of disease many adults fail to meet guidelines for physical activity. The most commonly cited reason for this failure to meet physical activity guidelines is a lack of time (185). Given that lack of time is such an important barrier to chronically increase physical activity levels we believe that exercise prescriptions which limit time commitment may be beneficial in increasing public health. As a result of this belief the following section will focus on studies investigating the effects low volume SIT and constant workload HIT rather than AIT as we believe these are modes of training, which have the ability to best reduce time commitment, and thus increase adherence and a widespread participation in the community.

Much of the current information on SIT and/or HIT has focused on the metabolic adaptations. Burgomaster *et al.*, (28) were the first to investigate SIT in comparison to ET and observed that 6 weeks SIT 3x per week produced similar improvements in aerobic exercise capacity as 6 weeks ET (5x per week, 40-60 minutes at 65% $\text{VO}_{2\text{peak}}$) (28). Subsequent studies have consistently shown that this increase in aerobic exercise capacity is associated with an increase in activity and content of mitochondrial enzymes which mirror those of ET (74). In addition to these changes in skeletal muscle oxidative capacity SIT has been shown to induce similar changes in whole body lipid oxidation (28). SIT has also been shown to have beneficial effects on insulin sensitivity. In previously sedentary individuals 2 weeks of SIT has been shown to increase insulin sensitivity (10). Further studies in type II diabetics have shown that an adapted HIT (10x 60s constant workload cycling at 90% maximal heart rate

interspersed with 60s rest, 3x per week for 2 weeks) can reduce the 24 hour blood glucose concentration profile (115).

Despite the growing body of information for the beneficial metabolic and health related effects of SIT and HIT there is very little research into the vascular effects of these potentially important training modes. To the author's knowledge Rakobowchuck et al. (149) is the only study to investigate the effect of SIT on vascular function. In this study 6 weeks of SIT (3x per week, 4-6 Wingate's interspersed by 4.5 minutes recovery) produced similar changes in FMD and peripheral artery stiffness as ET (5x per week, 40-60 minutes at 65% $\text{VO}_{2\text{peak}}$). We therefore believe that considerably more work is needed into the vascular effects of this training mode, especially given the beneficial vascular effects of AIT shown in a number of different groups (80, 183).

1.13 Resistance training

Resistance training (RT) has recently been added to the guidelines of the American College of Sports Medicine and the American Heart Association for physical activity and public health, because of its well-known effects on muscular strength, which help to promote and maintain health and independence (82). In addition to its effects on strength RT has also been shown to have a beneficial effect on insulin sensitivity (95, 123). The benefits of RT on insulin sensitivity have previously been attributed to the increase in muscle mass which accompanies RT (123, 175) (as skeletal muscle is the site primarily responsible for glucose uptake), but studies where differences in fat mass are taken into account or no differences in body composition are observed still produce increases in insulin sensitivity following RT (207). Studies have suggested that as well as changes in muscle mass, RT causes qualitative changes

in skeletal muscle such as increases in the content of the glucose transporter GLUT4 and components of the insulin signalling cascade within skeletal muscle (93, 108).

1.13.1 Effects of resistance exercise on vascular function

Although RT has been shown to have beneficial effects on skeletal muscle its effects on the vasculature are less known with many studies into a variety of vascular adaptations suggesting it has no impact or may even cause negative or harmful effects.

Early theories suggested that RT may cause an elevation in resting blood pressure; however a number of meta-analyses of the effect of RT have now suggested that RT may be an effective intervention to decrease resting systolic and diastolic blood pressure (43, 101, 102). Although small, these reductions could have an important impact on cardiovascular complications, as small reductions in BP decrease stroke and coronary heart disease risk.

Another RT induced adaptation which may bring negative effects on health is through its reported effect on arterial stiffness. A cross-sectional study has suggested that RT may elevate aortic stiffness (124). The potential for RT to elevate aortic stiffness is of importance as aortic stiffness is highly predictive of cardiovascular events (32). The potential for RT to elevate arterial stiffness has also been found following an RT intervention (41, 125), however not all studies suggest that RT increases arterial stiffness (31, 85, 147).

Studies investigating endothelial dependent dilation of conduit arteries in previously sedentary individuals have suggested that RT has no effect of NO bioavailability as FMD of the brachial artery does not change (148). This finding of unchanged endothelial function in

previously sedentary humans is supported by the observation in previously sedentary young animals that ACh mediated dilation of the femoral artery was unchanged by RT (81). In this study RT also did not lead to a change in eNOS content and ser¹¹⁷⁷ phosphorylation of the femoral artery. There are no studies in humans or animals investigating the effect of RT on skeletal muscle microvascular function.

Capillary density has been investigated following RT. Studies suggest that RT increases capillarization in proportion to fibre growth (76, 83, 119). Therefore although RT may stimulate angiogenesis (more capillaries will be needed for the larger muscle cells and total muscle mass) it does not have an effect on capillary density and as a result is unlikely to play a role in functional adaptations such as the increase in insulin sensitivity observed following RT.

1.14 Importance of measuring skeletal muscle microvascular enzyme contents

In the preceding sections a key role has become apparent for impairments in NO production within the microvasculature of skeletal muscle in the progression of microvascular dysfunction as it occurs in the sedentary state (inactivity) and obesity. It has also been explained that the ability of ET to improve the capacity to produce NO under basal and stimulated conditions may also play a key role in the improvements of the insulin and exercise induced microvascular vasodilatory capacity following ET. Knowledge of the adaptations to the enzymes that produce or quench NO, such as eNOS and NAD(P)Hox, would greatly add to our understanding of the mechanisms by which the sedentary state and

obesity lead to microvascular function loss and the mechanisms by which exercise training interventions restore the microvascular vasodilatory capacity.

Previous estimates of these enzymes have been made with Western Blot methodology applied to homogenates of human and rat muscle samples. Although Western blot methodology is useful, it does not reveal the relative distribution of these enzymes between muscle fibres and the microvasculature when applied to muscle homogenates. Western blots also do not provide information on whether changes in protein content in chronic disease or as a result of exercise training interventions are the result of changes in endothelial protein content or the result of changes in microvascular density.

There currently is no data on the eNOS content, eNOS ser¹¹⁷⁷ phosphorylation state, and NAD(P)Hox protein content in the endothelial layer of terminal arterioles and capillaries as they occur in rat and human muscles *in situ*. As changes in protein content and activity of these enzymes determine the vasodilatory capacity of the skeletal muscle microvasculature and the size of the capillary permeability surface area product for insulin, glucose, other nutrients and oxygen, it was clear that there was a need for the development of assays to specifically determine the protein content and phosphorylation state of the mentioned endothelial enzymes in the endothelial layer of the skeletal muscle microvasculature. This assay will then subsequently be used in the experimental chapters of this thesis to reveal the mechanisms behind the blunting of insulin and exercise induced vasodilation in the skeletal muscle microvasculature of sedentary and obese humans and rats with metabolic syndrome and also to investigate the beneficial effects of exercise training interventions.

1.15 Overview of the thesis

The studies in this thesis were either designed to investigate the effects of inactivity and obesity on skeletal muscle microvascular density and the protein content and phosphorylation state of microvascular endothelial enzymes that lead to NO production (eNOS) and NO quenching (NAD(P)Hox) or to investigate the effect of various training methods on the indicated variables and relate the results obtained to whole body insulin sensitivity and $\text{VO}_{2\text{max}}$.

Current methods to investigate microvascular enzymes controlling NO availability have a number of weaknesses which are explained in earlier subsections of chapter 1. Therefore in **Chapter 2** we describe the development of a new method to assess the enzymes controlling NO production and quenching. We present evidence that this novel immunofluorescence microscopy method can reliably and reproducibly quantify skeletal muscle eNOS content and ser¹¹⁷⁷ phosphorylation and NOX2 content. In addition the ability of the technique to investigate physiological changes is investigated by measuring eNOS content and eNOS ser¹¹⁷⁷ phosphorylation following 1 h of acute endurance-type exercise.

The OZR is a model of extreme obesity leading to the metabolic syndrome. Skeletal muscle insulin resistance in OZR is accompanied by an impaired hemodynamic response to insulin, which is proposed to contribute to the decreased glucose uptake observed in earlier studies during a hyperinsulinemic-euglycemic clamp. In **chapter 3** we aimed to use the immunofluorescence methods developed in **chapter 2** to investigate skeletal muscle microvascular eNOS content and eNOS ser¹¹⁷⁷ phosphorylation in response to a hyperinsulinemic euglycemic clamp in OZR to see if impairments in the content or

phosphorylation of eNOS contribute to the impaired glucose uptake. It was hypothesised that reduced microvascular eNOS content in the basal state and reduced eNOS phosphorylation during the clamp would contribute to the reduced glucose clearance seen in OZR. These changes are related to the time course of glucose clearance rates during hyperinsulinemic euglycemic clamps in the same rats from which the muscles are dissected at the end of clamp.

Sprint interval training (SIT) has received much attention recently as a time efficient exercise strategy which appears to mirror the metabolic adaptations observed during moderate intensity endurance training. However, SIT's effect on the skeletal muscle microvasculature has not been studied. Therefore in **chapter 4** we firstly aimed to make comparisons of the structural and endothelial enzymatic changes in skeletal muscle microvessels in response to ET and SIT in previously sedentary young males. Secondly, we aimed to investigate the effect of 1 h cycling on eNOS ser¹¹⁷⁷ phosphorylation both in the untrained state and after the two training interventions. Finally, the effects of SIT and ET on arterial stiffness and blood pressure were considered to investigate earlier claims of SIT's effectiveness within the macrovasculature. It was hypothesised that SIT and ET would produce similar changes in eNOS content and microvascular density.

Resistance training has recently been added to physical activity guidelines due to its effects on muscular strength and insulin sensitivity. It is suggested that qualitative changes within the skeletal muscle are responsible for the improvements in insulin sensitivity observed with RT. However, it is unknown whether changes in microvascular structure and/ or function play a role in the increased insulin sensitivity observed following RT. As such in **chapter 5** we aimed to investigate whether RT improved microvascular structure and endothelial enzymes

responsible for NO bioavailability, to investigate if changes in these factors parallel improvements in whole body insulin sensitivity. We hypothesised that RT would not or only minimally increase microvascular density and eNOS content in response to 6 weeks training, suggesting that these factors are not the primary drivers for the expected elevations in insulin sensitivity observed following 6 weeks of RT in young previously sedentary males.

In **chapter 4** it was shown that SIT was an effective method of improving microvascular density and eNOS content in lean sedentary participants. In the final chapter (**Chapter 6**) we conducted a pilot study to investigate the effects of a new more practical, time efficient constant workload SIT strategy. We investigated its effect on microvascular density and enzymes controlling NO production and quenching, and whether these changes translated into improved aerobic capacity, insulin sensitivity, and cardiovascular risk profile, in a young healthy obese previously sedentary group. We hypothesised that microvascular density and eNOS content would be increased in response to 4 weeks constant workload SIT, and that increases in NOX2 content in the basal state resulting from obesity would be reduced following training. Finally we hypothesised that the improvements in microvascular structure and enzymes would mirror improvements in aerobic capacity, insulin sensitivity and cardiovascular risk profile.

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Chapter 2

Immunofluorescence Microscopy to Assess Enzymes Controlling NO Availability and Microvascular Blood Flow in Muscle

2.1 Abstract

The net production of nitric oxide (NO) by the muscle microvascular endothelium is a key regulator of muscle microvascular blood flow. Here we describe the development of a method to quantify the protein content and phosphorylation of endothelial NO synthase (eNOS content and eNOS ser¹¹⁷⁷ phosphorylation) and NAD(P)H oxidase expression. Human muscle cryosections were stained using antibodies targeting eNOS, p-eNOS ser¹¹⁷⁷ and NOX2 in combination with markers of the endothelium and the sarcolemma. Quantitation was achieved by analysing fluorescence intensity within the area stained positive for the microvascular endothelium. Analysis was performed in duplicate and repeated 5 times to investigate coefficient of variation (CV). In addition, 8 healthy males (age 21±1years, BMI 24.4±1.0kg.m⁻²) completed 1 hour of cycling exercise at ~65% VO_{2max}. Muscle biopsies were taken from the *m. vastus lateralis* before and immediately after exercise and analysed using the new methods. The CV of all methods was between 6.5 and 9.5%. Acute exercise increased eNOS serine¹¹⁷⁷ phosphorylation (Fold change 1.29±0.05, *P* <0.05). These novel methodologies will allow direct investigations of the molecular mechanisms underpinning the microvascular responses to insulin and exercise, the impairments that occur in sedentary, obese and elderly individuals and the effect of lifestyle interventions.

2.2 Introduction

It is well established that exercise (8) and insulin (20) cause vasodilatation of feed arteries and arterioles and that both events contribute to increases in skeletal muscle blood flow and microvascular blood volume (31, 32). This microvascular response allows for exquisite control of blood flow distribution, and the delivery of oxygen and nutrients. Indeed, an early increase in microvascular volume in response to rises in insulin concentrations has been shown to play an important role in insulin mediated glucose uptake (1, 25, 30), while microvascular blood volume has been suggested to increase during low intensity exercise in the absence of changes in bulk blood flow (32).

As with feeding arteries, indirect evidence using NOS inhibitors suggests that the elevation in microvascular volume observed in response to insulin is NO-dependent (30, 31), and the response to exercise is at least part NO-dependent (14). As a result, impairments in microvascular volume in response to insulin or exercise observed in sedentary, obese and elderly individuals have been attributed at least in part to reductions in NO bioavailability (1, 2, 13, 25, 28, 33). Furthermore, an experimental reduction in microvascular NO bioavailability following NOS inhibition prevents exercise-induced angiogenesis in rats (23) and, therefore, reductions in NO bioavailability have been suggested as a potential cause of capillary rarefaction in ageing and obesity (11, 22). Finally, impairments in NO-dependent muscle microvascular function have also been suggested to precede macrovascular impairments. Given the importance of microvascular NO availability in the regulation of nutrient delivery and angiogenesis, and its importance as a target for therapeutic interventions (6, 18), the mechanisms regulating NO availability and microvascular perfusion are of great scientific and clinical importance.

NO bioavailability is determined by the balance between NO production and NO quenching by reactive oxygen species. As such reductions in both endothelial NO production (29) and/or increased NO quenching by reactive oxygen species (21) could explain reductions in NO bioavailability observed in aging, obesity and other pathologies (22, 26, 33). Current knowledge of the enzymes that determine endothelial NO bioavailability is primarily based on measurements of mRNA expression and protein content in isolated (larger) blood vessels from animal models (12, 17, 24). These studies have shown that the protein content and phosphorylation state of eNOS collectively determine NO production. Furthermore, the enzyme NAD(P)H oxidase (NAD(P)Hox) has been suggested to be the major source of superoxide (O_2^-) anion production in obesity (27) and hypertension (5). The superoxide anions thus generated are responsible for reductions of NO bioavailability via quenching of NO in a reaction that produces peroxynitrite. Studies measuring the protein content of these enzymes in human skeletal muscle have previously used Western blots or sandwich enzyme immunoassay techniques applied to extracts of whole muscle homogenates (15, 16). Importantly, these methodologies are not able to assess the protein content or phosphorylation state of eNOS in endothelial cells without potential contributions from the muscle fibres, nor can they provide information on changes in microvascular density.

In this study we therefore aimed to adapt immunofluorescence microscopy techniques previously applied to investigate endothelial protein expression in isolated blood vessels or isolated endothelial cells (4, 7, 9) for use on cryosections of percutaneous human muscle biopsies. These methods will allow measurement of the content of eNOS and NAD(P)H oxidase and eNOS ser¹¹⁷⁷ phosphorylation state (main activation site; (24)) in the endothelial layer of the microvasculature of human skeletal muscle. We also applied this

method to muscle biopsies obtained before and after 1 hour of dynamic cycling exercise to investigate the hypothesis that exercise in man leads to eNOS ser¹¹⁷⁷ phosphorylation in the muscle microvascular endothelium as previously observed in mouse arteries (34).

2.3 Materials and Methods

Muscle samples

Skeletal muscle samples used for the development of the immunofluorescence microscopy methods were obtained from the *m. vastus lateralis* of one human volunteer (age, 20 years; body mass index (BMI) 25 kg.m⁻²), using the percutaneous needle biopsy technique (3). Samples were blotted to remove excess blood and dissected free of fat and collagen. The samples were then embedded in Tissue-Tek OCT compound and frozen in liquid nitrogen cooled isopentane. Sample preparation and embedding for immunofluorescence took ~1-2min before samples were frozen in isopentane. Espina et al. (10) has previously shown eNOS ser¹¹⁷⁷ phosphorylation to be relatively stable after tissue procurement, as a result we believe this freezing time is adequate to avoid significant changes in eNOS ser¹¹⁷⁷ phosphorylation. Samples were stored at -80°C until analysis.

Skeletal muscle samples used to check the specificity and selectivity of the antibodies using blocking peptides and Western blotting were originally obtained from this one individual. However, as no band was observed on Western blots for each of the tested antibodies, we subsequently used a sample from the *m. vastus medialis* of a female obese type II diabetic individual (Age 60 years; body mass index 48.39 kg.m⁻²) undergoing total knee arthroplasty to successfully visualise the NOX2 protein band. The specificity of eNOS and phosphorylated eNOS ser¹¹⁷⁷ (p-eNOS ser¹¹⁷⁷) antibodies was tested in samples obtained from one male Wistar rat (Charles River, Kent, UK). The animal was housed on a controlled 12 hour light-dark cycle and fed standard rat chow and water ad libitum. The animal was killed by anaesthetic overdose (Alfaxalone 10 mg.ml⁻¹ dissolved in cyclodextrin; Vétquinol UK limited, Bucks, UK) diluted 1:2 with 0.9% saline and cervical dislocation. The *m. tibialis*

anterior was then dissected free and frozen in liquid nitrogen and stored at -80°C until further analysis.

To investigate whether endurance exercise activates muscle microvascular eNOS, 8 sedentary healthy males (age 21 ± 1 years, BMI 24.4 ± 1.0 kg.m⁻², VO_{2max} 39.6 ± 3.9 ml.kg.min⁻¹) completed 1 hour of cycling exercise at ~65% VO_{2max} . Before and immediately after exercise muscle biopsies were taken from the *m. vastus lateralis* as described above and eNOS content and p-eNOS ser¹¹⁷⁷ were assessed within the microvasculature.

Sample collection for the study was approved by the Black Country NHS Research Ethics Committee and the Coventry NHS Research Ethics Committee. Experiments on animals were performed in accordance with the UK animal (Scientific Procedures) Act 1986,

Antibodies and chemicals

Polyclonal antibody against NOX2 (a subunit of the NAD(P)Hox complex) was from Santa Cruz Biotechnology (Santa Cruz, CA) (gp91-phox/NOX2 (C-15), cat No. sc-5827); polyclonal antibody against p-eNOS ser¹¹⁷⁷ was from Cell Signalling Technology (Beverly, MA) (p-eNOS ser¹¹⁷⁷, cat No. 9570L); monoclonal antibody to eNOS was from Transduction Laboratories (Lexington, KY, cat No. 610297). Secondary antibodies were donkey anti-goat IgG₁ 594 (NOX2), goat anti-mouse IgG₁ 594 (eNOS) and goat anti-rabbit IgG₁ 594 (p-eNOS ser¹¹⁷⁷) (Invitrogen, Paisley, UK). Ulex Europaeus-FITC conjugate (UEA-I-FITC; Sigma-Aldrich, UK) was used to identify the microvascular endothelium and wheat germ agglutinin-350 (WGA-350; Invitrogen, UK) was used to identify the plasma membrane. Phosphate buffered saline (PBS, 137 mM sodium chloride, 3 mM potassium chloride, 8 mM disodium

hydrogen phosphate and 3 mM potassium dihydrogen phosphate, pH of 7.4.) was used for the dilution of all antibodies, reagents and washing steps.

Immunofluorescence staining

Serial 5µm cryosections were cut and collected onto room temperature uncoated glass slides. Sections were left to air dry at room temperature for 1 hour before treatment. Cross-sections were used for all immunofluorescence staining protocols. All subsequent immunofluorescence analysis was performed in duplicate (mean of 2 sections on one slide). Reproducibility data for eNOS, p-eNOS ser¹¹⁷⁷ and NOX2 (5 duplicate measures) was obtained.

Sections were fixed in acetone and ethanol (3:1) at room temperature for 5 minutes. Samples were then incubated overnight with primary antibodies against either eNOS or p-eNOS ser¹¹⁷⁷ or NOX2 at 4°C. Samples were further incubated for 30 minutes in appropriately labelled fluorescent conjugated secondary antibodies and UEA-I-FITC, which was used as an endothelial marker in all subsequent analyses. The cell membrane stain WGA-350 was then applied for 15 minutes. Following incubation, coverslips were mounted in a glycerol and mowiol 4-88 solution in 0.2 M Tris buffer (pH 8.5) with addition of 0.1% 1,4-diazobicyclo-[2,2,2]-octane (DABCO) anti-fade medium. Images were captured using a Nikon E600 Microscope with a 170W Xenon light source, coupled to a SPOT RT KE colour three shot camera (Diagnostic Instrument Inc., MI, USA). Images were taken at a magnification of 40x and a numerical aperture of 0.75. In order to capture the Alexa Fluor 594 fluorophore a Texas red (540-580 nm) excitation filter was used. UEA-I-FITC and WGA-350 were visualised using the FITC (465-495 nm) and DAPI UV (340-380 nm) excitation filters. Images were

captured using a semi automated filter wheel and controller. Camera settings (exposure time and gain) were adjusted to ensure that no pixel saturation of the fluorescent signal was present in either channel and identical camera settings were used throughout the image capture process.

Image analysis

Quantification of eNOS, p-eNOS ser¹¹⁷⁷ and NOX2 within the microvascular endothelium was achieved by analysing the fluorescence intensity of the signal within the area corresponding to the positive UEA-I-FITC staining. NOX2 fluorescence intensity was also quantified within the cell membrane using the area stained positive for the cell membrane using the corresponding WGA-350 image.

All image analyses were performed using Image-Pro Plus 5.1 software (Fig. 2.1.). Firstly, thresholding was used to create a mask of the area stained positive for the endothelium (UEA-I-FITC) or cell membrane (WGA-350) using routine parameter settings. Threshold levels were selected manually to optimise extraction of the positive signal from the background and then used for all images in one comparison or variation estimate. The endothelial/cell membrane outline was overlaid onto the corresponding eNOS, p-eNOS ser¹¹⁷⁷ or NOX2 image and fluorescence intensity of the eNOS, p-eNOS ser¹¹⁷⁷ or NOX2 staining was quantified within the endothelium or cell membrane specific area. Quantification of eNOS, p-eNOS ser¹¹⁷⁷ and NOX2 was performed on raw images, which were unaltered throughout the analysis. Mean fluorescent intensity of the signal within the outline was reported from 5 images per section resulting in ~181 vessels analysed from ~50 muscle fibres.

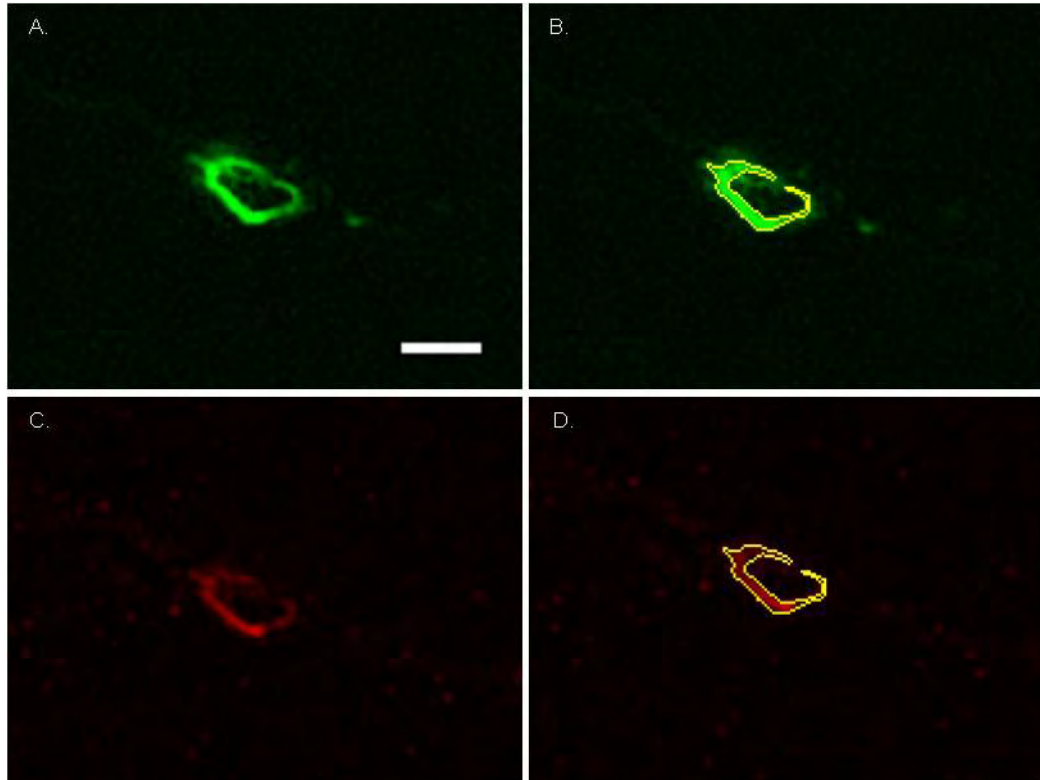


Figure 2.1. Image analysis for quantitative immunofluorescence.

Fluorescence microscopy images were captured and processed using image Pro Plus 5.1. Following image capture, (A) an outline of the endothelial image was created using the selection of a standardised signal intensity threshold tool (B). The endothelial outline was then transferred to the corresponding eNOS, p-eNOS ser¹¹⁷⁷ or NOX2 image (D). The staining intensity of the eNOS, p-eNOS ser¹¹⁷⁷ or NOX2 image was then quantified within the endothelial outline. The same technique was used to quantify NOX2 staining intensity within the cell membrane using the WGA-350 image, representing the cell membrane (bar = 5µm)

Antibody specificity

Blocking peptides of the respective antibodies were used to generate evidence that the antibodies used in this study in the immunofluorescence microscopy assays were selective and specific. The antibody against p-eNOS ser¹¹⁷⁷ was incubated overnight at 4°C with gentle agitation in the presence or absence of a ten-fold excess of p-eNOS ser¹¹⁷⁷ blocking peptide (Cell Signalling technology, cat No. 1827S). The antibody against NOX2 underwent the same treatment in the presence or absence of a 20-fold excess of gp91-phox (C-15) blocking peptide (Santa Cruz Biotechnology, cat No. sc-5827 P). The eNOS antibody (BD biosciences) was incubated overnight at 4°C with gentle agitation with a 20-fold excess of purified human eNOS recombinant protein (OriGene, Rockville, US, cat, No TP309228) as there is no specific blocking peptide for the latter. Following the overnight preincubation of these antibodies with their blocking peptides or in latter case intact human protein, the blocked antibody mixtures were used for immunofluorescence staining of muscle cross-sections as discussed above.

Selectivity and specificity of the used antibodies was further investigated by checking whether Western blots revealed a single protein band at the expected molecular weight. Western blotting was performed using tissue from the *m. tibialis anterior* of a male Wistar rat (eNOS and p-eNOS ser¹¹⁷⁷) or *m. vastus medialis* of a female obese type II diabetic individual (NOX2). Freeze dried muscle was added to homogenisation buffer containing 20mM Tris-HCL (pH7.5), 150mM NACL, 1mM Na₂EDTA, 1mM EGTA, protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Molecular Biochemicals). Samples were homogenised on ice and subsequently sonicated before being centrifuged at 10 000g for 20 min. The supernatant was collected and the protein content was determined (Pierce BCA

protein Assay Reagent). Cell lysates (100µg total protein for the detection of the eNOS and p-eNOS ser¹¹⁷⁷ band; 60µg for the NOX2 band) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with primary antibody against eNOS (1:1000), p-eNOS ser¹¹⁷⁷ (1:1000) and NOX2 (1:1000). Membranes were then exposed to appropriate horseradish peroxidase-conjugated secondary antibodies (Anti Mouse IgG, Anti rabbit IgG and Anti Goat IgG) for 1h at room temperature, diluted (1:2000). Bands were detected by enhanced chemiluminescence method (Amersham EC PlusTM western blotting detection reagents; GE Healthcare).

Antibody selectivity and specificity for eNOS (Transduction Laboratories) and NOX2 (Santa Cruz Biotechnology) were also checked using Basic Local Alignment Search tool (BLAST) analysis, run using UniProt.org software. Percentage alignment of the antibody sequence with the protein of interest is reported.

eNOS and p-eNOS ser¹¹⁷⁷ in pre- and post-exercise biopsies

The fluorescence signal was calculated from the mean of 2 pre- and 2 post-exercise sections of the same individual mounted on 1 slide. Each comparison was performed in duplicate. The reader was not blinded to condition as image analysis was automated removing the potential effects of investigator bias. Both eNOS content and p-eNOS ser¹¹⁷⁷ are presented as raw values and normalised to pre-training value and expressed as arbitrary units. In addition p-eNOS ser¹¹⁷⁷ is presented normalised to eNOS content to eliminate the potential confounding effects of differences in eNOS content pre and post exercise and as a ratio of p-eNOS ser¹¹⁷⁷ to eNOS content using the raw fluorescence intensity data (p-eNOS ser¹¹⁷⁷/eNOS).

Statistics

Image analysis was performed in duplicate (mean of 2 sections on 1 slide). The reproducibility of duplicate measures for protein content of eNOS and NOX2 and eNOS ser¹¹⁷⁷ phosphorylation were assessed by the range, mean percentage difference and coefficient of variation (CV) between duplicates. eNOS content and p-eNOS ser¹¹⁷⁷ were analysed using a paired t-test performed using statistical analysis software (SPSS for windows version 16.0 (SPSS, Chicago, IL)). Significance was set at $P \leq 0.05$. Data is presented as means \pm S.E.M.

2.4 Results

Localisation of staining

eNOS and p-eNOS ser¹¹⁷⁷ immunofluorescence was observed in the area stained by UEA-I-FITC, and p-eNOS ser¹¹⁷⁷ fluorescence was present at a lower intensity in the plasma membrane region of the muscle fibre. This demonstrates that these proteins are highly expressed in the microvascular endothelium (Fig. 2.2a, b.). NOX2 antibody immunofluorescence was observed in the areas stained by the sarcolemmal marker WGA-350 and endothelial marker UEA-I-FITC, indicating that NOX2 is present at both locations (Fig. 2.2c.).

Antibody specificity

Antigen specificity and selectivity of each of the used antibodies was confirmed by the absence of positive immunofluorescence staining when antibodies first had undergone an overnight preincubation with specific blocking peptides (p-eNOS ser¹¹⁷⁷ and NOX2) or the purified recombinant human protein (eNOS) (Fig. 2.3c, d, e.). Antigen specificity of the eNOS and p-eNOS ser¹¹⁷⁷ antibodies was also confirmed by the presence of a single band at the expected MW of approximately 140 kDa on the Western blot (Fig. 2.3a.), using tissue from *m. tibialis anterior* of a male Wistar rat. Antigen specificity of the NOX2 antibody was confirmed by the presence of a single band on the Western blot that migrated between approximately 50-60 kDa (Fig. 2.3b.), presumably representing unglycosylated NOX2 as previously reported (19), using tissue from the *m. vastus medialis* of a female obese type II diabetic.

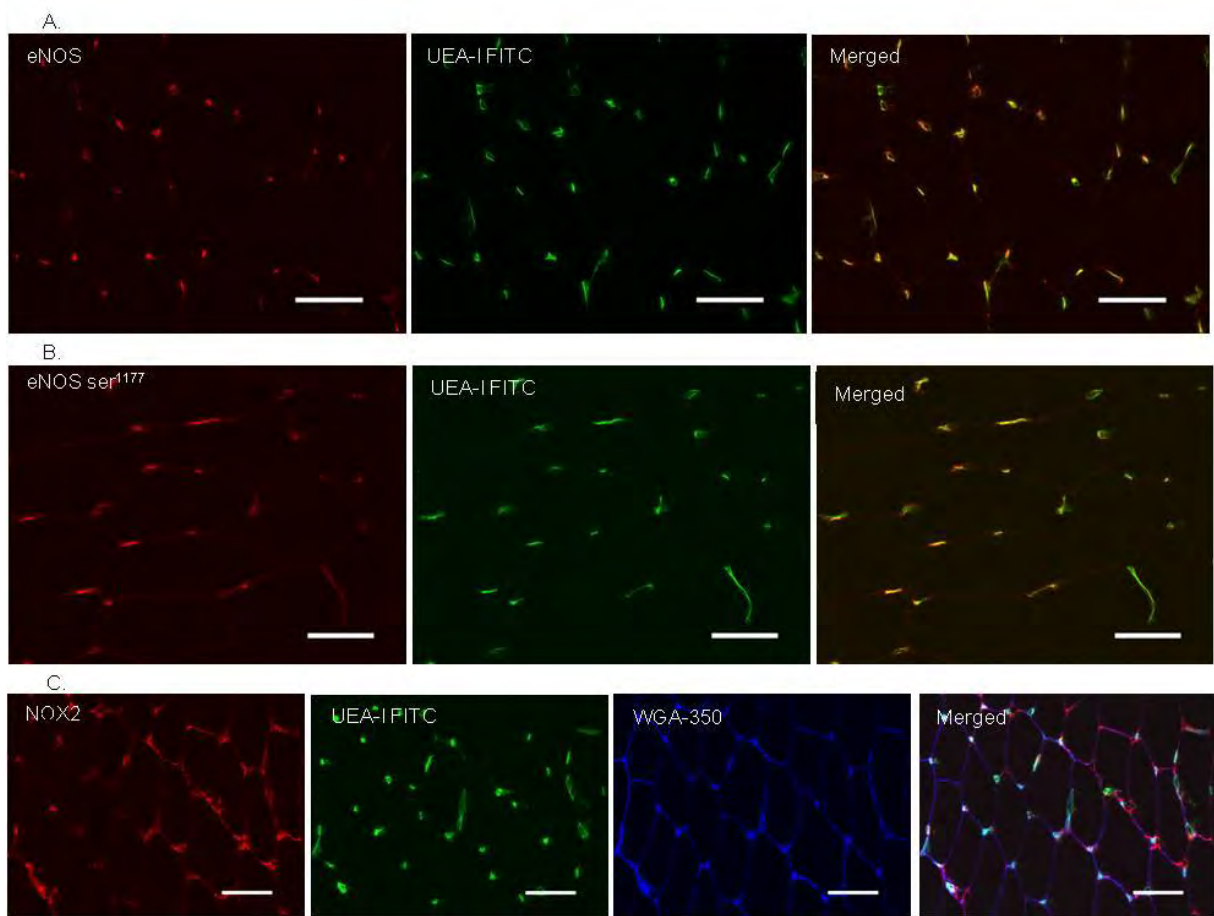


Figure 2.2. Detection of vascular enzymes within skeletal muscle microvasculature using immunofluorescence.

A, Fluorescence detection of eNOS in skeletal muscle microvascular endothelium (bars = 50μm). Immunostaining of eNOS (*red*) is combined with the vascular endothelial stain UEA-I-FITC lectin (*green*). Merged image shows that eNOS and UEA-I-FITC colocalise (*yellow*). B, Fluorescence detection of p-eNOS ser¹¹⁷⁷ in skeletal muscle microvascular endothelium (bars = 50μm). Immunostaining of p-eNOS ser¹¹⁷⁷ (*red*) is combined with the vascular endothelial stain UEA-I-FITC (*green*). Merged image shows that p-eNOS ser¹¹⁷⁷ colocalises with UEA-I-FITC (*yellow*). C, Fluorescence detection of NOX2 in the plasma membrane and microvascular endothelium of skeletal muscle (bars = 50μm). Immunostaining of NOX2 was combined with staining of the vascular endothelium and cell membrane with UEA-I-FITC

(*green*) and WGA-350 (*blue*), respectively. Merged image shows that NOX2 staining colocalises with the endothelial stain and the skeletal muscle plasma membrane.

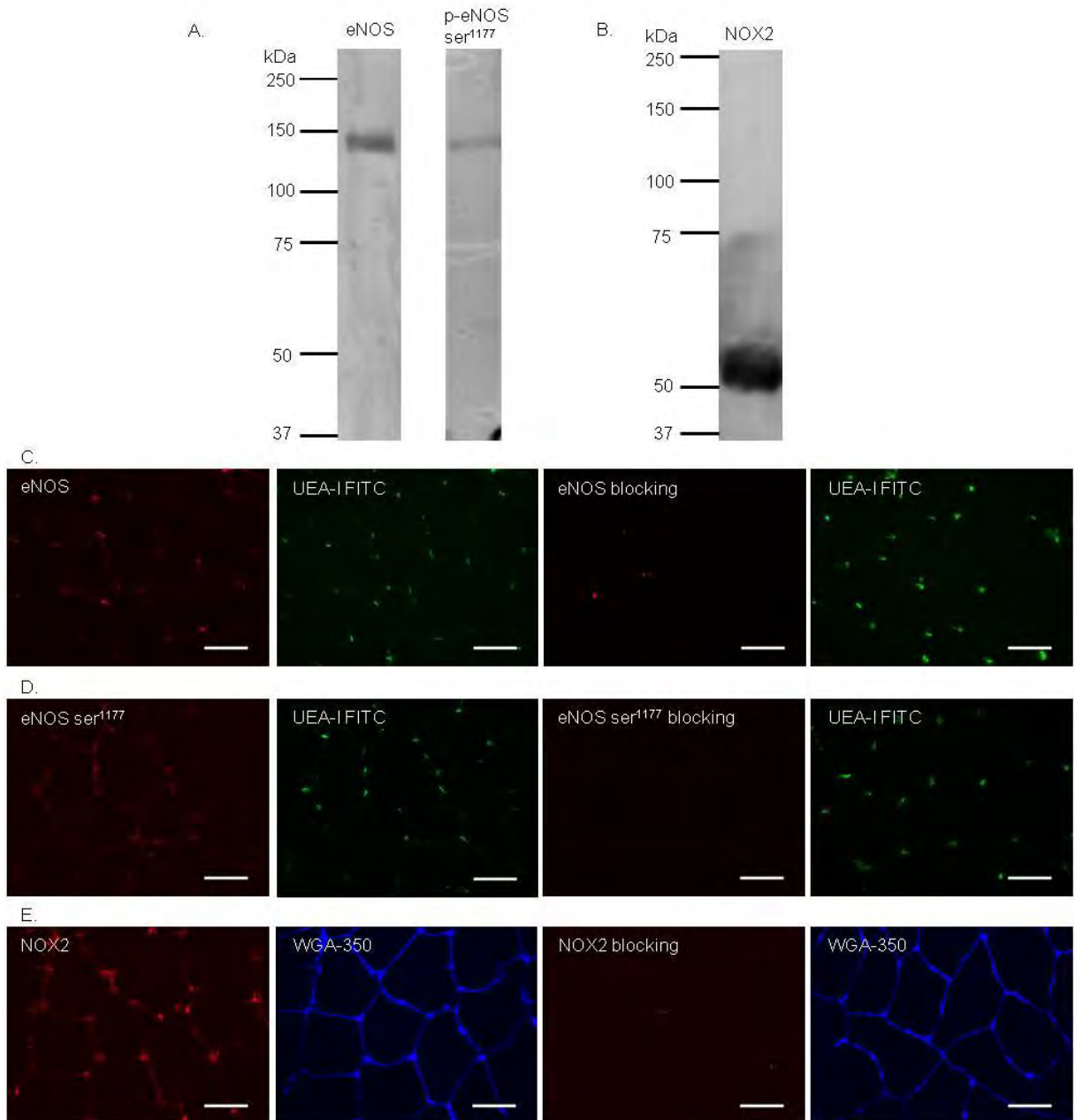


Figure 2.3. Specificity of antibodies used for immunofluorescence analysis using western blotting and blocking peptides.

A, eNOS and p-eNOS ser¹¹⁷⁷ was detected in rat *m. tibialis anterior* muscle at the expected molecular weight of 140 kDa. B, NOX2 was detected in human *m. vastus medialis* muscle at the expected molecular weight between 50 and 60 kDa. C, immunofluorescence detection of eNOS (red) in human skeletal muscle after preincubation of the eNOS antibody in the presence or absence of purified eNOS recombinant protein as a blocking protein. The vascular endothelium is stained with UEA-I-FITC (*green*). D, immunofluorescence detection of p-eNOS ser¹¹⁷⁷ (red) in human skeletal muscle after preincubation of the p-eNOS ser¹¹⁷⁷ antibody in the presence or absence of a specific blocking peptide. The vascular endothelium is stained with UEA-I-FITC (*green*). E, immunofluorescence detection of NOX2 (red) in human skeletal muscle after preincubation of the NOX2 antibody in the presence or absence of a specific blocking peptide. The cell membrane is stained with WGA-350 (*blue*). Bars = 50µm.

Results of BLAST analysis revealed that eNOS and NOX2 antibodies shared 100% sequence alignment with human eNOS and NOX2 respectively. NOX2 and eNOS antibodies also shared 98% and 90% sequence alignment with rat NOX2 and eNOS proteins respectively. p-eNOS ser¹¹⁷⁷ has been reported to share 100% sequence alignment with both human and rat eNOS phosphorylated at ser¹¹⁷⁷ (sequence alignment information from Cell Signalling Technology, Inc.).

Reproducibility of quantitative immunofluorescence

Immunofluorescence analysis of protein expression and phosphorylation was performed in duplicate. Reproducibility data for eNOS, p-eNOS ser¹¹⁷⁷ and NOX2 (5 duplicate measures) is displayed in Table 2.1. The CV for eNOS, p-eNOS ser¹¹⁷⁷ and NOX2 within the endothelium were 7%, 7% and 9.5%, respectively. The CV of NOX2 within the cell membrane was 6.5%.

eNOS following exercise

Total eNOS content within the microvasculature was unaffected by exercise (fluorescence intensity pre exercise 57 ± 5 AU vs. post exercise 58 ± 5 AU $P=0.708$; fold change from rest 1.01 ± 0.02 , $P=0.628$). eNOS ser¹¹⁷⁷ phosphorylation increased 29% within the microvascular endothelium following exercise (fluorescence intensity, pre exercise 50 ± 6 AU vs. post exercise 67 ± 9 AU $P < 0.05$; Fold change from rest 1.29 ± 0.05 , $P < 0.05$, Fig. 2.4.). When eNOS phosphorylation was presented as a ratio of p-eNOS ser¹¹⁷⁷ to eNOS content eNOS ser¹¹⁷⁷ phosphorylation was increased by 26% following acute exercise ($P < 0.05$, Fig. 2.4.).

Table 2.1. Reproducibility of protein content and eNOS phosphorylation state using immunofluorescence analysis

Variable	No. of Duplicate measurements	Range between duplicates (AU)	Mean Difference, %	Coefficient of variation, %
eNOS	6	56- 66	8	7
p-eNOS ser ¹¹⁷⁷	5	48-56	9	7
Endothelial NOX2	6	24-31	10	9.5
Cell Membrane NOX2	6	15-17	7	6.5

Range between duplicates measured in arbitrary unit (AU), mean percentage difference in signal intensity between duplicates and the coefficient of variation of signal intensity between duplicates are given; eNOS, endothelial nitric oxide synthase; p-eNOS ser¹¹⁷⁷, endothelial nitric oxide synthase phosphorylated on serine¹¹⁷⁷; NOX2, NAD(P)H oxidase subunit NOX2.

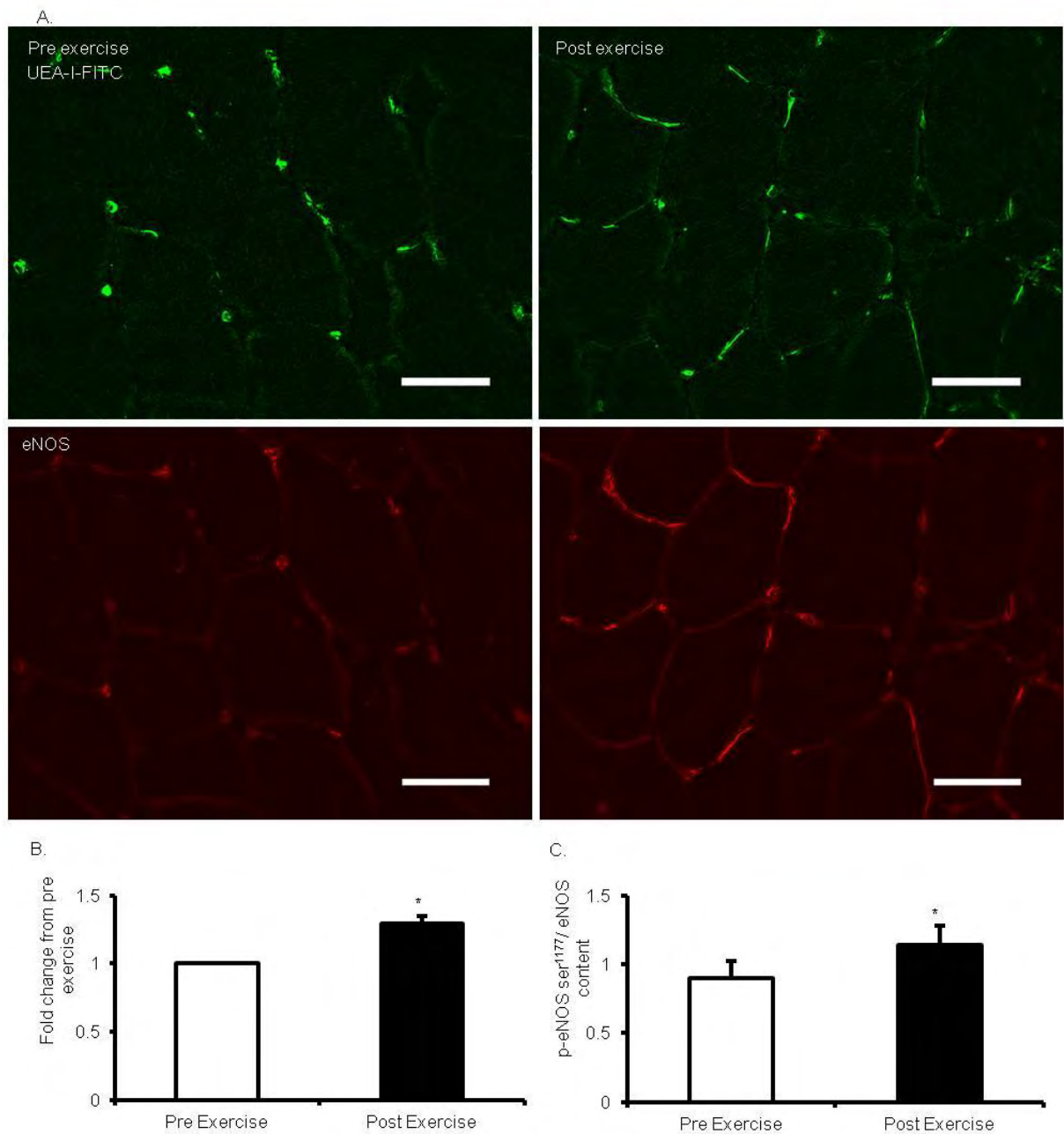


Figure 2.4. Evaluation of eNOS serine¹¹⁷⁷ phosphorylation (p-eNOS ser¹¹⁷⁷) pre and immediately post exercise in man.

A, representative immunofluorescence images of p-eNOS ser¹¹⁷⁷ pre- and immediately post-exercise. Immunostaining of p-eNOS ser¹¹⁷⁷ (red) with the vascular endothelial stain UEA-I-FITC (green). B, mean p-eNOS ser¹¹⁷⁷ within the skeletal muscle microvasculature significantly increases immediately post exercise. C, Mean p-eNOS ser¹¹⁷⁷ normalised to

eNOS protein content within the skeletal muscle microvasculature significantly increases immediately post exercise. * Significant difference between pre and immediately post exercise ($P < 0.05$). Values are means \pm S.E.M (bars = 50 μ m).

2.5 Discussion

This study describes a novel application of immunofluorescence microscopy to quantify the protein content and phosphorylation state of enzymes within the endothelium of human skeletal muscle microvasculature, which play a crucial role in production of NO (eNOS) and quenching of NO via superoxide anion production (NADPHox). The overall CV between duplicate measurements was <9.5% for all variables demonstrating that the technique is reproducible and the 1.29-fold increase in eNOS phosphorylation following exercise indicates that the technique can reliably detect changes in eNOS phosphorylation in response to physiological perturbation.

Measurements of vascular NOX2, eNOS and p-eNOS ser¹¹⁷⁷ have previously been made with Western blot methodology applied to aorta, arteries or resistance arterioles, primarily from animal models (12, 17). This has led to the current understanding of the mechanisms behind adaptations to pathologies and exercise training. However it is becoming increasingly apparent that the vasodilatory responsiveness of terminal arterioles and microvascular density in skeletal muscle is the major determinant of the supply of fuels and oxygen to the skeletal muscle fibres both at rest and during exercise (1, 11). In the present study, we first confirmed the presence of eNOS and p-eNOSser¹¹⁷⁷ in the muscle microvascular endothelium and NOX2 in the muscle microvascular endothelium and plasma membrane through the colocalisation of the enzyme with endothelial and plasma membrane markers. We then quantified the fluorescence intensity of each protein within the relevant locations as a measure of the protein content of each enzyme. The quantification method showed a good reproducibility as demonstrated by the CV which is lower than 10% for all analyses. This compares favourably with previous immunofluorescence approaches to quantify endothelial

enzyme expression in isolated endothelial cells (7). In addition the antibodies used in the development of the new assays were thoroughly tested for selectivity and specificity providing convincing evidence of this. However, we acknowledge that the most reliable method to assess antibody specificity would have been to perform measures in muscle from knockout animals, but tissue from such animals was not available.

Methods previously used to measure the protein content of vascular enzymes within skeletal muscle using whole muscle homogenates (Western blotting and sandwich enzyme immunoassays) do not reveal the relative distribution of these enzymes between muscle fibres and the microvasculature. These methods also do not provide information on whether changes in protein expression in chronic disease or in response to exercise training are the result of changes in endothelial protein content or of changes in microvascular density. The novel methodology described here, enables the expression and phosphorylation status of proteins to be analysed specifically within the endothelium of human skeletal muscle without confounding issues of variations in capillary density.

The ability of the method to generate novel information is demonstrated by the significant increase in endothelial eNOS ser¹¹⁷⁷ phosphorylation observed following 1 hour of cycling exercise, whereas no change in total eNOS content was detected. Previous studies have demonstrated eNOS phosphorylation in the aorta of rats following exercise (34), but this is the first time that increased eNOS phosphorylation has been observed in the microvasculature of either animal or human skeletal muscle. Elevated NO bioavailability during exercise has been suggested to play a role in microvascular exercise hyperaemia (14), although the full extent of its involvement is difficult to speculate on due to the observed redundancy of NO

seen in physiological systems (8). As such, the elevation in eNOS ser¹¹⁷⁷ phosphorylation observed in this study supports this suggestion with the underlying molecular mechanism. Furthermore, NO is also an important signalling mechanism for angiogenesis (23), and therefore, the increase in eNOS ser¹¹⁷⁷ phosphorylation within the microvasculature during exercise is likely to contribute to the angiogenic effect of endurance training.

We acknowledge that the analysis of protein content and enzyme phosphorylation status using immunofluorescence microscopy is semi-quantitative in nature, and only allows relative differences between two or more conditions to be assessed simultaneously. However, Western blots are also semi-quantitative and the CV of the novel method in our hands is comparable to those of Western blots. Therefore, this analytical approach has the potential to substantially improve our understanding of the role played by these microvascular endothelial enzymes in the mechanisms by which physiological stimuli increase eNOS phosphorylation and lead to insulin resistance and sarcopenia in chronic age and lifestyle related metabolic diseases. Furthermore, the novel methods could also be used to determine whether the well-known improvements in insulin sensitivity, glucose homeostasis and the reduction in sarcopenia that results from endurance and resistance training, is mediated at least in part by mechanisms that operate at the level of the microvascular endothelium in skeletal muscle (1, 25, 33).

Perspective

The novel immunofluorescence microscopy approach described in this study allows for reproducible measurements of the protein content and activation of microvascular enzymes, independent of changes in muscle fibre enzyme content and microvascular density. This method has the potential to generate novel insight into the molecular mechanisms determining

microvascular function and its role in the pathophysiology resulting from physical inactivity, obesity and ageing. The method can also be used to investigate the effect of lifestyle (diet and exercise) and pharmacological interventions aiming to improve microvascular endothelial function and metabolic health.

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Chapter 3

Impaired Insulin-Mediated ser¹¹⁷⁶ Phosphorylation of Endothelial Nitric Oxide Synthase in Skeletal Muscle Arterioles of Obese Zucker Rats

3.1 Abstract

Early studies in rat and man observed that insulin mediated NO-dependent increases in skeletal muscle microvascular volume precede increases in muscle glucose uptake and, therefore, hypothesised that insulin induced dilation of terminal arterioles leading to increased recruitment of capillaries is an important step in the control of skeletal muscle glucose uptake. Impairments in this mechanism have subsequently been seen in humans with obesity and type 2 diabetes and in obese Zucker rats (OZR), an animal model of the metabolic syndrome. In cultured endothelial cells insulin has been shown to activate endothelial nitric oxide synthase (eNOS) via insulin mediated serine phosphorylation. The aim of this study was to investigate the hypothesis that an impairment in eNOS ser¹¹⁷⁶ phosphorylation is instrumental to skeletal muscle insulin resistance in OZR. The *m. tibialis anterior* was dissected from 20 week old lean Zucker rats (LZR) and OZR in the fasted state (n=7) and 2 h after the start of a hyperinsulinaemic-euglycemic clamp (n=7). eNOS content and ser¹¹⁷⁶ phosphorylation were measured in the endothelial layer of skeletal muscle terminal arterioles and capillaries using quantitative immunofluorescence microscopy. Total eNOS content and eNOS ser¹¹⁷⁶ phosphorylation in the fasted state was not significantly different between LZR and OZR both in terminal arterioles and capillaries. Insulin stimulation significantly elevated terminal arteriole eNOS ser¹¹⁷⁶ phosphorylation in LZR (14%; $P < 0.05$), while causing a significant reduction in OZR (-28%; $P < 0.05$). Insulin did not significantly change capillary eNOS ser¹¹⁷⁶ phosphorylation in LZR (-7%), while reducing this variable in OZR (-31%, $P < 0.05$). Capillary density and number of capillaries per muscle fibre were also significantly lower in OZR compared to LZR ($P < 0.05$). The impairments in OZR coincided with a reduced glucose infusion rate area under the curve during the first 21 min of the hyperinsulinemic-euglycemic clamp and a reduced insulin sensitivity index (both $P < 0.05$). We conclude that

insulin-induced eNOS ser¹¹⁷⁶ phosphorylation is impaired in skeletal muscle arterioles of OZR, confirming the hypothesis that this molecular event is instrumental to skeletal muscle insulin resistance.

3.2 Introduction

The metabolic syndrome, defined as the combined presence of obesity, insulin resistance, hypertension and dyslipidemia affects 23.7% of American adults (31). The obese Zucker rat (OZR) experiences chronic hyperphagia due to a recessive mutation in the leptin gene (22). As a result it develops features similar to those observed in the human metabolic syndrome (12), and the OZR is therefore generally regarded as an animal model of the metabolic syndrome.

Wallis and colleagues (37) using hyperinsulinemic clamp conditions have previously observed that skeletal muscle insulin resistance in OZR is accompanied by an impaired hemodynamic response to insulin. This impaired hemodynamic response includes both a blunted increase in insulin stimulated femoral artery blood flow and recruitment of the skeletal muscle microvasculature (37). The latter was measured as a reduction in the extraction of 1-methylxanthine over the hindleg during the hyperinsulinemic clamp in the OZR, while the expected increase was seen in LZR. Impairments in the insulin stimulated recruitment of the skeletal muscle microvasculature (37) together with capillary rarefaction (11) have been suggested to be responsible for the large reduction in skeletal muscle glucose uptake observed in OZR (37) and in humans with obesity (6) and type 2 diabetes during hyperinsulinemic-euglycemic clamps (15). Both events contribute to a reduction in the capillary permeability surface area (PSA) available for delivery of insulin and glucose to the skeletal muscle fibres (4, 15, 16). Gudbjörnsdóttir et al (16) have shown that the capillary PSA is a major determinant for the increases in muscle glucose uptake that occur in man 90-120 min after the start of an OGTT and of a hyperinsulinemic-euglycemic clamp using low physiological concentrations of insulin.

Insulin induced NO-dependent vasodilatation of the skeletal muscle microvasculature occurs in rat muscle as early as 5-10 min after the start of a hyperinsulinemic-euglycemic clamp (33) and the vasodilation subsequently remains for 120 min (34, 36). Studies with cultured endothelial cells have shown that insulin acutely increases the synthesis rate of NO through activation of endothelial nitric oxide synthase (eNOS) by means of serine phosphorylation. Ser¹¹⁷⁶ is the main activation site in rats and ser¹¹⁷⁷ in humans (23, 30). Pre-treatment of rats with the NOS inhibitor L-NAME blocked the early microvascular recruitment in response to insulin infusion and partially inhibited the early increases in muscle glucose uptake (35). Therefore, reductions in eNOS content and/or reduced insulin induced eNOS phosphorylation, could be part of the mechanisms behind a reduced NO production and impairments in muscle microvascular vasodilatation and glucose uptake in OZR. Studies in isolated skeletal muscle resistance vessels of OZR have shown a decreased eNOS content (9), while the eNOS content of a whole muscle homogenate did not change and eNOS ser¹¹⁷⁶ phosphorylation was surprisingly elevated in OZR in the basal fasted state in comparison to LZR (13). However eNOS content or ser¹¹⁷⁶ phosphorylation in response to insulin has never been measured locally in the terminal arterioles of skeletal muscle, which is assumed by many to be the main insulin-induced vasodilator site, and thus, responsible for insulin induced increases in capillary permeability surface area and muscle microvascular blood volume (1, 2, 14, 28, 29, 34-36).

Therefore, the aim of the current study was to measure eNOS content and eNOS ser¹¹⁷⁶ phosphorylation in the fasted state and in response to insulin infusion in skeletal muscle terminal arterioles and capillaries of LZR and OZR, to investigate the hypothesis that the impaired glucose disposal in OZR during a hyperinsulinemic-euglycemic clamp is the result

of a failure of insulin to activate eNOS by ser¹¹⁷⁶ phosphorylation thus reducing capillary recruitment and muscle glucose uptake. This hypothesis will be investigated by measuring eNOS content and ser¹¹⁷⁶ phosphorylation in arterioles and capillaries in the *m. tibialis anterior* dissected from LZR and OZR before and 2 h after the start of a hyperinsulinemic-euglycemic clamp, while making continuous measurements of whole body glucose clearance and in the last 20 min an estimate of whole body insulin sensitivity. As insulin induced vasodilatation of the muscle microvasculature has been shown to occur as early as 5-10 min (32, 33), comparisons of whole body glucose clearance rates between LZR and OZR will focus on the early response (within the first 30 minutes) with the a priori hypothesis being that glucose infusion rate (GIR) will be substantially reduced in OZR.

3.3 Materials and Methods

Animals

The rat experiments were performed in the research laboratories of Astra Zeneca in Alderley Park (Cheshire, UK). Male 20 week old lean (Fa/?) and obese (fa/fa) Zucker rats (purchased from Charles River, France) were housed on a controlled 12 hour light-dark cycle and fed standard rat chow and water ad libitum. All experiments were performed in accordance with the UK animal (Scientific Procedures) Act 1986.

Experimental protocol

Rats were randomly assigned to the fasted group or insulin stimulated group (n=7 per group). The day before the experimental trial animals received a fixed ration of 16 or 10g (OZR and LZR, respectively) of rat chow at 16:00h, with the intention of ensuring animals were non-absorptive but not starved during the experimental protocol. Rats in the insulin stimulated condition underwent a hyperinsulinaemic-euglycemic clamp. Rats were anaesthetised (160-190 mg.kg⁻¹ Inactin I.P) and surgically prepared by placing catheters and probes for subsequent arterial and venous sampling and intravenous infusion and assessment of femoral artery blood flow and blood pressure. Following surgical procedures a 60 min recovery period was allowed, followed by a 120 min hyperinsulinaemic-euglycemic clamp. LZR were given 10 mU·min⁻¹·kg⁻¹ of insulin while OZR received 20 mU·min⁻¹·kg⁻¹ of insulin. The difference in infusion rate was chosen to achieve proportional increases in plasma insulin levels in a range that is normally seen by the LZR and OZR. Also for this reason euglycemia was maintained at 4 mM in the LZR and at 6 mM in the OZR via frequent on line measurements of the blood glucose concentration with an automated glucose analyser and small adjustments of the glucose infusion rate. Femoral artery blood flow was measured at 60 min using a perivascular

flowprobe (0.7 V) attached to a flow meter (T106 small animal blood flow meter; Transonic Systems Inc., NY, USA) placed around the animals femoral artery. Immediately following the hyperinsulinaemic-euglycemic clamp insulin stimulated animals were killed by anaesthetic overdose and cervical dislocation and the *m. tibialis anterior* was dissected free. Samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) and immediately frozen in liquid nitrogen cooled isopentane (Sigma-Aldrich, Dorset, UK). Samples were then stored at -80°C until analysis was performed. Control muscle was obtained from animals in the fasted state using the same procedures.

GIR ($\mu\text{mol.kg lean body mass}^{-1}.\text{min}^{-1}$) was calculated, and as plasma glucose concentration was kept constant is assumed to be equal to the disappearance rate of glucose from the blood into skeletal muscle and other tissues. Insulin sensitivity was calculated as GIR ($\mu\text{mol.kg lean body mass}^{-1}.\text{min}^{-1}$)/plasma insulin (pmol.L^{-1}) in the 60-80 min steady state period, using GIR and plasma insulin measurements at the corresponding time points. To investigate the early response of glucose uptake to insulin GIR area under the curve (calculated using the conventional trapezoid rule) for the first 21 minutes of the clamp procedure was measured.

Femoral artery vascular conductance (FVC) was calculated as femoral artery blood flow ($\text{ml}/\text{min}^{-1}$)/ arterial blood pressure (mmHg), in the fasted state and 60 min into the clamp in 4 of the 7 animals.

Quantitative immunofluorescence

Serial 5 μm sections of the *m. tibialis anterior* (TA) were cut at -30°C using a microtome and collected on to room temperature uncoated glass slides. Two sections from each condition

(lean fasted, obese fasted, lean insulin stimulated, obese insulin stimulated) were placed on each slide and analysis was performed in duplicate.

eNOS content and ser¹¹⁷⁶ phosphorylation of eNOS were assessed using the technique described by Cocks et al. (7) adapted for use in rats. The method was also modified to differentiate between skeletal muscle capillaries and arterioles. In brief sections were incubated with antibodies against either eNOS (Transduction Laboratories, Lexington, KY) or p-eNOS ser¹¹⁷⁷ (Cell Signalling Technology, Beverly, MA) (reported to share 100% sequence alignment with rat eNOS phosphorylated at ser¹¹⁷⁶, sequence alignment information from Cell Signalling Technology, Inc.) in combination with anti-alpha smooth muscle actin (SMA; Abcam, Cambridge, UK) as a marker to differentiate between arterioles and capillaries. Sections were then incubated with appropriately labelled secondary antibodies (Invitrogen, Paisley, UK), in combination with Fluorescein Griffonia (Bandeiraea) Simplicifolia I lectin (BSI-I, Vector labs, Burlingame, CA) as a marker of the endothelium. Following staining cover slips were applied using a glycerol and mowiol 4-88 solution.

Images were acquired as described in (7). Image analysis was performed as described in (7), but vessels were divided into capillaries and arterioles using the SMA image. Arteriole diameter was also determined on calibrated images using Image Pro Plus 5.1 software.

NOX2 content would have been an interesting measurement, but the antibody and methods previously described in human muscle (7) (gp91-phox/NOX2 (C-15); Santa Cruz Biotechnology, Santa Cruz, CA) did not work in rat muscle. As a result this measure was not conducted in the present study.

Capillary density

Sections were prepared as above, and then incubated with BSI-I (Vector labs, Burlingame, CA) and wheat germ agglutinin-350 (WGA-350; Invitrogen, UK) as markers of the endothelium and plasma membrane, respectively. Following staining cover slips were applied using a glycerol and mowiol 4-88 solution.

For analysis, slides were viewed using a Nikon E600 widefield fluorescence microscope using a 20 x 0.4 numerical aperture objective. Images were captured as described in (7).

Capillary density (caps/mm²) and number of capillaries per fibre (calculated as total number of capillaries/total number of muscle fibres analysed) were manually quantified using the BSI-I and WGA-350 images as markers. Fibre cross sectional area (units) was measured on calibrated images using ImagePro Plus 5.1 software.

Statistics

eNOS content and measures of capillarization and fibre area were analysed using an independent samples t-test. eNOS ser¹¹⁷⁶ phosphorylation was analysed using a two-way repeated measures ANOVA with the between group factor 'group' (lean versus obese) and within group factor 'stimulation' (fasted versus insulin-stimulated). Significant group*stimulation interactions were examined using an independent t-test due to the small sample size of the current study. Femoral artery blood flow and FVC was also analysed using a two-way repeated measures ANOVA with the between group factor 'group' (lean versus obese) and within group factor 'stimulation' (fasted versus insulin-stimulated). Significant group*stimulation interactions were examined using paired samples t-test (fasted versus

insulin stimulated), and independent samples t-test (lean versus obese) again due to the small sample size of the current study. Quantitative immunofluorescence (eNOS content and ser¹¹⁷⁶ phosphorylation) data from one slide was more than 2 standard deviations from the mean and, therefore, has been excluded as an outlier. All analyses were performed using statistical analysis software (SPSS for windows version 16.0, SPSS, Chicago, IL). Significance was set at $P \leq 0.05$. Data is presented as means \pm S.E.M.

3.4 Results

Glucose infusion rate (GIR), insulin sensitivity index and femoral artery blood flow

Plasma glucose and GIR during the hyperinsulinemic euglycemic clamp are given in Fig. 3.1. GIR area under the curve was greater in the LZR than in the OZR at 21 min (LZR 95.8 ± 14.1 , OZR 39.7 ± 17.9 ; $P < 0.05$). There was no significant difference for the mean GIR between the two groups in the 60-80 min period. The insulin sensitivity index during the last 20 min of the clamp was 6-fold higher for the LZR than for the OZR (Fig. 3.2; $P < 0.05$). There was no significant main effect of insulin on femoral artery blood flow at 60 min ($P = 0.306$) and no significant group*stimulation interaction was observed ($P = 0.257$) (Fig. 3.3a). A near significant group*stimulation interaction ($P = 0.052$) was observed in FVC at 60 min (Fig. 3.3b). Subsequent paired samples t-tests revealed FVC at 60 min in the LZR was significantly higher than in the fasted state LZR during the clamp ($P < 0.05$). No significant change from the basal fasted state was seen for FVC in the OZR ($P = 0.950$). However FVC was not significantly different between groups at 60 min ($P = 0.306$).

eNOS content and ser¹¹⁷⁶ phosphorylation

Skeletal muscle eNOS content in the endothelium did not differ significantly between LZR and OZR neither in skeletal muscle terminal arterioles ($P = 0.279$) nor in capillaries ($P = 0.125$) (Fig. 3.4 and 3.5). A significant group*stimulation interaction was observed in terminal arteriole eNOS ser¹¹⁷⁶ phosphorylation ($P < 0.05$) (Fig. 3.6). A subsequent independent samples t-test revealed that fasting eNOS ser¹¹⁷⁶ phosphorylation was not different between LZR and OZR ($P = 0.840$). However, there was a significant increase in eNOS ser¹¹⁷⁶ phosphorylation following insulin stimulation in the LZR ($P < 0.05$), whereas insulin stimulation significantly reduced eNOS ser¹¹⁷⁶ phosphorylation in OZR ($P < 0.05$). In

addition, following insulin stimulation eNOS ser¹¹⁷⁶ phosphorylation was significantly higher in the LZR than in OZR ($P < 0.05$). A significant group*stimulation interaction was observed in capillary eNOS ser¹¹⁷⁶ phosphorylation ($P < 0.05$) (Fig. 3.7). Subsequent independent samples t-tests revealed that fasting capillary eNOS ser¹¹⁷⁶ phosphorylation was not different between lean and obese animals ($P = 0.662$). Insulin stimulation did not change capillary eNOS ser¹¹⁷⁶ phosphorylation in LZR ($P = 0.339$), while in OZR insulin stimulation significantly reduced eNOS ser¹¹⁷⁶ phosphorylation ($P < 0.05$). Following insulin stimulation eNOS ser¹¹⁷⁶ phosphorylation in OZR was 23% of that in LZR, but the difference between the groups was not significant ($P = 0.072$). Mean arteriole diameter for assessment of eNOS and eNOS ser¹¹⁷⁶ phosphorylation was $8.9 \pm 0.1 \mu\text{m}$ and 7 ± 0.3 arterioles were analysed for each condition.

Fibre area and capillarization

No significant difference was observed in muscle fibre area between LZR and OZR (LZR $2377 \pm 237 \mu\text{m}^2$, OZR $2147 \pm 124 \mu\text{m}^2$; $P = 0.401$). There was a significant reduction in capillary density between lean and obese animals (LZR 1246 ± 109 capillaries/ mm^2 , OZR 962 ± 71 capillaries/ mm^2 ; $P < 0.05$). In addition the number of capillaries per fibre was significantly lower in OZR compared to the LZR (LZR 2.61 ± 0.11 capillaries/fibre, OZR 1.95 ± 0.08 capillaries/fibre; $P < 0.05$).

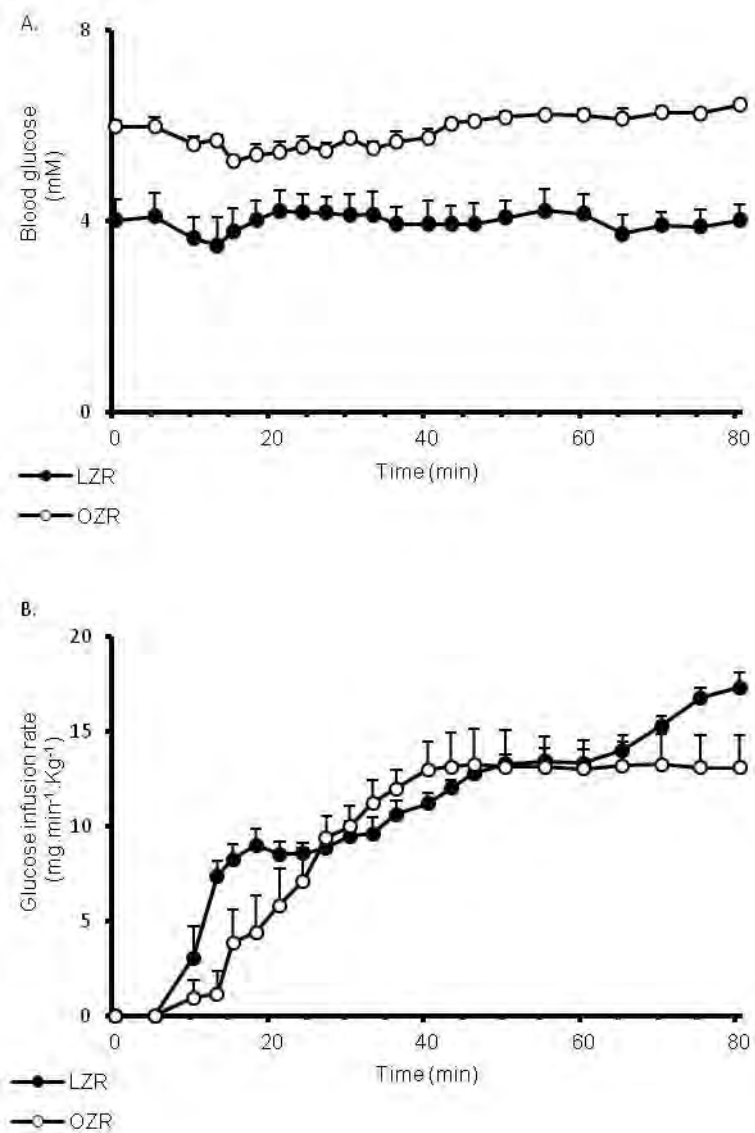


Figure 3.1. Blood glucose and glucose infusion rate in lean and obese Zucker rats

Blood glucose (A) and glucose infusion rate (B) during hyperinsulinaemic-euglycaemic clamp in lean (LZR) and obese (OZR) Zucker rats.

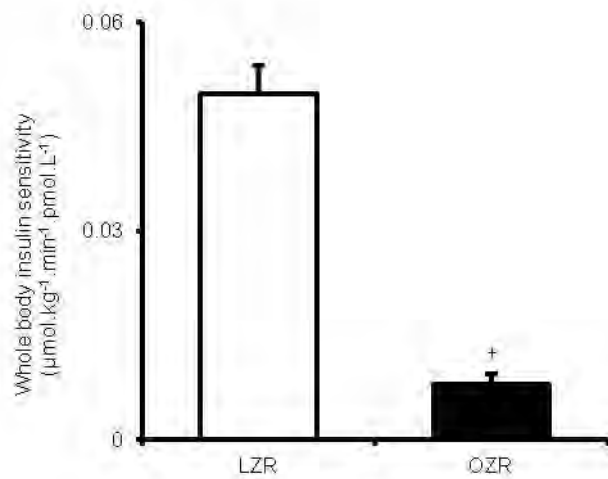


Figure 3.2. Insulin sensitivity in lean and obese Zucker rats

Insulin sensitivity in lean (LZR) and obese (LZR) Zucker rats. Insulin sensitivity was calculated as glucose infusion rate ($\mu\text{mol/lbmkg/min}$) / plasma insulin (pmol/L) at 60-80 min (at clamp) of the hyperinsulinaemic-euglycemic clamp. + $P < 0.05$ from LZR

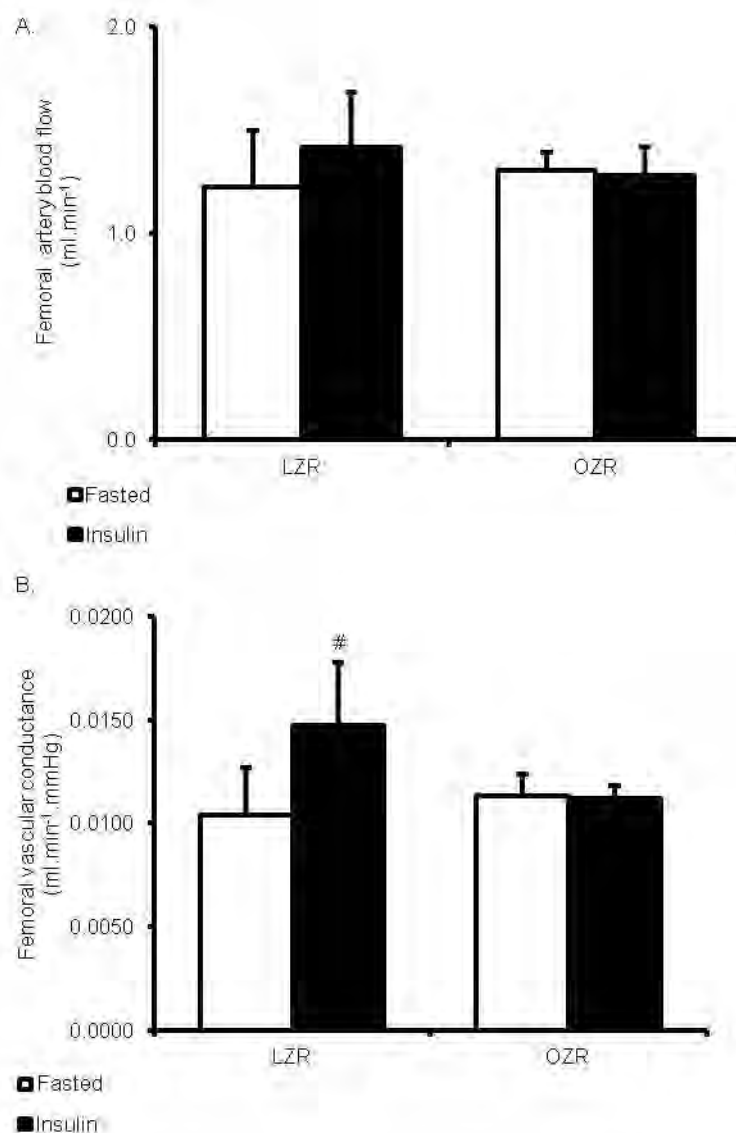


Figure 3.3. Femoral artery blood flow and vascular conductance in lean and obese Zucker rats

Change in femoral artery blood flow (A) and femoral vascular conductance (FVC) (B) from fasted to insulin-stimulated in lean (LZR) and obese (LZR) Zucker rats. FVC was calculated as femoral artery blood flow (ml.min⁻¹)/ arterial blood pressure (mmHg). Data was collected at 60 min after the start hyperinsulinaemic-euglycemic clamp. # $P < 0.05$ from LZR fasted

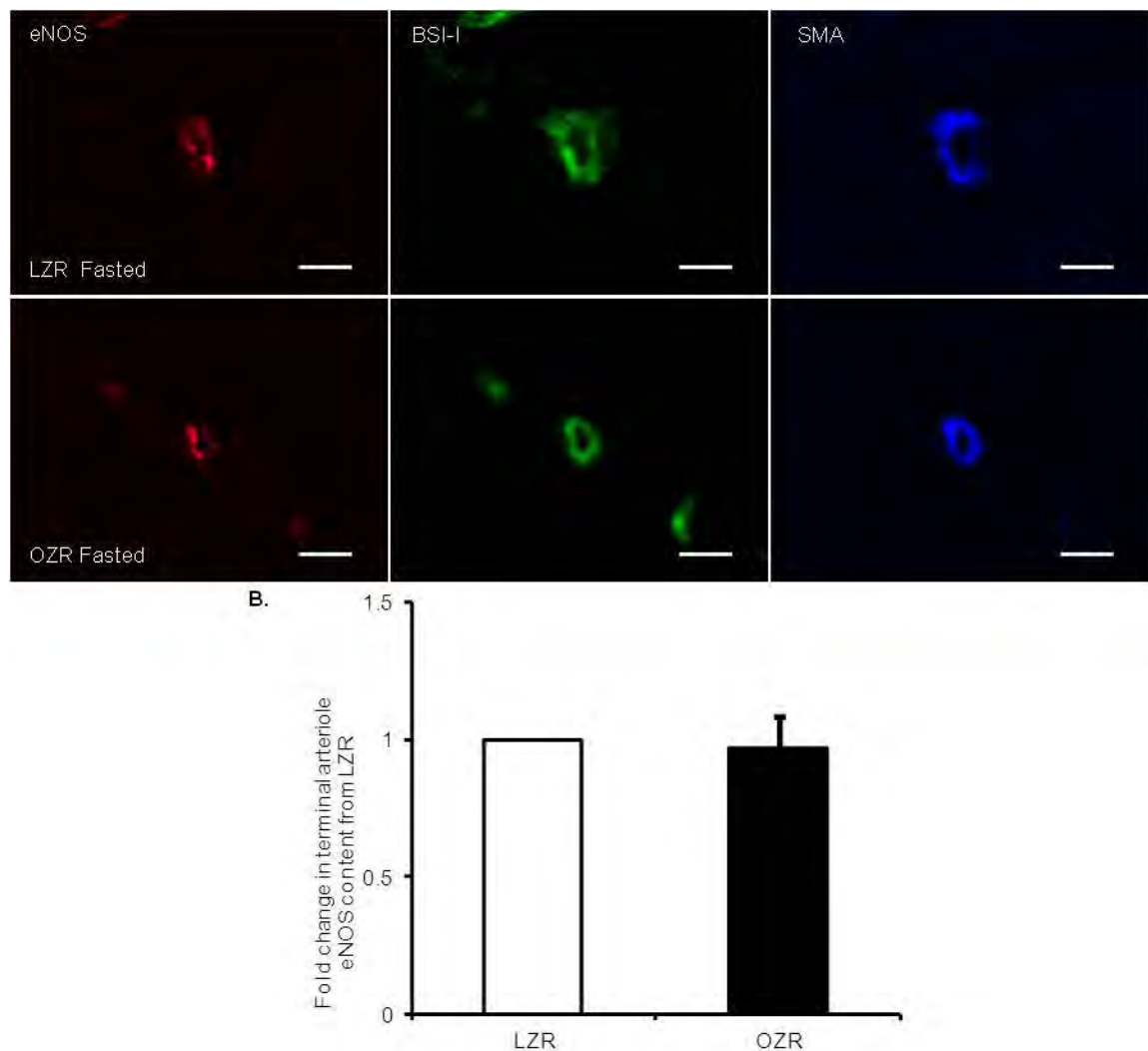


Figure 3.4. Terminal arteriole eNOS content in lean and obese Zucker rats

A. Widefield microscopy images of *m. Tibialis anterior* in lean (LZR) and obese (OZR) Zucker rats. The skeletal muscle microvascular endothelium was revealed using Fluorescein Griffonia (Bandeiraea) Simplicifolia I lectin (BSI-I) (green). Arterioles and capillaries were identified using anti smooth muscle actin (SMA) in combination with Alexa-Fluor 350 conjugated secondary antibody (blue). eNOS was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 10 μ m. B Mean fluorescence intensity of eNOS within terminal arterioles is summarized. The mean level of eNOS in LZR was assigned a value of 1, and the relative intensity of eNOS in OZR was calculated.

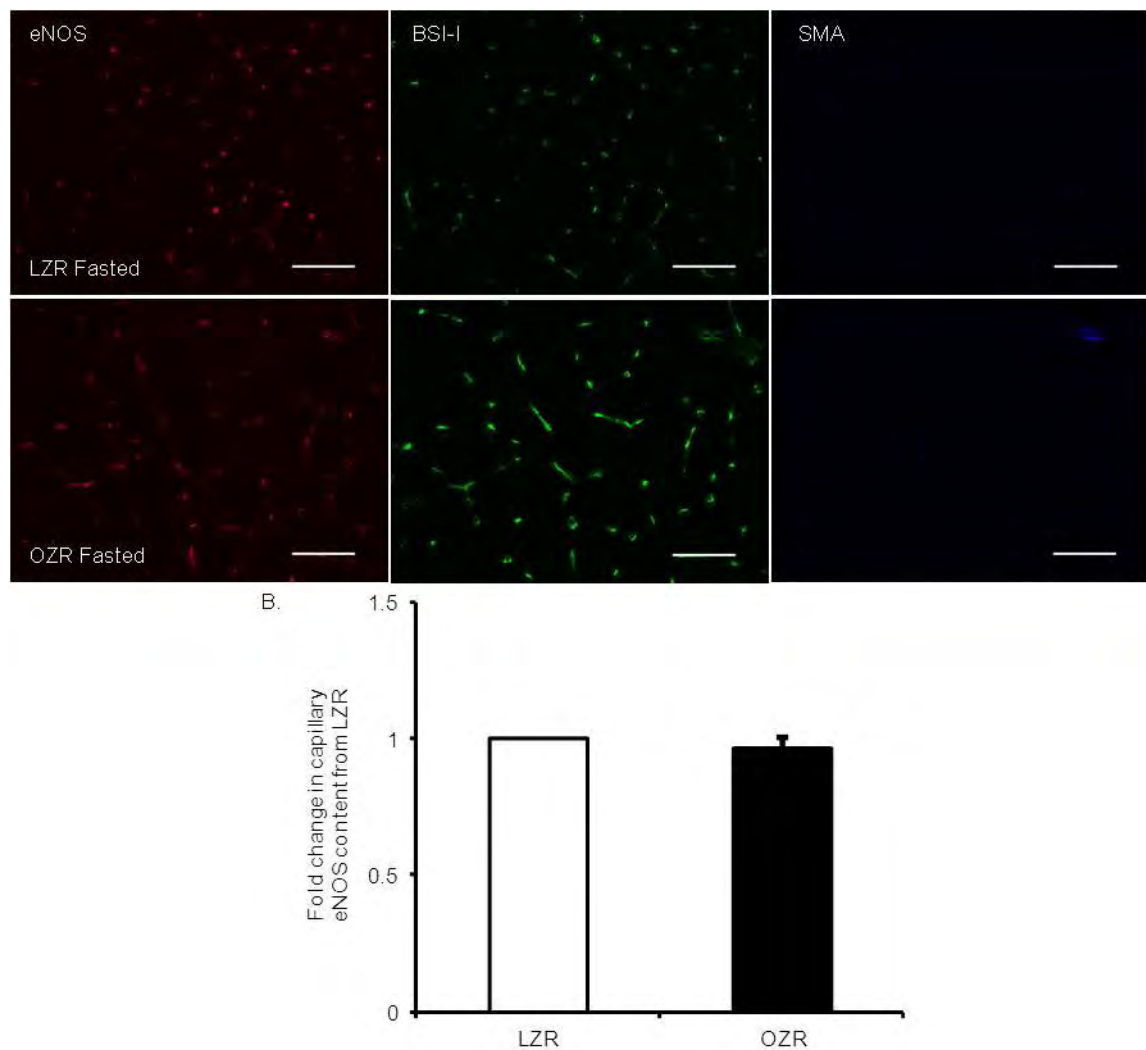


Figure 3.5. Capillary eNOS content in lean and obese Zucker rats

A. Widefield microscopy images of *m. Tibialis anterior* in lean (LZR) and obese (OZR) Zucker rats. The skeletal muscle microvascular endothelium was revealed using Fluorescein Griffonia (Bandeiraea) Simplicifolia I lectin (BSI-I) (green). Arterioles and capillaries were separated using anti smooth muscle actin (SMA) in combination with Alexa-Fluor 350 conjugated secondary antibody (blue). eNOS was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 50 μ m. B Mean fluorescence intensity of eNOS within capillaries is summarized. The mean level of eNOS in LZR was assigned a value of 1, and the relative intensity of eNOS in OZR was calculated.

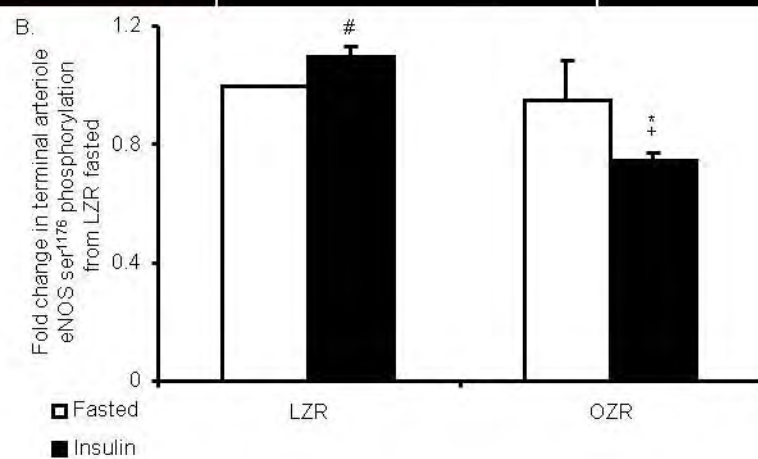
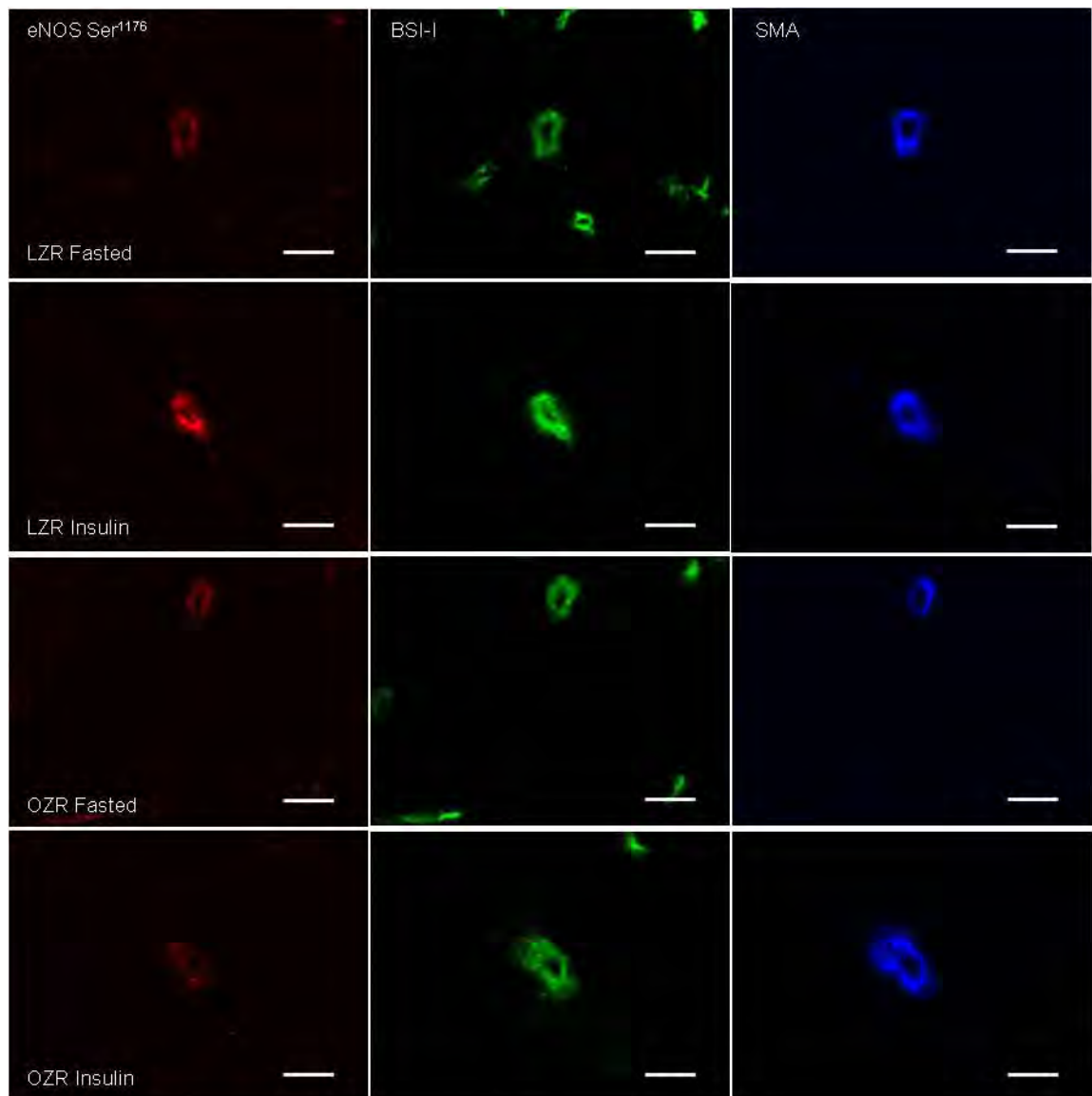


Figure 3.6. Fasted and insulin stimulated terminal arteriole eNOS ser¹¹⁷⁶ phosphorylation in lean and obese Zucker rats

A. Widefield microscopy images of *m. Tibialis anterior* in lean (LZR) and obese (OZR) Zucker rats. The skeletal muscle microvascular endothelium was revealed using Fluorescein Griffonia (Bandeiraea) Simplicifolia I lectin (BSI-I) (green). Arterioles and capillaries were separated using anti smooth muscle actin (SMA) in combination with Alexa-Fluor 350 conjugated secondary antibody (blue). eNOS ser¹¹⁷⁶ phosphorylation was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 10µm. B Mean fluorescence intensity of eNOS ser¹¹⁷⁶ phosphorylation within terminal arterioles is summarized. The mean level of eNOS ser¹¹⁷⁶ phosphorylation from LZR in the fasted state (LZR fasted) was assigned a value of 1, and the relative intensity of eNOS ser¹¹⁷⁶ phosphorylation in LZR post insulin (LZR insulin), OZR in the fasted state (OZR fasted) and OZR post insulin (OZR insulin) was calculated. # $P<0.05$ from LZR fasted, + $P<0.05$ from LZR insulin, * $P<0.05$ from OZR fasted

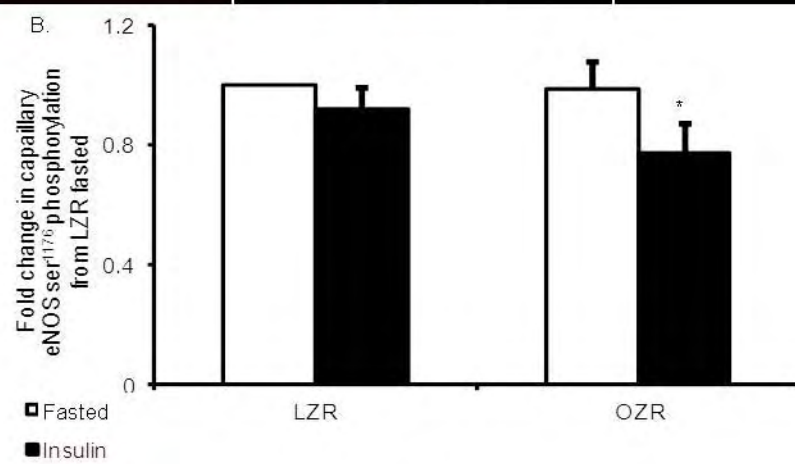
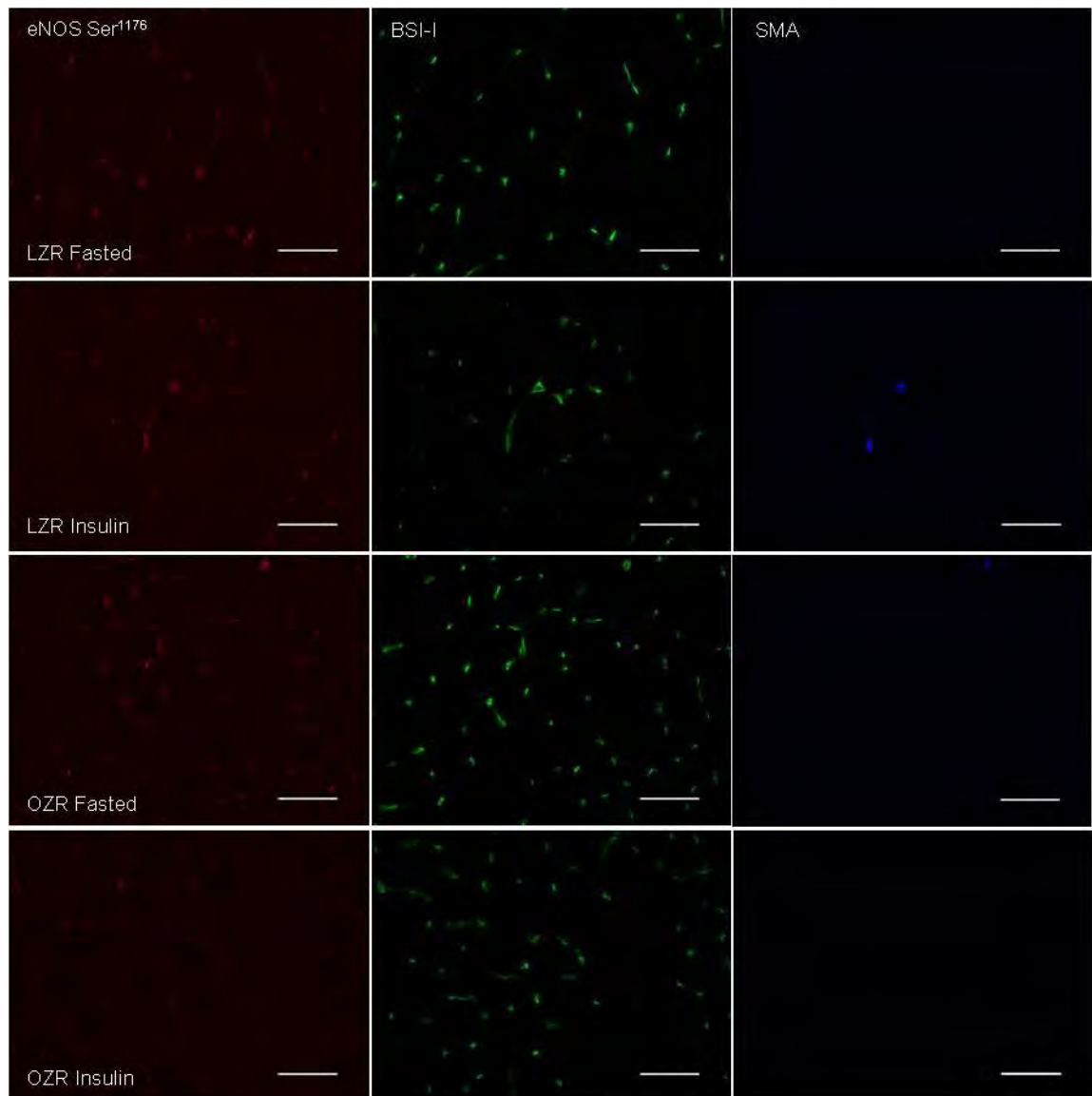


Figure 3.7. Fasted and insulin stimulated capillary eNOS ser¹¹⁷⁶ phosphorylation in lean and obese Zucker rats

A. Widefield microscopy images of *m.Tibialis anterior* in lean (LZR) and obese (OZR) Zucker rats. The skeletal muscle microvascular endothelium was revealed using Fluorescein Griffonia (Bandeiraea) Simplicifolia I lectin (BSI-I) (green). Arterioles and capillaries were separated using anti smooth muscle actin (SMA) in combination with Alexa-Fluor 350 conjugated secondary antibody (blue). eNOS ser¹¹⁷⁶ phosphorylation was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 50µm. B Mean fluorescence intensity of eNOS ser¹¹⁷⁶ phosphorylation within capillaries is summarized. The mean level of eNOS ser¹¹⁷⁶ phosphorylation from LZR in the fasted state (LZR basal) was assigned a value of 1, and the relative intensity of eNOS ser¹¹⁷⁶ phosphorylation in LZR post insulin (LZR insulin), OZR in the fasted state (OZR fasted) and OZR post insulin (OZR insulin) was calculated. * $P < 0.05$ from OZR fasted.

3.5 Discussion

The most important novel findings of the present study are 1) skeletal muscle eNOS content was not different between LZR and OZR in either arterioles or capillaries, 2) fasted eNOS ser¹¹⁷⁶ phosphorylation was not different between LZR and OZR in arterioles or capillaries, 3) skeletal muscle eNOS ser¹¹⁷⁶ phosphorylation in arterioles of LZR was significantly elevated following insulin stimulation, but was significantly reduced in OZR, 4) capillary eNOS ser¹¹⁷⁶ phosphorylation was unchanged in LZR, but was significantly reduced in OZR in response to insulin stimulation. As hypothesised these impairments in insulin stimulated eNOS ser¹¹⁷⁶ phosphorylation were accompanied by significant reductions in glucose clearance and whole body insulin sensitivity during the early stages and final 20 minutes, respectively, of a hyperinsulinemic-euglycemic clamp. These findings add an important *in vivo* signalling perspective to existing physiological data, disclosing at least part of the molecular mechanisms that contribute to impaired dilation of (terminal) arterioles and reduced glucose uptake in skeletal muscle of OZR.

The OZR in the present study were insulin resistant as shown by the substantial reduction in insulin sensitivity index (Fig. 3.2) compared to their lean littermates. However no difference was observed in GIR between LZR and OZR at the end of the clamp, which may be explained by the different insulin infusion rates in LZR and OZR and preferential channelling of glucose to tissues other than skeletal muscle in OZR. Different insulin infusion rates were chosen to generate increases in insulin concentration that are in the normal physiological range both for the LZR and OZR, with much higher concentrations occurring in the OZR. In the LZR a 4-fold increase in hind limb glucose uptake has been observed during a hyperinsulinaemic-euglycemic clamp (37). However, in OZR increases in hind limb glucose extraction were

minimal 2 h after the start of the hyperinsulinemic-euglycemic clamp (37) using the same insulin infusion rates as in our study, implying that the majority of the glucose in the OZR must be taken up by tissues other than skeletal muscle. Adipose tissue (26), liver (3) and skin (27) seem to be the major contributors to the clearance of the large increases in GIR observed in OZR in the 30-120 min period, with only minimal increases in glucose uptake occurring in skeletal muscle (37). Finally previous studies have suggested that increases in microvascular volume occur as early as 5 min into a hyperinsulinemic-euglycemic clamp and are therefore important in the early rise in glucose uptake. As such, the significant reduction in GIR in the OZR in the early phase (first 21 min) of the clamp seems to be due to impairment in microvascular recruitment. The reduced 1-methyl-xanthine extraction by the hindlimbs observed in the OZR in comparison to the LZR may also suggest that insulin infusion during the clamp in fact leads to the vasoconstriction of the muscle microvasculature in the OZR.

eNOS content was not different between LZR and OZR in either terminal arterioles or capillaries. This is the first observation of endothelial specific eNOS content from terminal arterioles or capillaries of OZR, and suggests that reductions in eNOS content do not contribute to the previously observed reductions in capillary surface area that is perfused in the fasting and insulin stimulated state in the hindlimbs of OZR (37).

Fasting eNOS ser¹¹⁷⁶ phosphorylation was also unaltered in OZR terminal arterioles and capillaries compared to their lean littermates. These results, therefore, suggest that reductions in eNOS ser¹¹⁷⁶ phosphorylation and any subsequent impairment in NO production also do not play a role in the previously observed reduction in capillary surface area that is perfused in the fasting state (37).

Although fasting eNOS ser¹¹⁷⁶ phosphorylation was unchanged we observed a significant impairment in terminal arteriole eNOS ser¹¹⁷⁶ phosphorylation in response to insulin in OZR. Insulin stimulation mediated an elevation in terminal arteriole eNOS ser¹¹⁷⁶ phosphorylation in LZR, however in OZR insulin stimulation surprisingly resulted in reduced eNOS ser¹¹⁷⁶ phosphorylation. The observation that insulin is able to reduce eNOS phosphorylation has previously been made in aortic endothelial cells isolated from ob/ob mice (19) exposed to high concentrations of insulin during in vitro incubations. As eNOS phosphorylation increases eNOS activity and subsequent NO production (23, 30) it is reasonable to assume that reduced eNOS ser¹¹⁷⁶ phosphorylation is instrumental in the failure of insulin to increase the capillary surface area that is perfused in OZR. As increases in microvascular volume and the capillary permeability surface area product are important for the ability of insulin to stimulate glucose uptake (1, 2, 16, 35), reduced eNOS ser¹¹⁷⁶ phosphorylation is likely to be an important mechanism in the impaired insulin sensitivity and early glucose clearance observed in OZR in the current study.

Similar to the observation in terminal arterioles, eNOS ser¹¹⁷⁶ phosphorylation was also reduced in skeletal muscle capillaries following insulin stimulation in OZR. However, unlike the observation made in terminal arterioles, insulin stimulation did not increase eNOS ser¹¹⁷⁶ phosphorylation in skeletal muscle capillaries in LZR. As muscle samples were collected 120 min after the start of the clamp, increases at earlier time points may well have been missed. As previous research has provided evidence for the existence of eNOS phosphatases (24), such enzymes might potentially contribute to subsequent inactivation (switching off of the signal) following prolonged insulin stimulation, but future research will be required to confirm this.

NO production and/ or bioavailability has been implicated in the trans-endothelial transport of insulin (2). Trans-endothelial transport of insulin across the endothelium of the muscle capillary bed has been suggested to be a second mechanism next to capillary recruitment exerting control on the delivery of insulin to the skeletal muscle fibres and is suggested to be NO-dependent (2). Therefore the reductions in the insulin induced eNOS ser¹¹⁷⁶ phosphorylation in muscle capillaries of OZR may potentially reduce trans-endothelial transport of insulin and this mechanism may well be part of the reduction in the capillary permeability surface area product available for delivery of insulin that has been observed in patients with type 2 diabetes (15).

NO production and/ or bioavailability has also been implicated and in the mechanisms leading to VEGF induced angiogenesis (9). Therefore, Frisbee et al. (10) have suggested a role for chronically reduced NO bioavailability in the capillary rarefaction that is seen in OZR. The impaired capillary eNOS ser¹¹⁷⁶ phosphorylation in response to insulin stimulation may therefore play a role in the reduced capillary density in OZR observed in the current study (9). As previously suggested (9), the capillary rarefaction in the current study will contribute to the insulin resistance in OZR as it will reduce the capillary permeability surface area product for insulin and glucose and thus reduce muscle glucose uptake (15).

Elevated plasma tumor necrosis factor- α (TNF α) (18) and plasma free fatty acids (FFA) (21) in OZR may be responsible for the impaired insulin mediated eNOS signalling. Acutely elevated TNF α (38) and FFA (5) have been shown to impair microvascular recruitment and skeletal muscle glucose uptake. *In vitro* it has been shown that elevated exposure of endothelial cells to FFA increases levels of diacylglycerols, ceramide and long chain fatty

acyl CoA (25). These fatty acid metabolites and TNF α activate serine kinases such as PKC, inhibitory KB kinase (IKK β) and Jun N-terminal kinase (JNK) inhibiting the insulin signalling cascade and eNOS phosphorylation (8, 25). Although the inhibitory effects of TNF α and FFA may explain impaired eNOS ser¹¹⁷⁶ phosphorylation in OZR they cannot explain the reduction observed in the present study. Vasoconstriction in response to insulin reducing shear stress may therefore be an additional important mechanism responsible for the reduction eNOS ser¹¹⁷⁶, as shear stress is one of the most important regulators of eNOS ser¹¹⁷⁶ phosphorylation (24). Unlike the Akt dependent insulin signalling cascade the MAP-kinase signalling pathway which is also stimulated by insulin is intact in OZR (17). Activation of the MAP-kinase pathway results in production of endothelin-1 (ET-1), a vasoconstrictor peptide (25) and this activation especially occurs at the high insulin levels that occur in the OZR (17, 25). Acute TNF α has been shown to inhibit insulin mediated vasodilatation but elevate vasoconstriction, reducing blood flow in skeletal muscle microvessels (8). In addition increased adrenergic activity and reactivity has been shown in OZR (12). As such, elevated vasoconstriction may reduce blood flow and shear stress in the OZR, and this could provide an explanation for the insulin induced reduction in eNOS ser¹¹⁷⁶ phosphorylation both in terminal arterioles and capillaries.

An obvious limitation of the current study is that muscle microvascular volume was not measured; as a result the physiological consequences of eNOS signalling in obesity and following insulin stimulation have been inferred from previous studies conducted in LZR and OZR (37). The present study did however show impairments in insulin induced reductions in femoral vascular conductance (Fig. 3.3). Although the time course of insulin-mediated vasodilatation is different between arteries (90-120 min) and the skeletal muscle

microvasculature (36) (5-10 min), insulin-mediated dilation of the macrovasculature is also NO dependent (33), and Wallis et al (37) have previously shown that increases in femoral artery blood flow are impaired in the arteries of OZR during a hyperinsulinemic-euglycemic clamp. A further limitation is that only eNOS activation by ser¹¹⁷⁶ phosphorylation was investigated. A recent study by Ritchie et al. (30) has shown that ser⁶¹⁵ is also phosphorylated by insulin, but phosphorylation at ser⁶¹⁵ alone was not sufficient to activate eNOS unlike ser¹¹⁷⁶ phosphorylation.

Finally it is well documented that NO bioavailability is not only a result of NO production by eNOS, but is a balance between NO production and NO quenching by superoxide anions ($\cdot\text{O}^{2-}$) (20). Due to methodological issues discussed earlier we were not able to measure NAD(P)H oxidase content of muscle terminal arterioles and capillaries. As elevations in NAD(P)H oxidase have previously been observed in endothelial scrapings of the macrovasculature in obese humans (32), future studies should also investigate the role that induction of this enzyme has on the impaired microvascular response to insulin in obesity and type 2 diabetes,.

In conclusion, the current study provides novel findings which suggest that impaired eNOS ser¹¹⁷⁶ phosphorylation plays a crucial role in the previously observed blunting of insulin mediated increases in muscle microvascular volume, and that these molecular mechanisms are instrumental to the reduction in insulin sensitivity and early uptake of glucose in skeletal muscle of OZR.

3.6 Acknowledgements This study was supported by funding from Astra Zeneca

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Chapter 4

Sprint Interval and Endurance Training are Equally Effective in Increasing Muscle Microvascular Density and eNOS Content in Sedentary Males

4.1 Abstract

Sprint interval training (SIT) has been proposed as a time efficient alternative to endurance training (ET) for increasing skeletal muscle oxidative capacity and improving certain cardiovascular functions. In this study we sought to make the first comparisons of the structural and endothelial enzymatic changes in skeletal muscle microvessels in response to ET and SIT. 16 Young sedentary males (age 21 ± 0.7 yr, BMI 23.8 ± 0.7 kg.m⁻²) were randomly assigned to 6 weeks of ET (40-60 min cycling at ~65% VO_{2peak}, 5 times per week) or SIT (4-6 Wingate tests, 3 times per week). Muscle biopsies were taken from the *m. vastus lateralis* before and following 60 min cycling at 65% VO_{2peak} to measure muscle microvascular endothelial eNOS content, eNOS serine¹¹⁷⁷ phosphorylation, NOX2 content and capillarization using quantitative immunofluorescence microscopy. Whole body insulin sensitivity, arterial stiffness, and blood pressure were also assessed. ET and SIT increased skeletal muscle microvascular eNOS content (ET 14%; $P < 0.05$, SIT 36%; $P < 0.05$), with a significantly greater increase observed following SIT ($P < 0.05$). 60 Min of moderate intensity exercise increased eNOS ser¹¹⁷⁷ phosphorylation in all instances ($P < 0.05$), however basal and post-exercise eNOS ser¹¹⁷⁷ phosphorylation was lower following both training modes. All microscopy measures of skeletal muscle capillarization ($P < 0.05$) were increased with SIT or ET, while neither endothelial nor membrane NOX2 were changed. Both training modes reduced aortic stiffness and increased whole body insulin sensitivity ($P < 0.05$). In conclusion, in sedentary males SIT and ET are effective in improving muscle microvascular density and eNOS protein content.

4.2 Introduction

It is well established that endothelial function is dependent on nitric oxide (NO) bioavailability (30), which in turn is determined by the balance between NO synthesis and scavenging by superoxide anions and related reactive oxygen species. As endothelial function plays a role in insulin sensitivity, blood pressure regulation and the molecular mechanisms leading to atherosclerosis (3, 4, 30, 44) an optimal balance between NO production and scavenging is important in the maintenance of a healthy phenotype. Experiments with isolated arteries and cultured endothelial cells have shown that endothelial nitric oxide synthase (eNOS) is the rate limiting enzyme for endothelial NO synthesis. The eNOS protein content and serine¹¹⁷⁷ (ser¹¹⁷⁷) phosphorylation state together determine eNOS activity and NO production. A major source of superoxide production and NO scavenging in the vascular wall is NAD(P)H oxidase (NAD(P)Hox) (7, 39), but substantial expression of this enzyme is assumed to only occur in obesity, cardiovascular pathology and ageing (7, 39). However, the lack of a validated method means that currently there is little information on the protein content of these enzymes in the endothelial layer of the microvasculature of human skeletal muscle.

There is substantial evidence in the literature that the adoption of a sedentary lifestyle, obesity, ageing and chronic diseases lead to an attenuation of endothelial NO production by reductions in eNOS expression and protein content (McAllister & Laughlin, 2006) and increases in NAD(P)Hox expression and protein content (Brandez and Kreuzer 2005, Silver et al., 2007) in the macrovasculature. Whether these adaptations also occur in the muscle microvasculature is currently unknown. In obese and elderly individuals and patients with type 2 diabetes and cardiovascular disease an attenuated NO production in the muscle

microvasculature has been implicated in the development of skeletal muscle insulin resistance (3, 4), anabolic resistance to insulin and amino acids leading to sarcopenia and reductions in muscle capillary density (44). An attenuated endothelial NO production has also been implicated in the reduction in exercise hyperemia that is known to occur in elderly humans (12, 38) with Spier et al. (40) providing evidence that this impairment indeed occurs in isolated muscle arterioles of old rats and can be restored by exercise training. Krentz et al. (26) have also made the observation that functional impairments in NO-dependent muscle microvascular function precede macrovascular impairments in humans and, therefore, suggested that the muscle microvasculature should be regarded as a primary target for therapeutic interventions.

Endurance training (ET) is recognised as an efficient means to increase eNOS gene expression, protein content, and NO production in feeding and resistance arteries (McAllister & Laughlin 2006), thereby increasing the vasodilatory response to insulin (3, 37) and flow mediated dilation, and reducing the risk for the development of hypertension and atherosclerosis (30, 42). ET is also the traditional means to increase the production of vascular endothelial growth factor (VEGF) in skeletal muscle which generates an NO dependent signal stimulating angiogenesis (1, 16, 21, 31, 45). However, it is not known whether ET increases eNOS content in the endothelial layer of the muscle microvasculature or its phosphorylation at ser¹¹⁷⁷ in the basal state or in response to moderate intensity cycling exercise in man. In addition the effect of ET on muscle microvascular NAD(P)Hox content has not been investigated.

Recently sprint interval training (SIT) has received much attention as it elicits similar muscle metabolic (increases in activity of mitochondrial enzymes, aerobic capacity and insulin sensitivity) and macrovascular adaptations (reduced arterial stiffness and blood pressure) as ET, despite a marked reduction in time commitment (2, 8, 17, 36). In rodent models, SIT has also been shown to increase aortic eNOS content (18). However to date, no studies have made comparisons between the effects of SIT and ET on the microvascular enzymes controlling NO production and skeletal muscle microvascular density in man.

The main aims of the present study were twofold. First, we sought to determine the effects of six weeks of traditional ET and SIT on skeletal muscle microvascular density and microvascular enzyme content (eNOS and NOX2) in previously sedentary men. We employed a novel method recently developed in our laboratory (10), to measure the protein content of these enzymes in the endothelial layer of the muscle microvasculature using quantitative immunofluorescence microscopy. Secondly, we aimed to investigate the effect of 1 h of continuous cycling exercise at 65% $\text{VO}_{2\text{peak}}$ on eNOS ser¹¹⁷⁷ phosphorylation both in the untrained state and after the two training interventions. eNOS ser¹¹⁷⁷ is the main activation site previously shown to be phosphorylated by endurance exercise in mouse arteries (48). Finally, the effects of SIT and ET on arterial stiffness and blood pressure were considered to investigate earlier claims that SIT is an effective means to improve macrovascular functions (24, 36). We hypothesized that microvascular density and eNOS content would increase in response to both modes of training, that eNOS ser¹¹⁷⁷ phosphorylation would be lower after training as the increase in microvascular density and metabolic training adaptations will reduce shear stress per microvessel and that NOX2 content would be unaltered due to the healthy nature of the participants.

4.3 Materials and Methods

The percutaneous muscle biopsies taken in this study before and after both training modes and under basal conditions and after 1 h of endurance exercise have been used both for the measurements described in this manuscript and for measurements of the content of intramuscular triglycerides (IMTG), perilipin-2 and perilipin-5 and usage of total IMTG and IMTG associated with perilipin-2 and perilipin-5 during endurance exercise. The latter are reported in a parallel manuscript (Shepherd SO, Cocks M, Tipton KD, Ransinghe AM, Barker TA, Burniston JG, Wagenmakers AJM and Shaw CS, unpublished data). Measures such as insulin sensitivity and $\text{VO}_{2\text{peak}}$ also made in the indicated human volunteers are relevant for the interpretation of both studies and are presented in both manuscripts.

Participants

16 young sedentary males (defined as performing less than 1 h of organised exercise per week; in sports clubs, university or commercial gyms or sports classes) participated in the study (Table 4.1). Participants were randomly assigned to either ET or SIT groups, in a matched fashion based on age and $\text{VO}_{2\text{peak}}$ ($n=8$). The participants gave written informed consent to a protocol adhering to the Declaration of Helsinki and approved by the Black Country NHS Research Ethics Committee.

Pre-training testing protocol

Participants first completed an incremental exercise test to exhaustion on an electromagnetically braked cycle ergometer to determine maximal aerobic power output (W_{max}) and $\text{VO}_{2\text{peak}}$ using an online gas collection system (Oxycon Pro, Viasys, Wuerzburg, Germany). Briefly, participants started cycling at 95 W for 3 min; following this the workload

was increased by 35 W every 3 min until volitional fatigue. $\text{VO}_{2\text{peak}}$ corresponds to the highest value achieved over a 15 second recording period. After sufficient rest, participants in the SIT group performed a Wingate test (30 s 'all out' effort against a resistance equivalent to 0.075kg body mass) to familiarise themselves with the SIT training protocol.

3-7 Days after the incremental exercise test and following an overnight fast, macrovascular function was assessed (blood pressure and arterial stiffness). Following this a 2 h oral glucose tolerance test was completed. The next day, again following an overnight fast a muscle biopsy was taken at rest from the *m. vastus lateralis*. Participants then performed 60 min of cycling on an electromagnetically braked cycle ergometer at 65% of pre-training $\text{VO}_{2\text{peak}}$. A second biopsy was taken immediately after exercise from the same leg as the pre-exercise biopsy.

Post-training testing protocol

Post-training $\text{VO}_{2\text{peak}}$ testing was performed the day before the final training session.

Approximately 48 hours after the final training session the post-training testing protocol was conducted with procedures, methods and timings identical in all respects to the pre-training testing protocol. Power output of the 60 min steady state cycling exercise bout was set at the same absolute intensity as during the pre-training exercise bout.

Arterial stiffness

Supine brachial artery blood pressure measurements were made in triplicate using an automated sphygmomanometer (Omron 7051T, Omron Corporation, Kyoto, Japan) following 15 minutes of supine rest. Central (carotid-femoral, cPWV) and peripheral (carotid-radial, pPWV) pulse wave velocity (PWV) were assessed using a semi automated device and

software (SphygmoCor, AtCor Medical, Sydney, Australia). PWV is defined as the speed of travel of a pulse between two sites along the arterial branch (34) and is a measure of artery stiffness. A single high fidelity applanation tonometer was used to obtain a proximal (carotid artery) and distal (radial or femoral artery) pulse, recorded sequentially over 10 waveforms. Simultaneously the QRS complex was measured using electrocardiography (ECG). The time between the R wave of the ECG and the foot of the proximal waveform is subtracted from the time between the R wave and the foot of the distal waveform to obtain the pulse transit time. To determine the distance used for PWV the distance from the proximal measurement site (carotid artery) to the suprasternal notch was subtracted from the distance between the distal (radial/ femoral artery) measurement site and the suprasternal notch using an anthropometric measuring tape. PWV measurements were made in triplicate. The day to day variability measured as co-efficient of variation for cPWV and pPWV in our laboratory was 4% and 4%, respectively. Radial artery pressure waveforms were acquired (SphygmoCor, AtCor Medical, Sydney, Australia) and the aortic waveform reconstructed using a validated transfer function (34). Augmentation index (AI_x), an assessment of systemic wave reflection and thus arterial stiffness (27), was then determined using pulse wave analysis (PWA). To control for the potentially confounding influence of heart rate (47) AI_x was normalised to a heart rate of 75 bpm ($AI_x@75bpm$). Resting heart rate was obtained during PWA using the SphygmoCor software. PWA measurements were made in triplicate. The day to day variability measured as co-efficient of variation in our laboratory was 8% for $AI_x@75bpm$.

Oral glucose tolerance test and Matsuda insulin sensitivity index

Following the insertion of a cannula into an antecubital vein, a baseline 25 ml blood sample was taken. Participants then ingested a 25% glucose beverage containing 75g glucose made

up in 300 ml of water. Further blood samples (10 ml) were collected at 30, 60, 90 and 120 minutes while participants rested, following the procedure originally proposed by Matsuda & DeFronzo (1999) for the assessment of whole body insulin sensitivity. Plasma was separated by centrifugation (10 minutes at 3000 rpm) and stored at -80°C until analysis. Plasma insulin concentrations were determined by enzyme linked immuno-sorbent assay (ELISA) using a commercially available kit (Invitrogen, UK). Plasma glucose concentrations were analysed using an automated analyzer (IL ILab 650 Chemistry Analyzer, Diamond Diagnostics, USA). Area under the curve (AUC) for insulin and glucose during the oral glucose tolerance test was calculated using the conventional trapezoid rule. The Matsuda index, a variable which is generally accepted and widely used as a non-invasive alternative to measure whole body insulin sensitivity has been shown to have a high correlation with insulin sensitivity measured with the hyperglycemic euglycemic clamp (28), which is the gold standard method. The Matsuda index was calculated using the formula:

$$\text{Matsuda index} = \frac{10000}{\sqrt{(\text{FPG} - \text{FPI}) \cdot (\text{mean OGTT insulin}) (\text{mean OGTT glucose})}}$$

Where FPG is fasting plasma glucose concentration (mg.dl⁻¹), FPI is fasting plasma insulin concentration (μU.ml⁻¹), and mean OGTT glucose and insulin is the mean plasma glucose (mg.dl⁻¹) and insulin (μU.ml⁻¹) concentration of all samples taken during the oral glucose tolerance test (0, 30, 60, 90 and 120 min).

Muscle biopsy

While participants rested a muscle biopsy was taken from the lateral portion of the *m. vastus lateralis*, approximately 25-50% of the distance from the lateral joint line and the greater trochanter under local anaesthesia (1% lidocaine) using the percutaneous needle biopsy

technique (6) as recently described (41). Post exercise biopsies were taken from an incision ~2 cm proximal to the first incision. Excess blood and visible collagen or fat were removed before samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) and immediately frozen in liquid nitrogen cooled isopentane (Sigma-Aldrich, Dorset, UK). Samples were then stored at -80°C until analysis was performed.

Dietary controls

24 Hours prior to and throughout the 2 days of the pre- and post-training testing protocol participant's diets were standardised. Using commercially available diet analysis software (WISP) 3 day diet diaries were logged and average daily energy intake was calculated. The standard diet was matched to the participant's habitual energy intake and was composed of 50% carbohydrate, 35% fat and 15% protein.

Training

Training programs were initiated 48 hours after completion of the pre-training testing protocol. Training for the ET group consisted of 40-60 min continuous cycling on an electromagnetically braked cycle ergometer at an intensity eliciting 65% $\text{VO}_{2\text{peak}}$. Participants trained 5 times a week. Following 3 weeks of training a second incremental exercise test was conducted and workload was changed accordingly. The duration of the sessions was increased from 40 min during the first 10 sessions, to 50 minutes for sessions 11- 20 and 60 min for sessions 21-30. The SIT group performed repeated Wingate tests (8), interspersed with 4.5 min recovery (cycling at 30 W). The number of Wingate tests performed each session was increased from 4 for the first 6 sessions to 5 during sessions 7-12 and 6 during session 13-18.

All training sessions were supervised by members of the research team and significant encouragement was provided during the Wingate tests.

Quantitative immunofluorescence

Serial 5µm sections were cut at -30°C using a microtome and collected on to room temperature uncoated glass slides. Pre- and post-training sections were placed on the same slide for analysis of eNOS and NOX2 content. For analysis of p-eNOS ser¹¹⁷⁷ pre- and post-training and pre- and post-exercise sections were placed on the same slide. Two sections from each condition (pre- or post-training and -exercise) were placed on each slide and analysis was performed in duplicate. Transverse orientated samples were used for all analysis. Sections were left to dry for 1 hour before treatment.

The procedures for immunofluorescence staining and subsequent image analysis have been described in detail by Cocks et al. (10). Briefly, sections were incubated with antibodies against eNOS (Transduction Laboratories, Lexington, KY), p-eNOS ser¹¹⁷⁷ (Cell Signalling Technology, Beverly, MA) and NOX2 (Santa Cruz Biotechnology, Santa Cruz, CA), which have been previously validated for immunofluorescence (10). Sections were then incubated with appropriately labelled secondary antibodies (Invitrogen, Paisley, UK), in combination with Ulex Europaeus-FITC conjugated (UEA-I-FITC; Sigma-Aldrich, UK) and wheat germ agglutinin-350 (WGA-350; Invitrogen, UK) as markers of the endothelium and plasma membrane, respectively.

For analysis, slides were viewed using a Nikon E600 microscope using a 40x 0.75 numerical aperture objective. Images were captured using a SPOT RT KE colour three shot CCD camera

(Diagnostic Instrument Inc., MI, USA). Camera exposure time and gain was adjusted so that no pixel saturation was present in any channel and identical camera settings were used for all images within each participant. Image analysis was performed using Image Pro Plus 5.1 software (Media Cybernetics Inc, Bethesda, MD, USA). Endothelial specific fluorescence was determined using the UEA-I FITC (endothelial marker) image, which was extracted and overlaid onto the corresponding eNOS, p-eNOS ser¹¹⁷⁷ or NOX2 image. Cell membrane specific fluorescence for NOX2 was determined using WGA-350 as a stain to create an outline of the cell membrane. The latter was extracted and overlaid onto the corresponding NOX2 image. Fluorescence intensity of the eNOS, p-eNOS ser¹¹⁷⁷ or NOX2 signal was quantified within the endothelium or cell membrane specific area. The reader was not blinded to condition as image analysis was automated removing the potential effects of investigator bias (10). The mean fluorescence levels of pre-training and pre-training pre-exercise (p-eNOS ser¹¹⁷⁷ only) samples were normalised to a value of 1, and the relative level of post-training and post-exercise (p-eNOS ser¹¹⁷⁷ only) samples were calculated.

Capillarization

Capillaries were quantified manually using the UEA-I FITC and WGA-350 images as capillary and sarcolemmal markers, respectively. The following indexes were measured (20): 1) number of capillaries around a fibre (capillary contacts), 2) capillary-to-fibre ratio on an individual-fibre basis, 3) the number of capillaries sharing a fibre (sharing factor) and 4) capillary density. Quantitation of capillarization was performed only on transverse fibres (longitudinal fibres were discounted from the analysis). In line with previous studies assessing capillarization, at least 50 complete fibres (pre 52±1, post 52±1 fibres per subject)

were included in each analysis (35). Fibre cross sectional area and perimeter were measured on calibrated images using ImagePro Plus 5.1 software.

Statistics

All variables except p-eNOS ser¹¹⁷⁷ were analysed using a two-way mixed analysis of variance (ANOVA), with the between factor 'group' (ET vs. HIIT) and repeated factor 'training status' (pre-training vs. post-training). p-eNOS ser¹¹⁷⁷ was analysed using a three-way mixed ANOVA with the factors 'group' (ET vs. SIT), 'training status' (pre- vs. post-training) and 'exercise time' (0 versus 60 min). All analyses were performed using statistical analysis software (SPSS for windows version 16.0 [SPSS, Chicago, IL]). Significance was set at $P \leq 0.05$. Data is presented as means \pm S.E.M. All measures of arterial stiffness were measured in 15 participants due to an unacceptable pulse recording in one participant. Due to unsuccessful UEA-I FITC staining in one participant, p-eNOS ser¹¹⁷⁷ and NOX2 within the endothelium is presented for 15 participants. The primary aim of the study was to compare the effects of ET and SIT on muscle microvascular eNOS content and microvascular density. The study was powered to detect between group (SIT versus ET) differences in the increase in these variables in response to training. G*Power 3.1 software (G*Power Software Inc., Kiel, Germany) was used to calculate the required sample size. The study was designed to detect a between group effect of $f=0.30$, representative of a medium sized effect (11) adopting an alpha of 0.05 and power of 0.80. In light of the paucity of studies investigating the effects of training on microvascular eNOS content we deemed an f of 0.30 to be a physiologically relevant difference.

4.4 Results

Training effect

Training increased $\text{VO}_{2\text{peak}}$ (ET 15%, SIT 8%) and W_{max} (ET 16%, SIT 9%) with main effect of training ($P < 0.05$; Table 4.1) and no difference between groups. Resting heart rate was reduced in both SIT and ET groups following training (main effect of training, $P < 0.05$; Table 4.1). Mean and diastolic blood pressures were both reduced by training, with no difference between groups ($P < 0.05$; Table 4.1). Systolic blood pressure was not altered by either training method ($P = 0.211$; Table 4.1).

Insulin sensitivity

The Matsuda insulin sensitivity index was significantly improved by ET (31%) and SIT (27%), respectively, with no difference between training methods (main effect of training, $P < 0.05$; Table 4.1). Both glucose and insulin area under the curve (AUC) were reduced by both methods of training (main effect of training, $P < 0.05$; Table 4.1).

eNOS content and eNOS ser¹¹⁷⁷ phosphorylation

A main effect of training and group on eNOS content was observed ($P < 0.05$), while there also was a significant interaction ($P < 0.05$). When within group differences were examined both SIT and ET significantly increased eNOS content (36%, $P < 0.05$, 16%, $P < 0.05$), and when between group differences were probed eNOS content post SIT was significantly greater than eNOS content post ET ($P < 0.05$) (Fig. 4.1). 1 h moderate intensity exercise significantly increased eNOS ser¹¹⁷⁷ phosphorylation both before and after training (main effect of time, $P < 0.05$). Both pre- and post-exercise eNOS ser¹¹⁷⁷ phosphorylation was lower following training (main effect of training, $P < 0.05$), with no difference between groups. There was no

Table 4.1. Subject characteristics, insulin sensitivity, hemodynamic and peak oxygen uptake pre and post 6 weeks of training.

Variable	Endurance		Sprint interval	
	Pre training	Post training	Pre training	Post training
Age (yr)	21±1	-	22±1	-
Height (cm)	177±3	-	174±2	-
Weight (kg)	71±4	71±5	75±3	75±3
BMI (kg.m ⁻²)	22.6±1.6	22.6±1.2	24.8±0.8	24.8±0.9
VO _{2peak} (ml.kg ⁻¹ .min ⁻¹)	41.7±4.1	48.2±5.0*	41.9±1.8	45.1±2.3*
W _{max} (W)	218±11	253±16*	221±11	241±14*
ISI Matsuda	3.7±0.5	4.7±0.7*	3.9±0.3	5.8±0.4*
Glucose AUC (mmol.L ⁻¹ .120min ⁻¹)	16835±992	14783±950*	17634±710	14551±398*
Insulin AUC (mmol.L ⁻¹ .120min ⁻¹)	8434±880	6813±711*	8847±1140	5792±688*
Resting heart rate (bpm)	60±2	57±2*	65±2	61±3*
Mean arterial pressure (mmHg)	78±2	76±3*	81±3	77±3*
Systolic blood pressure (mmHg)	114±4	113±5	117±3	115±3
Diastolic blood pressure (mmHg)	61±3	57±3*	62±3	59±3*

Values are means ± S.E.M., n=8 per group. * $P < 0.05$, main effect of training

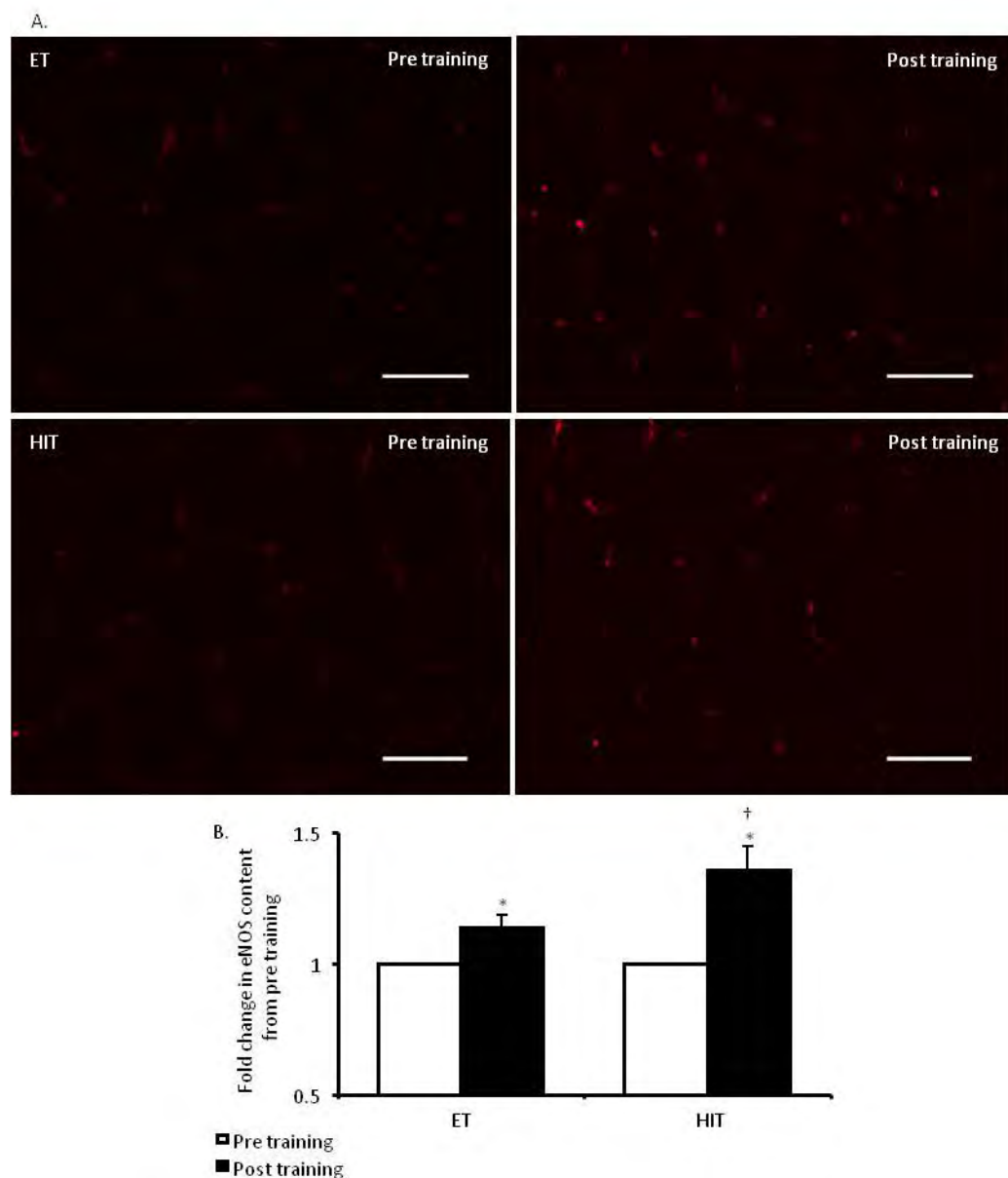


Figure 4.1. Effects of endurance training (ET) and sprint interval training (SIT) on eNOS content.

A. Widefield microscopy images of skeletal muscle pre (left) and post (right) endurance training (top) and high intensity interval training (bottom). Skeletal muscle eNOS expression was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 50 μ m. B Mean fluorescence intensity of eNOS is summarized. The mean level of eNOS pre training was assigned a value of 1, and the relative intensity of eNOS post training was calculated (ET

n = 8, SIT n = 8). * $P < 0.05$, different from pre training. + $P < 0.05$, different from ET post training

effect of group or training on the change in phosphorylation from pre- to post-exercise (Fig. 4.2) meaning that the relative increase in eNOS ser¹¹⁷⁷ phosphorylation was unchanged by training.

NOX2 content

Microvascular and sarcolemma-associated NOX2 expression was unaltered by training, with no difference between groups (microvascular, $P=0.805$; membrane, $P=0.085$) (Fig. 4.3).

Capillarization

Muscle fibre cross sectional area and sharing factor were unchanged by training, with no difference between groups (Fibre area, $P=0.202$; Sharing factor, $P=0.085$). Capillary contacts increased by 20% and 21% in the ET and SIT groups, respectively, with no difference between groups (main effect of training, $P < 0.05$). The capillary-to-fibre ratio on an individual-fibre basis was increased in both ET and SIT by 22% and 24%, respectively, with no difference between groups (main effect of training, $P < 0.05$). Finally, capillary density was increased 32% in the ET group and 27% in the SIT group, with no difference between groups (main effect of training, $P < 0.05$, Table 4.2). Representative images from pre- and post-training in both ET and SIT groups are presented in Fig. 4.4.

Arterial stiffness

AIx@75bpm was significantly decreased following training, with no difference observed between training methods (main effect of training, $P < 0.05$; Fig. 4.5a). cPWV was decreased by 5% and 7% following ET and SIT, respectively (main effect of training, $P < 0.05$; Fig.

4.5b), whereas pPWV was not significantly altered following either training mode ($P = 0.404$; Fig. 4.5c).

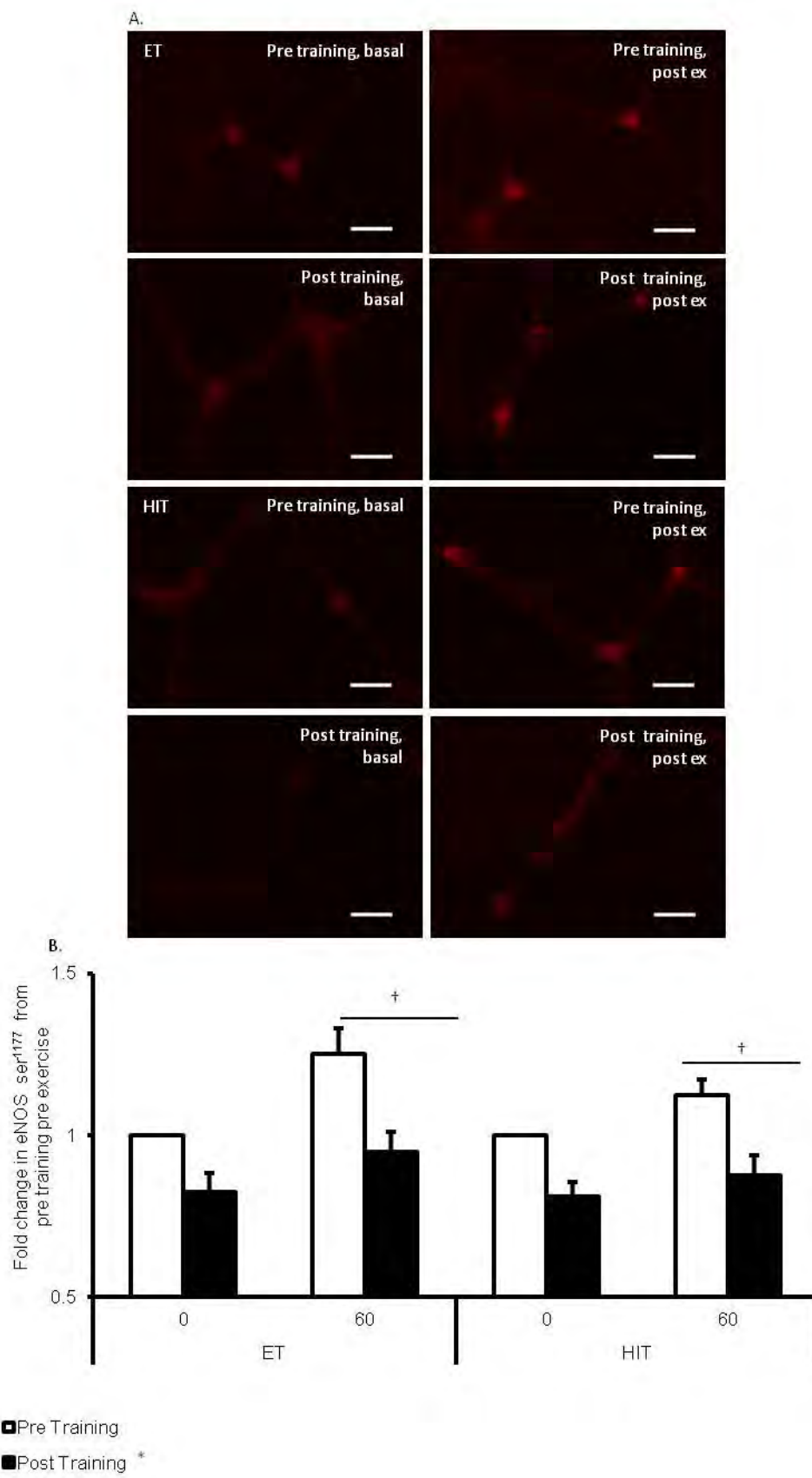


Figure 4.2. Effects of acute exercise and endurance training (ET) and sprint interval training (SIT) on eNOS serine¹¹⁷⁷ phosphorylation.

A. Widefield microscopy images of skeletal muscle pre training pre exercise (pre, pre), post training pre exercise (Pre, post), pre training post exercise (Pre, post) and post training post exercise (Post, post) in endurance training (top) and high intensity interval training (bottom). Skeletal muscle eNOS serine¹¹⁷⁷ (ser¹¹⁷⁷) phosphorylation was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 5µm. B Mean fluorescence intensity of eNOS ser¹¹⁷⁷ is summarized (ET n = 7, SIT n = 8). The mean level of eNOS ser¹¹⁷⁷ pre training pre exercise was assigned a value of 1, and the relative intensity of eNOS ser¹¹⁷⁷ post training or post exercise was calculated. * $P < 0.05$, Main effect of training. + $P < 0.05$, Main effect of time.

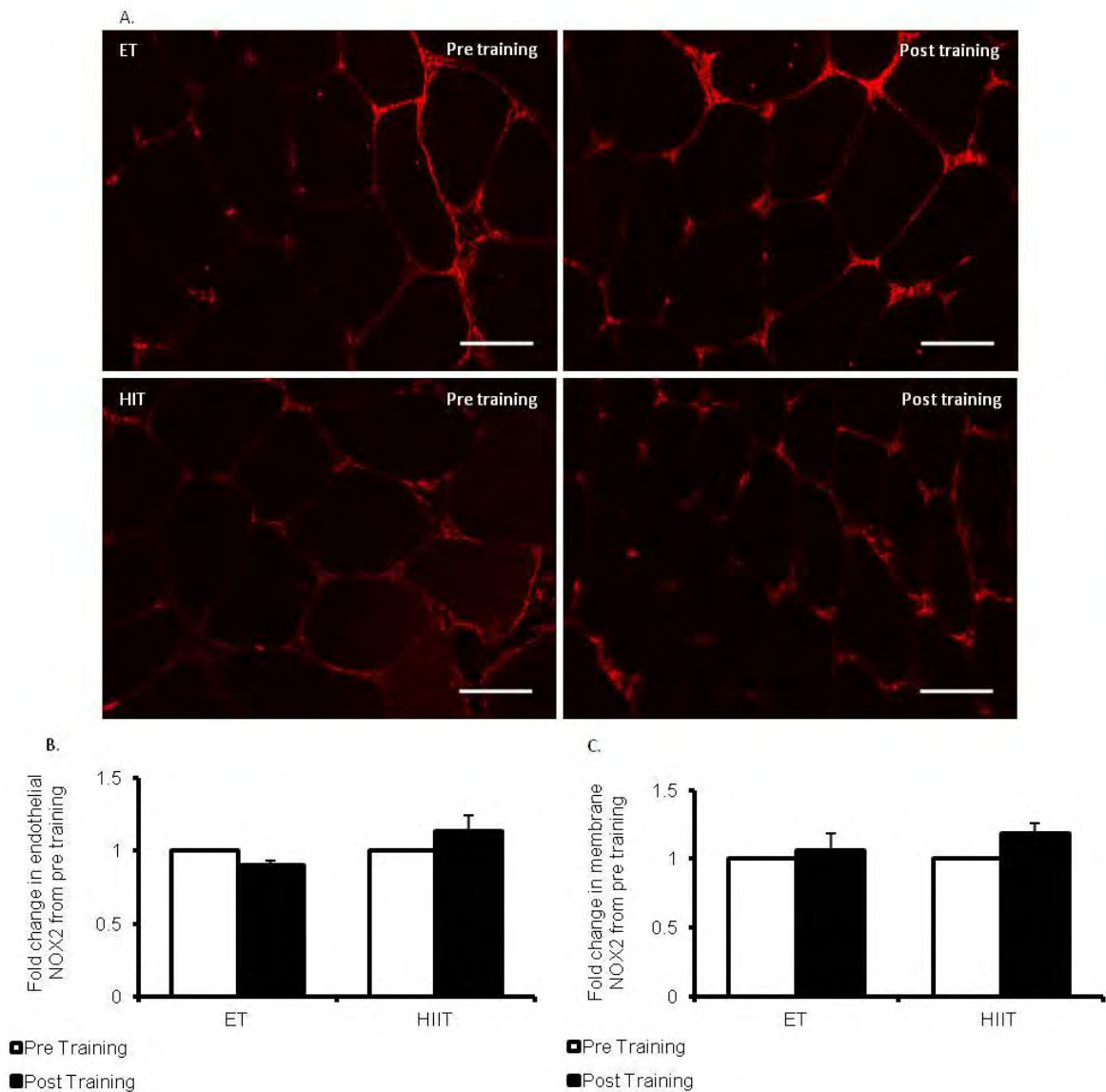


Figure 4.3. Effects of endurance training (ET) and sprint interval training (SIT) on NOX2 content.

A. Widefield microscopy images of skeletal muscle pre (left) and post (right) endurance training (top) and high intensity interval training (bottom). Skeletal muscle NOX2 content was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 50 μ m. B Mean fluorescence intensity of NOX2 within the endothelium is summarized (ET n = 7, SIT n = 8). C Mean fluorescence intensity of NOX2 within the muscle membrane is summarized

(ET n = 7, SIT n = 8). The mean level of NOX2 pre training was assigned a value of 1, and the relative intensity of NOX2 post training was calculated.

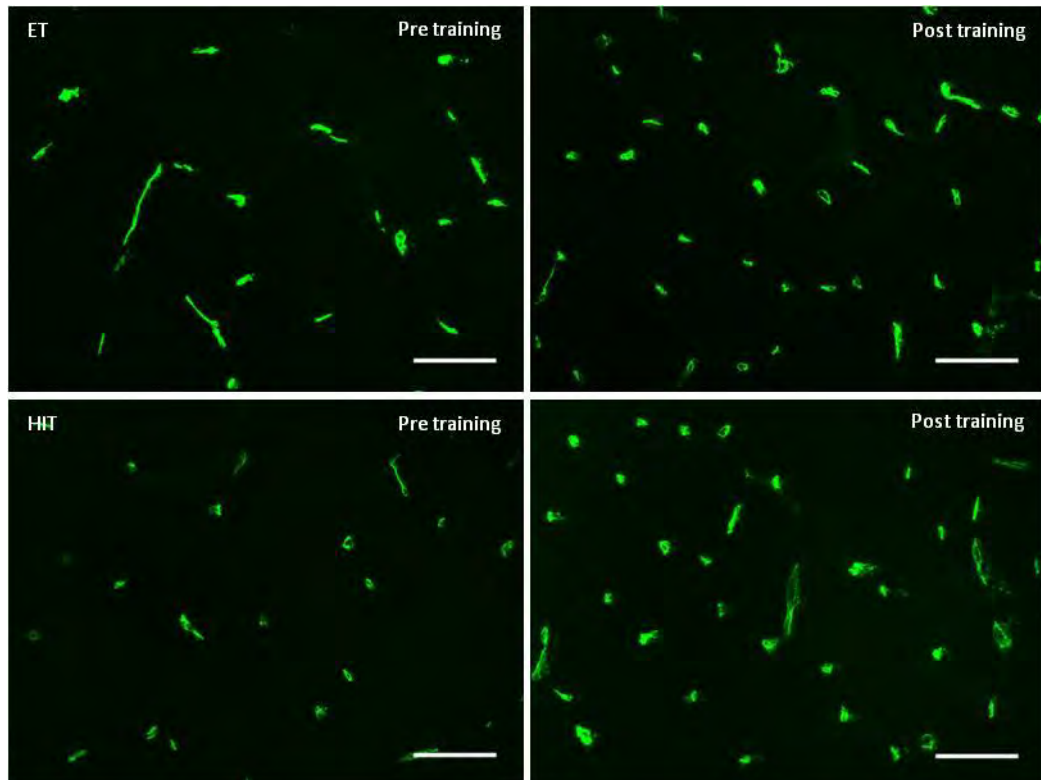


Figure 4.4. Effects of endurance training (ET) and sprint interval training (SIT) on skeletal muscle capillarization.

Composite widefield microscopy images of skeletal muscle pre (left) and post (right) endurance training (top) and high intensity interval training (bottom). Skeletal muscle microvessels were visualised using Ulex Europaeus-FITC conjugated lectin (green) and the skeletal muscle membrane was revealed using wheat germ agglutinin-350 (blue). Bar = 50 μ m.

Table 4.2. Capillarization pre and post training.

Variable	Endurance		Sprint interval	
	Pre training	Post training	Pre training	Post training
FA (mm ²)	5131±525	4607±398	4437±172	4339±332
CC	5.07±0.47	6.07±0.55*	4.53±0.23	5.50±0.35*
C/F _I	1.90±0.20	2.32±0.23*	1.66±0.10	2.07±0.15*
SF	2.69±0.06	2.68±0.03	2.78±0.02	2.71±0.03
CD (caps/ mm ²)	663.0±26.5	872.9±33.6*	642.0±34.2	816.3±24.0*

Values are means ± S.E.M. * $P < 0.05$, main effect of training. FA, fibre cross sectional area, SF, sharing factor, CD, capillary density, CC, capillary contacts, C/F_I, capillary-to-fibre ratio on an individual-fibre basis.

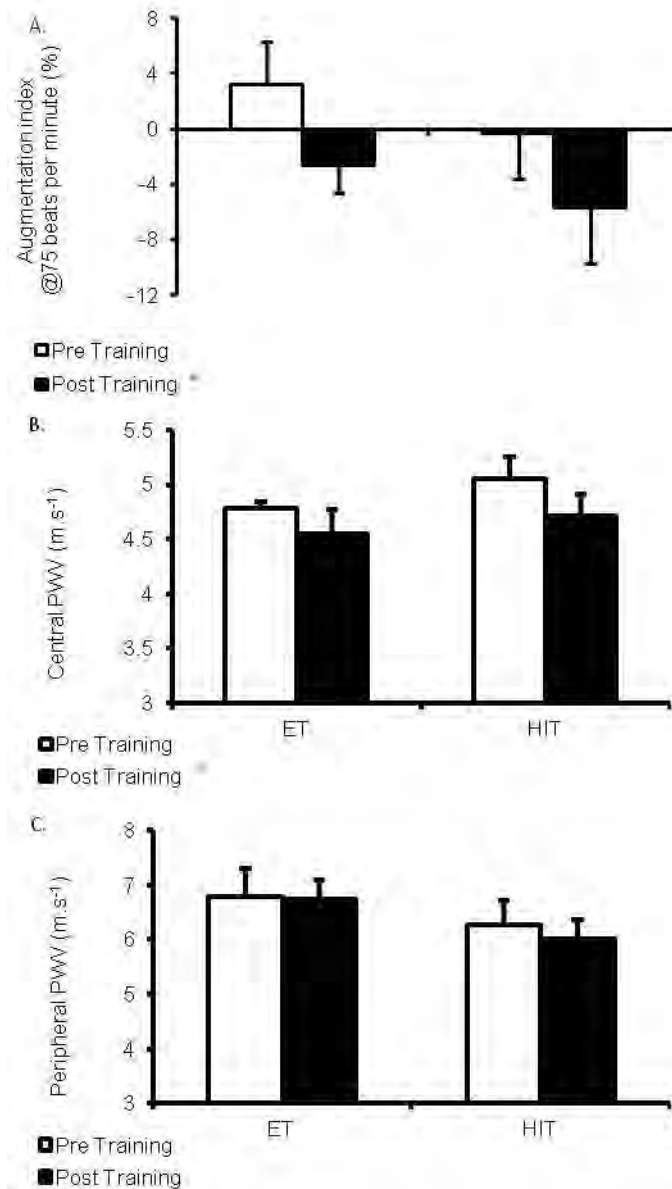


Figure 4.5. Effect of endurance training (ET) and sprint intensity interval training (SIT) on systemic wave reflections and central and peripheral artery stiffness.

A. systemic wave reflections measured using Augmentation index normalized to 75 bpm (AIx@75bpm) following ET and RT. B. Central artery (aortic) stiffness measured using pulse wave velocity (PWV) following ET and SIT. C. Peripheral artery (brachial artery) stiffness measured using pulse wave velocity following ET and SIT. * $P < 0.05$, Main effect of training.

4.5 Discussion

The most important novel findings of the present study are that: 1) 6 weeks of ET and SIT in young previously sedentary adult males increased skeletal muscle capillarization to a similar extent, 2) SIT increased the eNOS protein content of the muscle microvascular endothelium more than ET, 3) 1 h of moderate intensity exercise increased eNOS ser¹¹⁷⁷ phosphorylation irrespective of training status, but with lower pre- and post-exercise eNOS phosphorylation occurring following both modes of training, 4) neither intervention as expected induced a change in the protein expression of NOX2. Finally our results confirm previous reports showing that both ET and SIT significantly decrease arterial stiffness, brachial artery blood pressure and increase insulin sensitivity. Our integrative approach suggests that SIT is a time efficient alternative training mode for increasing aerobic exercise capacity and changing cardiovascular function in a direction consistent with health benefits in young healthy males.

Aerobic capacity and capillarization

The 6 week ET and SIT interventions both induced significant improvements in VO_{2peak} . These findings are in agreement with a number of recent studies demonstrating that SIT and ET both induce improvements in aerobic capacity (8, 17). ET has long been known to improve capillarization of several leg skeletal muscles (1, 22). This study is the first to show that SIT improves all measures of capillarization within the *m. vastus lateralis* to a similar extent as ET in healthy sedentary young men. Future studies will be required to investigate if this effect also occurs in other muscles. The observation of an increased VO_{2peak} and muscle capillarization is very important as they occur in the SIT group despite a marked reduction in total exercise duration of the training sessions and total workload of individual training sessions and the cumulative training intervention. Skeletal muscle angiogenesis has been

suggested to be the result of several stimuli including contractile activity, shear stress resulting from exercise hyperaemia, low O₂ tension and metabolic activity (16, 45).

Increases in capillarization and mitochondrial density are well described adaptations to endurance training and both contribute to the increases in aerobic exercise capacity (5). This study is the first to show that enhanced muscle capillarization is also an adaptation that contributes to the increase in aerobic exercise capacity with SIT, while the parallel study executed in the same individuals also showed similar increases in the SIT and ET group in mitochondrial density (Shepherd SO, Cocks M, Tipton KD, Ransinghe A, Barker T, Burniston JG, Wagenmakers AJM and Shaw CS, unpublished data). This implies that SIT and ET increase 2 of the major determinants of aerobic exercise capacity at the muscle level and are equally effective in the current study despite a massive difference in the nature of the exercise. The elevated capillarization following the 2 training modes may also contribute to the improved delivery of nutrients and hormones to the muscle fibres and therefore contribute to the improvements in insulin sensitivity.

Microvascular enzyme content

This study utilised a new quantitative immunofluorescence microscopy method recently developed in our laboratory (10) to study changes with ET and SIT in the protein content of enzymes that control NO production in the microvascular endothelium of skeletal muscle. Analytical methods such as Western blotting and sandwich enzyme immunoassay techniques which are applied to extracts of whole muscle homogenates do not reveal the relative distribution of these enzymes between muscle fibres and the microvessels and also do not provide information on whether changes in protein expression following training are the result

of changes in endothelial protein content or the result of changes in microvascular density. The method used in the present study is the first to generate important information on changes in the protein content and phosphorylation of eNOS and NAD(P)Hox in response to training interventions within the endothelial layer of the microvasculature of human skeletal muscle.

eNOS content

6 Weeks of ET and SIT significantly increased the protein content of eNOS within the microvascular endothelium of human skeletal muscle (Fig. 4.1) with a significantly larger increase occurring following SIT (36%) than following ET (16%). The effect of ET in our study in humans is in line with previous work in rats showing that endurance training increases the eNOS content, measured with Western blots applied to isolated 2nd-5th order arterioles isolated from the gastrocnemius muscle (29). This increase in eNOS content may potentially lead to increases in NO production upon stimulation by insulin, exercise induced shear stress and exercise induced VEGF production as previously hypothesised (21, 40, 43, 44). However, future studies making parallel measurements of eNOS content, eNOS ser¹¹⁷⁷ phosphorylation and muscle microvascular blood volume and flow will be required to confirm that higher eNOS content has functional consequences for muscle microvascular blood flow regulation.

The larger improvement in microvascular eNOS content following SIT is in agreement with previous work in the macrovasculature showing that SIT increased eNOS content of the aorta more than ET in low aerobic capacity rats (18). Given the greater microvascular eNOS expression induced by SIT, it cannot be excluded that SIT is more effective than ET at

improving the vasodilatory response of the muscle microvasculature to increases in insulin (meal ingestion) and exercise induced shear stress.

eNOS phosphorylation

This is the first study to report changes in microvascular eNOS ser¹¹⁷⁷ phosphorylation induced by acute exercise before and after 6 weeks ET and 6 weeks SIT. We show that pre- and post-exercise microvascular eNOS ser¹¹⁷⁷ phosphorylation was reduced by both modes of training. Shear stress in cultured endothelial cells has been suggested to be one of the most important regulators of eNOS ser¹¹⁷⁷ phosphorylation (32). Therefore, the reduction in microvascular eNOS ser¹¹⁷⁷ phosphorylation following training that we observe may potentially be the result of a reduction of the size of the shear stress stimulus. As the absolute intensity of the acute cycling exercise was the same before and after training (1 h at 65% of pretraining $\text{VO}_{2\text{peak}}$) and the muscle capillary density was higher post-training (Fig. 4.2) it is likely that blood flow per capillary will be reduced after training both at rest and during cycling exercise. Previous one leg knee extensor training studies (25) have shown that the femoral venous blood flow was lower in the trained than the non-trained leg during exercise at 65% of the $\text{VO}_{2\text{peak}}$ for the knee extensors, implying that total local muscle blood flow may be lower as well. It should be noted that the relative increase in eNOS ser¹¹⁷⁷ phosphorylation in response to acute exercise was unaltered by training (Fig. 4.2). Further studies are required to fully understand the mechanisms behind ET and SIT induced changes in eNOS phosphorylation, capillary density and skeletal muscle perfusion during exercise.

NOX2 content

ET and SIT did not lead to changes in the protein content of the membrane associated subunit of NAD(P)Hox NOX2 in the skeletal muscle microvasculature (Fig. 4.3b). As in the previous study (10) NOX2 was also observed to be present in the sarcolemma (Fig. 4.3c) and again SIT and ET did not change the protein content in the sarcolemma. Despite being sedentary, it is unlikely that the young healthy individuals in the present study exhibited high levels of NAD(P)Hox, which may explain the absence of a training effect. NAD(P)Hox is an inducible enzyme and its expression is increased by obesity (39), inflammation and cardiovascular disease (7). This suggests that any changes observed in microvascular function with training, at least in lean sedentary young men, is mediated by increases in eNOS protein content, rather than a reduction in the scavenging of NO by superoxide anions resulting from a training induced decrease in NAD(P)Hox content.

Arterial stiffness

The present study is the first to show that ET and SIT induce similar changes in central artery stiffness in young healthy sedentary males. Traditional moderate intensity ET has previously been shown to improve central arterial stiffness (13, 23). However to date, SIT studies have failed to show any training induced improvements in central artery stiffness (36, 46). This discrepancy may be due to the following factors. Firstly in the study by Whyte et al. (2010) training duration was only 2 weeks in comparison to the 6 weeks intervention employed in the current study. Secondly, Rakobowchuk et al. (2008), used a different method to measure central stiffness than those employed in the current study (cPWV vs. carotid artery distensibility) (36). A considerable variability has been observed between different

methodologies to measure central arterial stiffness (9, 19), and this may also contribute to the discrepancy between the studies.

No change was observed in our study in pPWV following either ET or SIT. This is in agreement with an earlier study that has shown that 16 weeks of ET in middle aged men had no effect on stiffness of muscular peripheral arteries of the leg (19). However, in contrast to the current study another study investigating the effects of 6 weeks SIT in young sedentary males has observed reductions in stiffness of the popliteal artery stiffness (36). Differences in arteries used (prone to develop stiffness or not) are likely to explain the differences between these studies (14).

Our study is the first to investigate the effect of SIT on AIx and compare the effects of 6 weeks ET and SIT and shows that both were equally effective in improving AIx, an assessment of systemic wave reflection and thus arterial stiffness (33). The only other study that has investigated the effect of ET was a study of Edwards et al in coronary artery disease patients, which showed that 12 weeks of ET improved AIx (15). However, to the authors' knowledge this is the first time that AIx have been measured following a period of sustained exercise training in a young healthy sedentary group and that a comparison has been made between the effects of ET and SIT.

Limitations

We decided to not include an untrained control group with repeated measurements in this study for comparison. This would have strengthened the design, but reduced the feasibility of completing the study (e.g. due to costs, time demands and recruitment difficulties). The study

was powered to detect a medium effect size between groups for muscle microvascular eNOS content and muscle microvascular density, as a result it should be noted that the sample size of the study may not have been high enough to detect between group differences in other variables displaying a larger variability (e.g. increases in $\text{VO}_{2\text{peak}}$ and insulin sensitivity). Translational studies with a larger number of participants are required before it can be concluded that SIT and ET are similarly effective in inducing adaptation in all the variables investigated in this study. We also acknowledge that the conclusions made in this study should also be restricted to previously sedentary young adult males. Finally, the results of the current study and others (24, 36) indicate that both SIT and ET improve a number of cardiovascular functions, but additional larger scale and longer term studies are required before it can be concluded that SIT and ET are equally effective in reducing cardiovascular disease risk in a variety of populations.

In conclusion this study generates novel information that 6 weeks of SIT is equally effective as 6 weeks of ET in increasing skeletal muscle microvascular density. The study also shows for the first time that ET and SIT both lead to significant increases in microvascular eNOS content, with SIT leading to a greater increase than ET. The study finally generates the novel information that the structural adaptations that occur in the muscle microvasculature in response to SIT and ET leads to a reduced eNOS ser¹¹⁷⁷ phosphorylation state both at rest and in response to 1 h of endurance exercise at 65% $\text{VO}_{2\text{peak}}$. The increase in microvascular eNOS may contribute to the increases in insulin sensitivity observed following both modes of exercise observed in this study and several earlier studies. The SIT intervention used in this study involved a maximum time commitment of 1.5 h per week, while the ET involved 5 h of exercise per week leading to the conclusion that SIT is a time efficient alternative to achieve

these effects in young previously sedentary men. Together with previous reports that SIT and ET elicit similar muscle metabolic (increases in activity of mitochondrial enzymes and aerobic capacity (8, 17)), and macrovascular adaptations (24, 36) suggests that SIT is a time efficient alternative training mode to simulate many of the known effects of ET on fitness, the macrovasculature and the metabolic function of skeletal muscle and its microvasculature.

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Chapter 5

Effect of resistance training on microvascular density and eNOS content in skeletal muscle of sedentary men

5.1. Abstract

Increases in muscle microvascular density and eNOS content are fundamental adaptations to traditional endurance training (ET) and are mechanistically linked to skeletal muscle insulin sensitivity and maximal aerobic oxygen consumption ($\text{VO}_{2\text{max}}$) during exercise. Apart from the well known effects of resistance training (RT) on muscle mass, strength and insulin action, it is currently unknown whether in sedentary males RT also increases muscle microvascular density and eNOS content. Eight previously sedentary males (age 20 ± 0.4 y, BMI 24.5 ± 0.9 kg.m⁻²) completed 6 weeks of whole body progressive RT 3 times per week. Muscle biopsies were taken from the *m. vastus lateralis* and microvascular density, eNOS content, eNOS Ser¹¹⁷⁷ phosphorylation and NOX2 content were assessed pre- and post-RT using quantitative immunofluorescence microscopy. Whole body insulin sensitivity (measured as Matsuda Index), microvascular filtration capacity (a functional measure of the total available endothelial surface area) and arterial stiffness (augmentation index and central and peripheral pulse wave velocity) were also measured pre- and post-RT. Measures of microvascular density, microvascular filtration capacity, microvascular eNOS content, basal eNOS phosphorylation and endothelial NOX2 content did not change from pre-RT to post-RT. RT increased insulin sensitivity ($P < 0.05$) and reduced resting blood pressure and augmentation index ($P < 0.05$), but did not change central or peripheral pulse wave velocity. In conclusion RT did not change any measure of microvascular structure or function, indicating that adaptations to the microvasculature do not make a major contribution to the well-known insulin sensitizing effect of RT.

5.2. Introduction

Resistance training (RT) has recently been added to the guidelines for physical activity and public health of the American College of Sports Medicine (ACSM) and the American Heart Association (19). The main reasons for these recommendations are the established effects of RT on insulin sensitivity (whole body and muscle) and muscular strength, variables that are strongly related to promotion and maintenance of independence and health throughout the lifespan (19).

The elevation in insulin-mediated glucose uptake and therefore insulin sensitivity observed following resistance training has been attributed to elevations in muscle mass and qualitative changes within the muscle (14) involving increases in GLUT 4 and key proteins in the insulin signalling cascade (22, 25, 45). However, it is becoming increasingly apparent that in addition to qualitative changes within the skeletal muscle adaptations to the muscle microvasculature also play important roles in determining insulin sensitivity (2, 3). It has long been known that traditional endurance-based training (ET) leads to increases in skeletal muscle capillary density (1), and recent research from our laboratory (11) has shown that increases in endothelial eNOS content are also part of the muscle microvascular adaptations to ET. Increases in microvascular eNOS content are important as eNOS is responsible for insulin stimulated NO production and local microvascular vasodilatation (41). These variables and eNOS ser¹¹⁷⁷ phosphorylation (main eNOS activation site; (31)) by insulin and exercise-induced shear stress are therefore important determinants of the local microvascular blood flow and the capillary surface area available for glucose uptake (2, 3, 12, 28, 35, 42). As a result, these microvascular adaptations are potentially important in the increased insulin sensitivity (15) and aerobic exercise capacity (4) seen following endurance-training,

increasing local blood flow and capillary surface area available for delivery of insulin, blood borne fuels and oxygen to the muscle.

Today no studies have investigated the effect of resistance training (RT) on muscle microvascular density and eNOS content in man to obtain insight into whether such adaptations can play a role in the RT-induced increases in insulin sensitivity. As previously explained (12) NO bioavailability in the microvasculature is determined by the balance between production of NO by eNOS (36, 37, 39), and the scavenging of NO by superoxide anions (O_2^-). A major source of superoxide production and NO scavenging in the vascular wall is NAD(P)H oxidase (NAD(P)Hox) (6, 38), but substantial expression of this enzyme is assumed to only occur in obesity, cardiovascular pathology and ageing (6, 38).

As there is a paucity of data on the effect of RT on the skeletal muscle microvasculature we first sought to determine the effects of six weeks RT on skeletal muscle microvascular density and microvascular enzyme contents (eNOS and NOX2) in previously sedentary men. We employed a novel method recently developed in our laboratory (12), to measure the protein content of these enzymes in the endothelial layer of the muscle microvasculature using quantitative immunofluorescence microscopy. Secondly, we aimed to investigate the effect of 6 weeks RT on microvascular eNOS ser¹¹⁷ phosphorylation in the resting fasted state. Finally, the effects of 6 weeks RT on arterial stiffness and blood pressure were considered to investigate earlier claims that RT may have a negative effect on arterial stiffness (30). We hypothesized that microvascular density and eNOS content would not or only minimally increase in response to 6 weeks RT, implying that different mechanisms contribute to the insulin sensitising effects of RT and ET.

5.3. Materials and Methods

The percutaneous muscle biopsies taken in this study have been used both for the measurements described in this manuscript and for measurements of the content of intramuscular triglycerides (IMTG) and perilipin-2 and perilipin-5. The latter are reported in a parallel manuscript (Shepherd SO, Cocks M, Tipton KD, Ransinghe AM, Barker TA, Burniston JG, Wagenmakers AJM and Shaw CS, unpublished data). Measures such as insulin sensitivity made in the indicated human volunteers are relevant for the interpretation of both studies and are presented in both manuscripts.

Participants

Eight healthy sedentary males participated in the study (Table 5.1). The sedentary state was defined as performing less than 1 hour per week of structured physical activity (e.g. sports club, commercial gym or exercise class). Following recruitment all participants provided written informed consent, using a protocol adhering to the Declaration of Helsinki and approved by the Black Country NHS Research Ethics Committee.

Familiarisation and 1RM

Participants were first familiarised with all the resistance training equipment and instructed on correct lifting technique. Eight motion guided resistance exercise machines (Cybex International Inc., MA, USA) targeting both the upper and lower body were used (leg press, leg extension, seated leg curls, chest press, lat pull down, shoulder press, arm curls, and arm extensions). In order to determine the initial load for the training period, 1RM was determined on all eight machines, 1RM was determined using the method of Kraemer and Fry (24). Before determination of 1 RM participants completed a warm up consisting of 6

repetitions at an estimate of 50% 1RM, 4 repetitions at approximately 70% 1RM, and 2 repetitions at 80% 1RM, with 2 min recovery between sets. Subsequently, each exercise was performed at the estimate of 1RM. Repetitions were valid if the load was lifted through the full range of motion in a controlled manner without assistance. Following this first attempt the load was increased based on the maximum number of repetitions performed for the previous load, using the following equation:

This was continued until only 1 repetition was possible, this load was determined to be 1RM. Post training 1RM was completed prior to the final training session, 1RM was determined on the leg press, chest press, leg extension and shoulder press, as a measure of training effect.

Experimental protocol

Experimental testing took place over 2 days and included measures of vascular function, insulin sensitivity (day 1) and a muscle biopsy (day 2). Pre- and post-training testing was identical in all respects and all testing procedures were conducted at least 48 hours after the last exercise bout to exclude the effects of acute exercise. On all occasions testing was performed following 24 hours standardised diet and following an overnight fast. Standard diets were matched to the participant's average daily energy intake, calculated using 3 day diet diaries. The composition of the standard diet was 50% carbohydrate, 35% fat and 15% protein.

Experimental procedures

Arterial stiffness

Supine blood pressure was measured in triplicate using an automated sphygmomanometer (Omron 7051T, Omron Corporation, Kyoto, Japan) following 15 minutes of supine rest. Pulse wave analysis was conducted using a semi-automated device and software (SphygmoCor, AtCor Medical, Sydney, Australia). Central (carotid- femoral, cPWV) and peripheral (carotid-radial, pPWV) pulse wave velocity were assessed using a semi-automated device and software (SphygmoCor, AtCor Medical, Sydney, Australia) using the protocol described in (11). Briefly, pulse transit time was calculated by concurrent measurement of the QRS complex obtained using electrocardiography and arterial waveforms collected using applanation tonometry. Distance was measure using an anthropometric measuring tape between specific anatomical locations. Tonometry measures were collected from the femoral and carotid arteries for cPWV, and radial and carotid arteries for pPWV. All measurements were made in triplicate. A series of arterial waveforms were collected from the radial artery using applanation tonometry. The brachial artery blood pressure was then used to calibrate the waveforms to create a complete cardiac cycle. Using a generalised transfer function (32) the aortic waveform was reconstructed. Augmentation index (AIx) an assessment of systemic wave reflection and thus arterial stiffness (26) was calculated from the resulting aortic waveform. To control for the influence of heart rate on AIx, AIx was normalised to a heart rate of 75 beats per minute (AIx@75) (43). All measurements were made in triplicate.

Venous occlusion plethysmography

Microvascular filtration capacity (Kf) was measured using venous occlusion plethysmography. The system and small cumulative venous congestion plethysmography

protocol is based on the previously established system developed by Gamble et al. (16, 17). The measurements were made in a quiet temperature controlled room following a supine rest of at least 30 minutes. Measurements were made with the left calf elevated to the level of the heart and a congestion pressure cuff placed around the left thigh. Changes in calf circumference were measured using a passive inductive transducer with an accuracy of ± 5 μm , in response to five 10 mmHg cumulative congestion pressure steps. The maximum pressure never exceeded the participant's diastolic pressure.

Changes in pressure in excess of the ambient venous pressure caused a rapid volume response attributable to venous filling. Following this rapid volume response a slow steady state volume change occurred, reflecting fluid filtration (J_v). Slope of the J_v response was then measured over the last 2 minutes of the pressure step to ensure vascular filling had occurred. The slope of the regression line attained by plotting J_v at each pressure represented K_f .

Oral glucose tolerance test and Matsuda insulin sensitivity index

Following the insertion of a cannula into an antecubital vein, a baseline 25 ml blood sample was taken. Participants then ingested a 25% glucose beverage containing 75g glucose made up in 300 ml of water. Further blood samples (10 ml) were collected at 30, 60, 90 and 120 minutes while participants rested, following the procedure originally proposed by Matsuda & DeFronzo (27) for the assessment of whole body insulin sensitivity. Plasma was separated by centrifugation (10 minutes at 3000 rpm) and stored at -80°C until analysis. Plasma insulin concentrations were determined by enzyme linked immuno-sorbent assay (ELISA) using a commercially available kit (Invitrogen, UK). Plasma glucose concentrations were analysed using an automated analyzer (IL ILab 650 Chemistry Analyzer, Diamond Diagnostics, USA).

Area under the curve (AUC) for insulin and glucose during the oral glucose tolerance test was calculated using the conventional trapezoid rule. The Matsuda index, a variable which is generally accepted and widely used as a non-invasive alternative to measure whole body insulin sensitivity has been shown to have a high correlation with insulin sensitivity measured with the hyperglycemic euglycemic clamp (27), which is the gold standard method. The Matsuda index was calculated using the formula:

$$\text{Matsuda index} = \frac{10000}{\sqrt{(\text{FPG} - \text{FPI}) \cdot (\text{mean OGTT insulin}) (\text{mean OGTT glucose})}}$$

Where FPG is fasting plasma glucose concentration (mg.dl⁻¹), FPI is fasting plasma insulin concentration (μU.ml⁻¹), and mean OGTT glucose and insulin is the mean plasma glucose (mg.dl⁻¹) and insulin (μU.ml⁻¹) concentration of all samples taken during the oral glucose tolerance test (0, 30, 60, 90 and 120 min).

Muscle biopsy

On day 2 following an overnight fast a muscle biopsy was taken from the lateral portion of the *m. vastus lateralis*, approximately 25-50% of the distance from the lateral joint line and the greater trochanter under local anaesthesia (1% lidocaine) using the percutaneous needle biopsy technique (5) as recently described (40). Excess blood and visible collagen or fat were removed before samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) and immediately frozen in liquid nitrogen cooled isopentane (Sigma-Aldrich, Dorset, UK). Samples were then stored at -80°C until analysis was performed.

Training

Participants trained three times per week completing a minimum of 16 sessions and maximum of 18 during the 6 weeks. Participants completed the training sessions in the School of Sport and Exercise Sciences at the University of Birmingham under the instruction of the research team. Eight motion guided resistance exercise machines (discussed above) targeting both the upper and lower body were used. During the first week participants completed 3 sets of 10-12 repetitions at 50%, 60% and finally 70% 1RM. Following the first week participants completed 2 sets of 10-12 repetitions, the third set was then performed to volitional fatigue. Loads of 80% 1RM were used. To ensure progression, load was increased by 5 lb following successful completion of 3 sets of 12 lifts.

Quantitative immunofluorescence

The immunofluorescence staining protocol and quantification has been described previously by Cocks et al. (12). Briefly, samples orientated to provide muscle fibre cross-sections were cut (5µm) and placed on glass slides. Both pre- and post-training samples within an individual were placed on the same slide. Sections were fixed in acetone and ethanol (3:1). Following fixation sections were incubated overnight with antibodies for the following: NOX2 (Santa Cruz Biotechnology, Santa Cruz, CA, gp91-phox/NOX2 (C-15), cat No. sc-5827), eNOS (Transduction laboratories, Lexington, KY, cat No. 610297) or p-eNOS ser¹¹⁷⁷ (Cell Signalling Technology Beverly, MA, p-eNOS ser¹¹⁷⁷, cat No. 9570L). Sections were then incubated with appropriately labelled secondary antibodies (Invitrogen, Paisley, UK) and the endothelial marker Ulex Europaeus-FITC conjugated (UEA-I-FITC; Sigma-Aldrich, UK). Finally sections were incubated with the cell membrane marker wheat germ agglutinin-350 (WGA-350; Invitrogen, UK).

For image capture, muscle sections were viewed using a Nikon E600 microscope using a 40x 0.75 numerical aperture objective, illuminated with a 170W Xenon light source. Images were captured using a SPOT RT KE colour three shot camera (Diagnostic Instrument Inc., MI, USA) coupled to the microscope. Camera exposure time and gain was adjusted so no pixel saturation was present in any channel and identical camera settings were used for all images within each participant.

Once captured images were analysed using Image Pro Plus 5.1 software (Media Cybernetics Inc, Bethesda, MD, USA). Endothelial specific fluorescence was determined using the UEA-I FITC (endothelial marker) image, which was extracted and overlaid onto the corresponding eNOS, p-eNOS ser¹¹⁷⁷ or NOX2 image. Cell membrane specific fluorescence for NOX2 was determined using WGA-350 as a stain to create an outline of the cell membrane. The latter was extracted and overlaid onto the corresponding NOX2 image. Fluorescence intensity of the eNOS, p-eNOS ser¹¹⁷⁷ or NOX2 signal was quantified within the endothelium or cell membrane specific area. The reader was not blinded to condition as image analysis was automated removing the potential effects of investigator bias (12). Values were normalised to pre-training values.

Capillarization

Capillaries were quantified manually using the UEA-I and WGA-350 images as markers of capillaries and muscle fibre membrane. The following indexes were measured as previously described (21): 1) number of capillaries around a fibre (capillary contacts (CC)), 2) capillary-to-fibre ratio on an individual-fibre basis (C/F_1), the number of capillaries sharing a fibre (sharing factor (SF)), 4) capillary density (CD) and 5) capillary-fibre-perimeter exchange

(CFPE) index. Fibre cross sectional area and perimeter were measured using ImagePro Plus 5.1.

Statistics

Statistical analyses were performed using SPSS for windows version 16.0 (SPSS, Chicago, IL)). All variables were analysed using paired samples t-tests for comparison. Significance was set at $P \leq 0.05$. Data is presented as means \pm S.E.M. The primary aim of the study was to compare the effects of RT on muscle microvascular eNOS content. G*Power 3.1 software (G*Power Software Inc., Kiel, Germany) was used to calculate the required sample size. The study was designed to detect an effect size of $d_z=0.98$, representative of a large sized effect (13) adopting an alpha of 0.05 and power of 0.80. We deemed a d_z of 0.98 to be a physiologically relevant difference as we previously observed an effect of this size following 6 weeks of ET and sprint interval training (SIT) in sedentary individuals.

5.4. Results

Training effects

Participants as expected exhibited significant gains in strength following RT. 1RM increased by 43%, 33%, 32% and 38% for the leg press, leg extension, chest press and shoulder press, respectively (all variables $P < 0.05$; Table 5.1). Resting heart rate was unchanged by training ($P = 0.119$; Table 5.1). Brachial mean arterial pressure (MAP), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were all reduced following training ($P < 0.05$; Table 5.1).

Insulin sensitivity

The Matsuda insulin sensitivity index was significantly increased by 31% following RT ($P < 0.05$; Table 5.1). Both glucose and insulin area under the curve (AUC) were reduced following training ($P < 0.05$; Table 5.1).

Microvascular enzymes

Skeletal muscle endothelial specific eNOS content and ser¹¹⁷⁷ phosphorylation was not significantly different between pre- and post-RT (eNOS content: $P = 0.091$, Fig. 5.1; eNOS ser¹¹⁷⁷ phosphorylation: $P = 0.075$; Fig. 5.2). Skeletal muscle endothelial specific and membrane specific NOX2 content also was not changed by RT (endothelial specific: $P = 0.319$; membrane specific $P = 0.164$; Fig. 5.3).

Filtration capacity and capillarization

K_f was unaltered by training ($P = 0.333$; Fig. 5.4). Fibre cross sectional area and perimeter were not changed by RT in either type I or II fibres (fibre cross sectional area: $P = 0.827$; perimeter: $P = 0.625$). Sharing factor was not changed by RT ($P = 0.408$) and no significant

difference was found for capillary density ($P = 0.715$). Type I fibres had significantly higher capillary contacts, capillary-to-fibre ratio on an individual-fibre basis and capillary-fibre-perimeter exchange than type II fibres both before and after training ($P < 0.05$), but no significant differences were found in these variables as a result of RT (CC: $P = 0.716$; C/F_I: $P = 0.598$; CFPE, $P = 0.654$; Table 5.2).

Arterial stiffness

AIx@75bpm was significantly decreased following training ($P < 0.05$; Fig. 5.5a). However, neither cPWV nor pPWV were affected by training (cPWV $P = 0.934$, pPWV $P = 0.708$; Fig. 5.5b and c).

Table 5.1. Subject characteristics, insulin sensitivity and hemodynamics pre and post 6 weeks of resistance training.

Variable	Pre training	Post training
Age (yr)	20±1	-
Height (cm)	172±3	-
Weight (Kg)	72.6±2.4	71.1±2.2
BMI (kg.m ⁻²)	24.4±0.9	24.2±0.8
ISI Matsuda	2.8±0.2	3.7±0.3*
Glucose AUC (mmol.L ⁻¹ .120min ⁻¹)	923.7±64.6	843.9±73.1*
Insulin AUC (mmol.L ⁻¹ .120min ⁻¹)	8914.4±513.1	7308.1±652.5*
Resting heart rate (bpm)	67±4	63±2
Mean arterial pressure (mmHg)	81±2	75±1*
Systolic blood pressure (mmHg)	115±2	109±3*
Diastolic blood pressure (mmHg)	64±2	57±2*
Leg press 1RM (lb)	427 ± 21	612 ± 34*
Chest press 1RM (lb)	119 ± 6	157 ± 7*
Leg extension 1RM (lb)	263 ± 10	349 ± 12*
Shoulder press 1RM (lb)	103 ± 4	142 ± 5*

Values are means ± S.E.M. * $P < 0.05$. 1RM, 1 repetition maximum

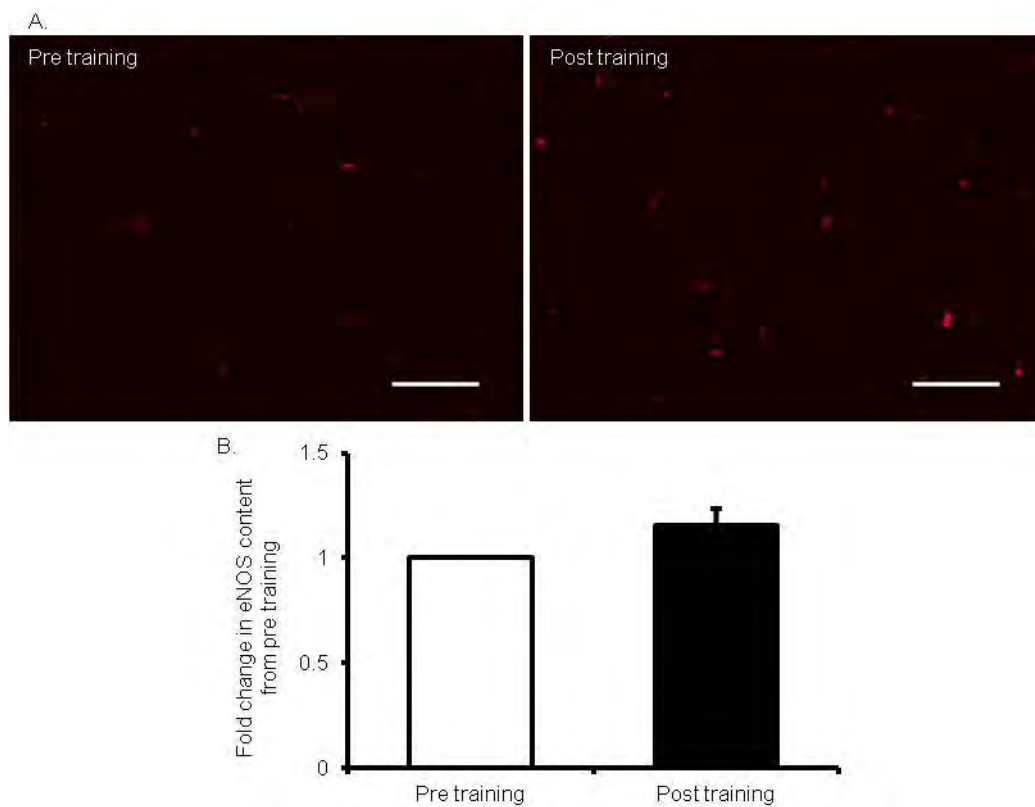


Figure 5.1. Effect of resistance training (RT) on eNOS content.

A. Widefield microscopy images of skeletal muscle pre- (left) and post-RT (right). Skeletal muscle eNOS expression was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 50 μ m. B Mean fluorescence intensity of eNOS is indicated. The mean level of eNOS pre-training was assigned a value of 1, and the relative intensity of eNOS post-training was calculated.

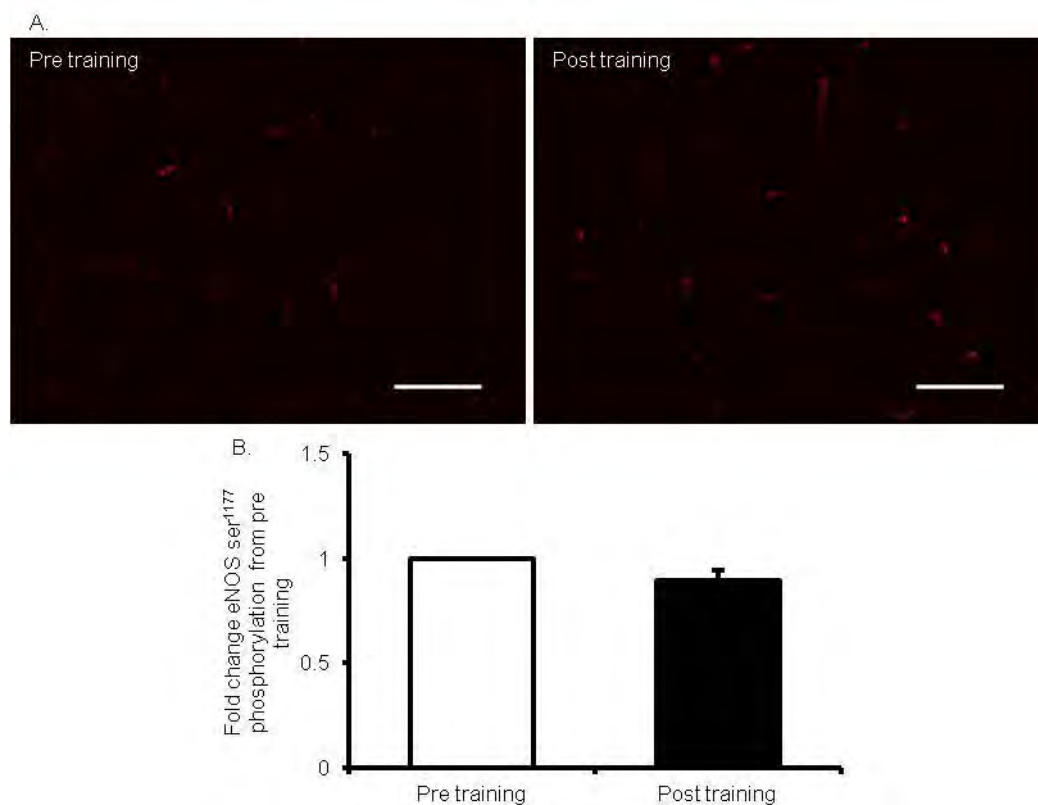


Figure 5.2. Effect of resistance training (RT) on basal eNOS serine¹¹⁷⁷ phosphorylation.

A. Widefield microscopy images of skeletal muscle pre- (left) and post-RT (right). Skeletal muscle eNOS serine¹¹⁷⁷ phosphorylation was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 5 μ m. B Mean fluorescence intensity of eNOS serine¹¹⁷⁷ is indicated. The mean level of eNOS serine¹¹⁷⁷ pre-training was assigned a value of 1, and the relative intensity of eNOS serine¹¹⁷⁷ post training was calculated.

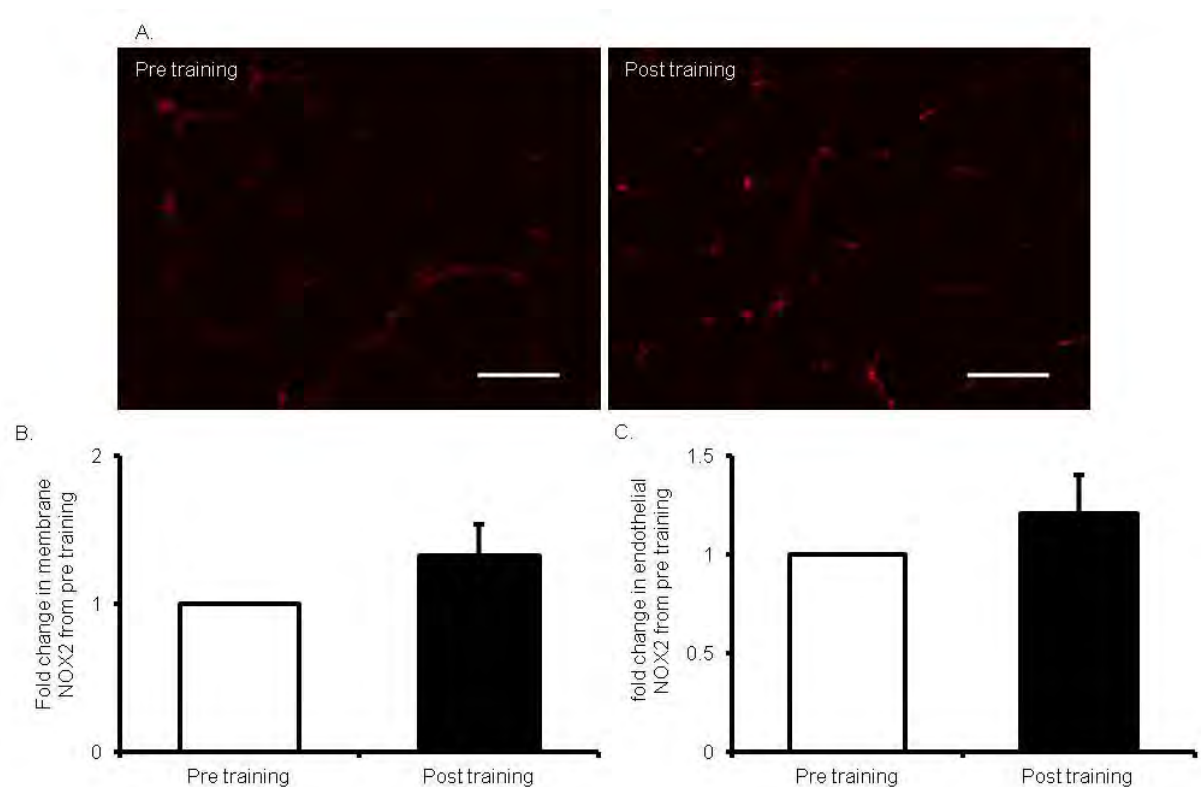


Figure 5.3. Effects of resistance training (RT) on NOX2 content.

A. Widefield microscopy images of skeletal muscle pre- (left) and post-RT (right). Skeletal muscle NOX2 content was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 50 μ m. B Mean fluorescence intensity of NOX2 within the muscle membrane is summarized. C Mean fluorescence intensity of NOX2 within the endothelium is indicated. The mean level of NOX2 pre-training was assigned a value of 1, and the relative intensity of NOX2 post-training was calculated.

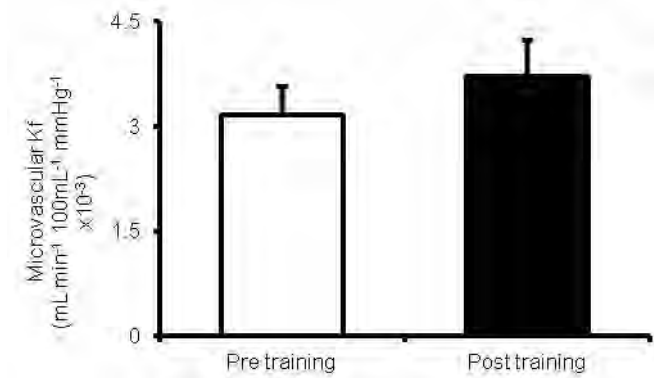


Figure 5.4. Effect of resistance training on calf microvascular filtration capacity (K_f).

Calf microvascular K_f measured using venous occlusion plethysmography. Fluid filtration (J_v) was measured over the final 2 minutes of 5x 5 minute cumulative pressure steps (10mmHg). J_v was then plotted against pressure and the resulting slope of the line gave a measure of K_f . K_f is a measure proportional to the capillary surface area available for exchange.

Table 5.2. Capillarization pre and post resistance training.

Variable	Pre training	Post training
Overall FA (mm ²)	3694±206	3713±258
Type I FA (mm ²)	3473±232	3674±341
Type II FA (mm ²)	3761±263	3681±253
Overall Perimeter (mm ²)	250.3±7.5	251.3±8.0
Type I Perimeter (mm ²)	243.1±7.6	253.2±11.1
Type II Perimeter (mm ²)	252.7±9.4	250.2±7.9
Overall CC	4.21±0.29	4.27±0.19
Type I CC	4.61±0.28	4.73±0.20
Type II CC	4.02±0.33	4.08±0.19
Overall C/F _I	1.53±0.13	1.56±0.09
Type I C/F _I	1.68±0.13	1.77±0.10
Type II C/F _I	1.45±0.14	1.48±0.09
Overall CFPE	6.07±0.42	6.23±0.29
Type I CFPE	7.03±0.54	7.03±0.28
Type II CFPE	5.66±0.43	5.92±0.26
SF	2.82±0.05	2.80±0.03
CD (caps/ mm ²)	701.4±42.4	713.0±40.6

Values are means ± S.E.M. FA, fiber cross sectional area; SF, sharing factor; CD, capillary density; CC, capillary contacts; C/F_I, capillary-to-fibre ratio on an individual-fibre basis; CFPE, capillary-fibre-perimeter exchange.

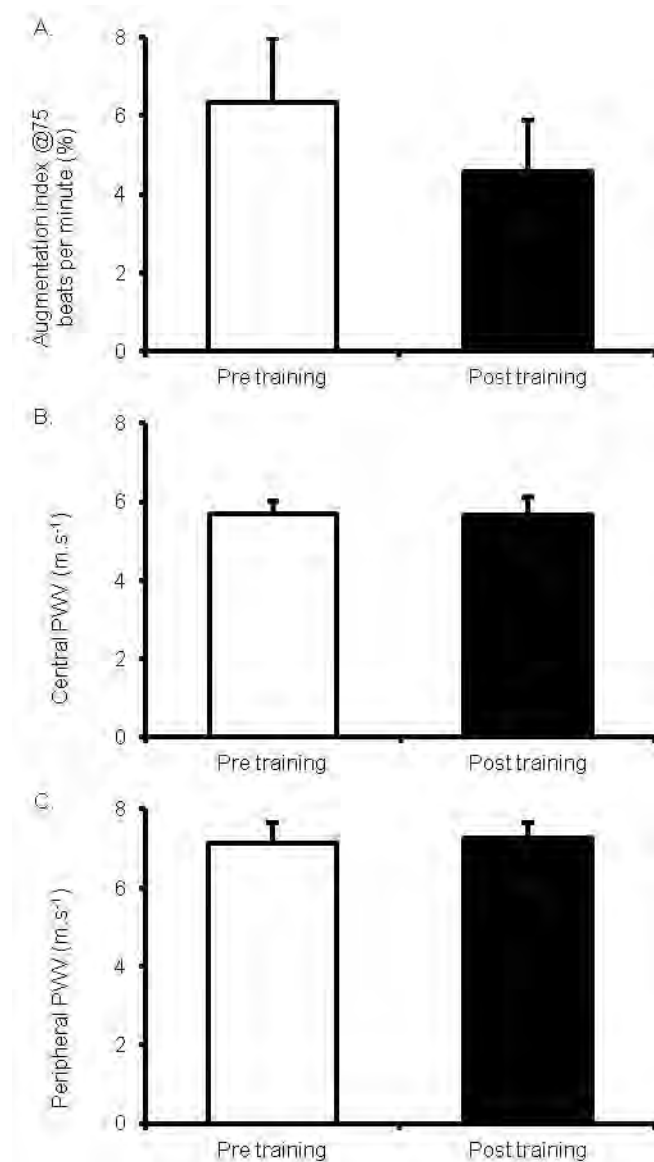


Figure 5.5. Effect of resistance training (RT) on augmentation index and central and peripheral artery stiffness.

A. systemic stiffness measured through augmentation index normalized to 75 bpm following RT. B. Central artery (aortic) stiffness measured using pulse wave velocity (PWV) following RT. C. Peripheral artery (brachial artery) stiffness measured using pulse wave velocity following RT. * $P < 0.05$.

5.5. Discussion

The most important findings of the present study are that: 1) 6 weeks of RT in young previously sedentary males did not increase skeletal muscle capillarization, 2) RT did not increase eNOS protein content of the muscle microvascular endothelium, 3) Basal eNOS ser¹¹⁷⁷ phosphorylation was unchanged by RT, 4) As expected RT did not induce a change in the protein content of NOX2, 5) despite the lack of microvascular adaptations RT increased insulin sensitivity by 32% ($P < 0.05$). Finally our results contrast previous suggestions that RT increases central arterial stiffness (30) and show that AIx a measure of systemic stiffness was reduced by 28% ($P < 0.05$) following RT. Our results suggest that increases in skeletal muscle capillary density and/or microvascular eNOS content are not the primary drivers for the elevations in insulin sensitivity observed following 6 weeks RT in young previously sedentary males.

Insulin sensitivity, capillary density and eNOS content

Results from previous studies investigating the effect of RT on capillary density have produced similar results to the current study suggesting that RT is not effective at increasing capillary density, however unlike the present study RT has previously been shown to increase the number of capillaries per fibre (18, 29). This rise in number of capillaries following RT was suggested to be proportional to fibre growth, so that no reduction in diffusion distances would occur in the enlarged fibres. The lack of change in number of capillaries per fibre in the current study might be explained by the fact that the 6 weeks RT intervention did not lead to significant muscle fibre hypertrophy in the sampled muscle. Further confirmation of the absence of an increase in capillary density is offered by the absence of a change in microvascular filtration capacity, a functional measure of capillary surface area available for

diffusion of plasma water. K_f was previously shown to correlate with capillary density (9). This is the first study to investigate the effects of RT on K_f , but results support previous findings in strength trained athletes showing no elevation in K_f in this group compared to sedentary individuals (7).

It has been suggested that capillary density (23) and microvascular K_f (10) are key determinants of insulin sensitivity due to their role in capillary surface area available for exchange of insulin and glucose, thus regulating insulin and glucose delivery to the muscle. However as neither capillary density nor K_f were increased following RT it appears that improvements in delivery of insulin and glucose to the muscle through increased surface area do not play a role in the RT induced elevation in insulin sensitivity observed in the current study and previous studies.

Key to insulin-induced increases in microvascular blood flow is an increase in NO production by eNOS (41). Increases in eNOS content in response to training interventions as we previously observed following 6 weeks of ET in previously sedentary individuals (11), therefore, may contribute to the increase in insulin sensitivity that is seen after ET. However, RT did not lead to increases in muscle microvascular eNOS content, implying that such increases cannot be responsible for the increased insulin sensitivity observed following RT. It cannot be ruled out though that other adaptations of the microvasculature, such as increased insulin-induced eNOS phosphorylation or reduced NO quenching by reactive oxygen species may cause increases in insulin mediated NO bioavailability.

Previous research by our group and others has suggested that ET is effective at increasing microvascular density ((1, 11)) and eNOS content (11)). These results suggest that the mechanisms leading to improved insulin sensitivity are different between ET and RT, with microvascular adaptations playing a greater role in the response to ET than RT. We, therefore, suggest that increases in muscle mass leading to an increased capacity to clear glucose and a higher protein expression of intermediates of the insulin signalling cascade which has been observed before (22, 25, 45) contribute to increase in insulin sensitivity following RT. It is also possible that the ability of insulin to activate the insulin signalling cascade in skeletal muscle is increased via a mechanism that is not currently known. As such, a combination of ET and RT may prove to be the most effective training approach as it may increase muscle insulin sensitivity via multiple mechanisms, but also increase muscle mass and strength, which is particularly relevant to maintain an independent lifestyle in the rapidly growing ageing population.

Although significance was not reached there was a trend for an increase in eNOS content ($P = 0.091$). Although this may suggest that the study was underpowered a previous study investigating the changes in microvascular eNOS content following ET and SIT using the same criteria and number of individuals did produce a significant increase (11).

eNOS phosphorylation

RT did not change basal eNOS ser¹¹⁷⁷ phosphorylation, although there was a trend towards a decrease ($P = 0.075$). In **chapter 4** (11) a reduction in eNOS ser¹¹⁷⁷ phosphorylation was observed in sedentary individuals following 6 weeks ET and SIT, which we attributed to a reduction in shear stress mediated by a training-induced increase in capillary density. As there

is no change in capillary density following RT, the absence of an effect on eNOS ser¹¹⁷⁷ phosphorylation is as expected.

NOX2

No change was seen in the protein content of the membrane bound subunit of the NAD(P)Hox complex NOX2 in either the skeletal muscle sarcolemma or in the muscle microvascular endothelium. The lack of response of NOX2 to RT in present study either seems to be the result of the nature of the exercise stimulus, or a consequence of the young healthy nature of the participants, who despite being sedentary are unlikely to have elevated levels of NAD(P)Hox before the intervention. In **chapter 4** (11) we also did not find a change in NAD(P)Hox following 6 weeks sprint interval (SIT) and ET, while 4 weeks SIT did reduce NAD(P)Hox in sedentary obese individuals (**chapter 6**).

Arterial stiffness

In contrast to a recent meta-analysis which suggested that high intensity RT is associated with increased central artery stiffness (30) the current study showed no change in aortic or peripheral artery stiffness following high intensity RT. The discrepancy between studies is likely the result of different RT protocols performed (concentric lifting protocols, high volume and high intensity RT versus eccentric lifting protocols). Studies using similar high intensity RT protocols to the current study also did not observe a change in arterial stiffness (8, 33, 34). This suggests that when ACSM guidelines for high intensity RT are followed arterial stiffness is not adversely affected; however, unlike exercise modes such as ET and HIT, RT does not have a beneficial effect on arterial stiffness.

Unlike local artery stiffness (aorta and brachial artery), augmentation index (AIx) was reduced following training. AIx is a measure of the contribution wave reflections make to the arterial pressure waveform (44). As such, AIx is a measure of systemic stiffness as the amplitude and timing of wave reflections depend on small and large arteries. Systemic stiffness is an important measure as it partly determines left ventricular workload, and therefore is of significant clinical importance (44). The current finding however is in contrast with two previous studies investigating RTs effect on systemic stiffness in young healthy participants, which suggested that RT has no effect on systemic stiffness (8, 20). An explanation for these differences is unclear as the current study uses the same protocol for measuring AIx and a similar RT protocol (8, 20). Because of the potential clinical implications more detailed future studies are required in young healthy adults, elderly individuals and patient populations to solve this discrepancy.

In conclusion this study generates novel information that 6 weeks RT in previously sedentary young males does not increase microvascular density or eNOS content and ser¹¹⁷⁷ phosphorylation state. Despite the lack of microvascular adaptations insulin sensitivity was increased by RT suggesting that microvascular adaptations are not responsible for the improvement in insulin sensitivity. Together with previous reports investigating the effect of ET (11) and RT (22, 25, 45), we suggest that the combination of both training modes may lead to optimal metabolic and health benefits, as different mechanisms appear to contribute to the increase in insulin sensitivity. ET in addition will be a powerful means to increase muscle oxidative capacity, capillarity and microvascular responsiveness, while RT has unique effects on muscle mass and strength.

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Chapter 6

Effect of Constant Workload Sprint Interval Training on Muscle Microvascular Density and eNOS Content in Sedentary Obese Men: a Pilot Study

6.1 Abstract

Previous research has shown that structural and functional impairments in the muscle microvasculature play an important role in the mechanisms by which obesity leads to endothelial function loss, characterised by reductions in NO bioavailability and insulin induced microvascular vasodilation. Sprint interval training (SIT) has recently been proposed as a time efficient alternative to traditional endurance based training, leading to similar increases in muscle microvascular density and larger increases in microvascular endothelial eNOS content in young previously sedentary individuals. In this pilot study, we aimed to investigate whether SIT has the same effects on the muscle microvasculature in obese males, and whether these changes translate into improved aerobic capacity and whole body insulin sensitivity as previously observed in sedentary young men. Six young obese males (age 23 ± 3 y, BMI 35.5 ± 1.1 kg.m⁻²) volunteered to participate. Participants performed 4-7 intervals (constant workload of 200% Watt_{max} with 2 minutes active recovery between intervals) 3 times per week for 4 weeks. Biopsies were taken before and after training from the *m. vastus lateralis* for quantitative immunofluorescence microscopy measurements of microvascular density and content and ser¹¹⁷⁷ phosphorylation state of eNOS and NAD(P)H oxidase (NOX2), in addition to a series of relevant functional measurements. SIT significantly ($P < 0.05$) increased eNOS protein content of skeletal muscle arterioles (10%) and capillaries (13%). SIT also increased basal ser¹¹⁷⁷ phosphorylation (arterioles 15% and capillaries 7%, both $P < 0.05$) and reduced skeletal muscle microvascular NOX2 content (15%; $P < 0.05$). SIT did not change microvascular density, but led to a significant increase in microvascular filtration capacity (18%; $P < 0.05$), a functional measure of the accessible capillary surface area. SIT in parallel significantly ($P < 0.05$) increased maximal aerobic capacity (VO_{2max}) (9%), whole body insulin sensitivity (18%) and reduced arterial stiffness (11%). Finally, SIT significantly

reduced fasting plasma free fatty acids. In conclusion, this pilot study in obese individuals shows that 4 weeks of constant workload SIT is an effective intervention to improve the content of endothelial enzymes controlling NO bioavailability and these molecular adaptations are paralleled by increases in whole body insulin sensitivity, microvascular filtration capacity and VO_{2max} , while reducing cardiovascular risk factors. All these changes are consistent with potential long-term health benefits of SIT in this high risk group.

6.2 Introduction

Obesity has become a global epidemic with 200 million men and 300 million women over 20 (1) classified as obese worldwide (Body mass index (BMI) > 30 kg.m⁻²) (32). The rapid increase in obesity prevalence is regarded to be instrumental to the worldwide increase in the prevalence of cardiovascular disease and metabolic syndrome (1) and the obesity epidemic, therefore is currently regarded as a major economic, social and health burden. If recent trends continue the number of obese adults is expected to grow to 1.12 billion by 2030 (32).

A growing body of literature suggests that reductions in muscle microvascular density in obese individuals and animal models (24) in combination with impairments in the vasodilatory responsiveness of the muscle microvasculature to physiological stimuli (insulin, VEGF and exercise-induced shear stress) are instrumental to the development of functional impairments and in the longer term chronic diseases in the obese population (4-6, 15, 20, 21, 52). Suggestions have also been made that microvascular impairments may be an early and important factor in the later development of macrovascular function loss (33).

It is well established that the insulin dependent dilation of the muscle microvasculature controls glucose uptake in skeletal muscle and depends on adequate increases in nitric oxide (NO) bioavailability (22, 39). The latter is determined by the balance between insulin induced increases in NO synthesis and in obesity (see next paragraph) scavenging of NO by superoxide anions and related reactive oxygen species.

Experiments with isolated arteries and cultured endothelial cells have shown that the rate limiting enzyme for endothelial NO synthesis is endothelial nitric oxide synthase (eNOS). The protein content and serine¹¹⁷⁷ phosphorylation state together determine total eNOS

activity and endothelial NO production (42). A major source of superoxide anion production and NO scavenging in the vascular wall is NAD(P)H oxidase (NAD(P)Hox) (9, 46), and substantial expression of this enzyme is assumed to occur in obesity and cardiovascular pathology (9, 46). Because of the lack of a method to measure the protein expression of these enzymes in the muscle microvascular endothelium it is currently unknown if there is an imbalance between eNOS protein expression and phosphorylation and NAD(P)Hox protein expression in obese individuals.

Hard evidence exists that traditional endurance-based training (ET) delays or prevents the onset of obesity related chronic diseases (34). However, the majority of the adult population does not meet the current recommendations to perform a minimum of 150 minutes of moderate intensity endurance exercise per week to thus sustain metabolic fitness and health (27). 'Lack of time' is cited as the major reason for the wide-spread failure to adhere to this exercise recommendation (50). In an earlier study (17) we showed that 6 weeks SIT using repeated Wingate's ("all out" cycling for 30 seconds) was more effective than traditional ET to increase muscle microvascular eNOS content and equally effective at increasing muscle microvascular density. We, therefore, decided to perform the current pilot study to investigate whether SIT might also be a time efficient alternative to ET to improve microvascular enzyme expression and function in obese individuals and whether this leads to parallel metabolic and functional adaptations that in the long-term might reduce the risk for development of cardiovascular disease and metabolic syndrome.

Previous studies (11, 17, 44) investigating SIT have used "all out" cycling, in the form of repeated 30s Wingate tests. However, this method of training is very demanding and requires

high levels of motivation to deliver maximal effort in each session and over prolonged intervention periods. In addition it also requires specialised cycle ergometers and may therefore not be a practical training method for the majority of the obese population. These criticisms have led others to develop high intensity interval (HIT) protocols which use constant loads (36, 49), as these have been suggested to remove the demanding character of “all out” SIT. As such, in the present study we have developed a SIT protocol designed to maintain the anaerobic nature of “all out” SIT whilst utilizing the benefits of constant workload HIT.

The main aims of the current study were twofold. First, we sought to determine whether 4 weeks constant workload SIT increased skeletal muscle microvascular density and microvascular filtration capacity, a functional measure of the accessible capillary surface area in muscle, in previously sedentary obese males. Secondly, we aim to investigate the effects of constant workload SIT on skeletal muscle microvascular endothelial enzyme content and phosphorylation (eNOS content and ser¹¹⁷⁷ phosphorylation and NOX2 content). As in previous chapters we hypothesise that these microvascular adaptations lead to parallel increases in aerobic capacity and insulin sensitivity. In addition this is the first study to investigate whether cardiovascular risk factors are reduced by SIT. We employed quantitative immunofluorescence microscopy, a recently developed technique to assess protein content and phosphorylation of the indicated enzymes within the endothelial layer of the skeletal muscle microvasculature. The method was modified from previous studies (17, 18) to allow for separation of arterioles and capillaries (eNOS and eNOS ser¹¹⁷⁷ phosphorylation only), as it is terminal arterioles that control capillary perfusion. Finally, the effects of constant workload SIT on arterial stiffness and blood pressure were also investigated. We hypothesise

that microvascular density and function would increase in response to constant workload SIT, that eNOS content would be increased in both arterioles and capillaries following training and that NOX2 content would be reduced. Finally we hypothesise that the improvements in microvascular function and structure would be paralleled by improvements in aerobic capacity and insulin sensitivity and a reduction in cardiovascular risk factors.

6.3 Materials and methods

The percutaneous muscle biopsies taken in this study have been used both for the measurements described in this manuscript and for measurements of the content of intramuscular triglycerides (IMTG), perilipin-2 and perilipin-5. The latter are reported in a parallel thesis (Shepherd SO, University of Birmingham, 2012). Measures such as $\text{VO}_{2\text{peak}}$, insulin sensitivity and the cardiovascular risk factors are relevant for the interpretation of both studies and are presented in both theses.

Participants

Six young sedentary obese males, defined by a BMI above 30 kg.m^{-2} and currently participating in less than 1 hour structured physical activity per week completed the study (Table 1). Participants were free of cardiovascular and metabolic disease and other contraindications to participate in exercise training interventions, ascertained through a medical screening process. All participants gave written informed consent to a protocol adhering to the *Declaration of Helsinki* and approved by the Black Country NHS Research Ethics Committee.

Pre training testing protocol

Participants first completed an incremental exercise test to exhaustion on an electromagnetically braked cycle ergometer to determine Watt max (W_{max}) and $\text{VO}_{2\text{peak}}$ (as described in **chapter 4**). Following sufficient rest participants were familiarised to the SIT protocol, for this participants completed 1x30s repetition of the SIT protocol ($200\% W_{\text{max}}$).

3-7 days after the incremental exercise test and following an overnight fast, vascular functions were assessed (blood pressure, arterial stiffness, microvascular filtration capacity), these were followed by a resting muscle biopsy, oral glucose tolerance test (OGTT) and finally body composition assessment using DXA.

Post training procedures

Post-training $\text{VO}_{2\text{peak}}$ testing was performed the day before the final training session. Approximately 48 hours after the final training session the post-training testing protocol was conducted with procedures, methods and timings identical in all respects to the pre-training testing protocol.

Arterial stiffness

Supine blood pressure was measured using an automated sphygmomanometer (Omron 7051T, Omron Corporation, Kyoto, Japan) following 15 minutes of supine rest. Systemic wave reflection was then investigated using pulse wave analysis conducted using a semi automated device and software (SphygmoCor, AtCor Medical, Sydney, Australia), using this augmentation index (AIx) was calculated as described previously (**Chapter 4**). To control for the influence of heart rate on AIx, AIx was normalised to a heart rate of 75 beats per minute (AIx@75) (53). Central (carotid- femoral, cPWV) and peripheral (carotid- radial, pPWV) artery stiffness were investigated by pulse wave velocity, assessed using a semi automated device and software (SphygmoCor, AtCor Medical, Sydney, Australia) as previously described (**Chapter 4**). All measurements were made in triplicate.

Venous occlusion plethysmography

Microvascular filtration capacity (K_f) was measured through venous occlusion plethysmography using the principles described previously (**Chapter 4**). However the method was adapted to use a mercury-in-silastic strain gauge and semi automated inflation pump (Hokanson, Inc). Strain gauge and pressure cuff signal were sampled at 1000Hz and stored for offline assessment of K_f .

Muscle biopsy

A resting muscle biopsy was taken from the lateral portion of the *m. vastus lateralis* using the percutaneous needle biopsy technique under local anaesthetic (1% lidocaine) as recently described (48). Excess blood and visible collagen or fat were removed before samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) and immediately frozen in liquid nitrogen cooled isopentane (Sigma-Aldrich, Dorset, UK). Samples were then stored at -80°C until analysis was performed.

Oral glucose tolerance test and Matsuda insulin sensitivity index

Following the insertion of a cannula into an antecubital vein, a resting 25ml blood sample was taken; participants then ingested a 25% glucose beverage containing 75g glucose made up in 300ml of water. Further blood samples were collected at 30, 60, 90 and 120 minutes while participants rested. Plasma samples were separated by centrifugation (10 minutes at 1600g) and stored at -80°C until analysis. Plasma insulin concentrations were determined by enzyme linked immuno-sorbent assay (ELISA) using a commercially available kit (Invitrogen, UK). Plasma glucose concentrations were analysed using an automated analyzer (IL ILab 650 Chemistry Analyzer, Diamond Diagnostics, USA).

Area under the curve (AUC) for insulin and glucose during the oral glucose tolerance test was calculated using the conventional trapezoid rule. The Matsuda index, a variable which is generally accepted and widely used as a non-invasive measure of whole body insulin sensitivity was calculated using the formula:

$$\text{Matsuda ISI} = \frac{10000}{\sqrt{(\text{FPG} \cdot \text{FPI}) \cdot (\text{mean OGTT insulin}) \cdot (\text{mean OGTT glucose})}}$$

Where FPG is fasting plasma glucose concentration ($\text{mg} \cdot \text{dl}^{-1}$), FPI is fasting plasma insulin concentration ($\mu\text{U} \cdot \text{ml}^{-1}$), and mean OGTT glucose and insulin is the mean plasma glucose ($\text{mg} \cdot \text{dl}^{-1}$) and insulin ($\mu\text{U} \cdot \text{ml}^{-1}$) concentration of all samples taken during the oral glucose tolerance test (0, 30, 60, 90 and 120 min).

Body composition

Percentage body fat was assessed by dual-energy X-ray absorptiometry (DXA-Hologic Discovery W with Hologic QDR software for windows XP version 12.4.2).

Dietary control

24 hours prior to both the pre and post training experimental trial participant's diets were standardised. Diets were matched to participants average daily energy intake based on 3 day diet diaries analysed using a computer system (WISP) and composed of 50% carbohydrate, 35% fat and 15% protein.

Training

Training was initiated ~48 hours after the muscle biopsy. Following a 2 minute warm up at 50W participants performed repeated 30s high intensity cycling bouts at a workload

corresponding to 200% W_{max}. High intensity bouts were interspersed with 120s of cycling at 30W for recovery. Participants completed 4 intervals for the first 3 sessions; this was then increased by 1 repetition every 3 sessions, participants did 12 sessions in total, completing 7 intervals during the final training session.

We chose a workload corresponding to 200% W_{max} as previous unpublished work by ourselves showed that Wingate based SIT elicited a mean power output of equivalent to 200% W_{max} as determined by progressive exercise test to exhaustion. Thus, to closely match the mean workload of Wingate based SIT we suggested 200% W_{max}. We believe the alternative SIT protocol developed for this study maintains both the anaerobic nature and the time saving benefits of Wingate based SIT whilst avoiding the demanding nature of Wingate based SIT as discussed earlier.

Cardiovascular risk profile

An automatic analyzer (IL ILab 650 Chemistry Analyzer, Diamond Diagnostics, USA) was used to determine non-esterified fatty acid (NEFA), triglyceride and total cholesterol concentrations using commercially available kits (NEFA: Randox Laboratories Ltd, Co. Antrim, UK, triglyceride and cholesterol: Instrumentation Laboratory Ltd UK, Warrington, UK).

Immunofluorescence

Quantitative immunofluorescence

NOX2 content in the skeletal muscle microvascular endothelium and membrane was assessed using the immunofluorescence staining protocol and quantification technique described

previously (**Chapter 2**). The immunofluorescence staining protocol and quantification technique used for eNOS content and eNOS ser¹¹⁷⁷ phosphorylation has been adapted from the method previously described in **chapter 2** to allow for differentiation between skeletal muscle capillaries and arterioles.

Serial 5µm transverse sections were cut and collected on to room temperature uncoated glass slides. Pre and post training samples from each participant were placed on the same slide, and slides were analysed in duplicate.

Sections were fixed in acetone and ethanol (3:1). Sections were then incubated with antibodies against either eNOS (Transduction Laboratories, Lexington, KY), p-eNOS ser¹¹⁷⁷ (Cell Signalling Technology, Beverly, MA) in combination with anti-α smooth muscle actin (SMA; abcam, Cambridge, UK) as a marker to differentiate between arterioles and capillaries. Sections were then incubated with appropriately labelled secondary antibodies (Invitrogen, Paisley, UK), in combination with Ulex Europaeus-FITC conjugated (UEA-I-FITC; Sigma-Aldrich, UK) as a marker of the endothelium. Following staining coverslips were applied using a glycerol and mowiol 4-88 solution.

Images were acquired with an inverted confocal microscope (Leica DMIRE2, Leica Microsystems) with a 40x oil immersion objective. FITC fluorescence was excited with a 488 nm line of the argon laser and detected with 500-570 nm emission. Alexa fluor 546 and 633 fluorophore were excited with 543 nm and 633 nm lines of the Helium-Neon laser and 550-650 nm and 650-730 nm emission, respectively. Identical settings were used for all image capture within each participant.

Image analysis was performed using Image Pro Plus 5.1 software. Blood vessels were divided into either capillaries or arterioles using the α SMA image. The endothelial (UEA-I-FITC) outline was overlaid onto the corresponding eNOS or p-eNOS ser¹¹⁷⁷ image. Fluorescence intensity of the eNOS or p-eNOS ser¹¹⁷⁷ signal was quantified within the endothelial specific area. Arteriole diameter was also determined on calibrated images using Image Pro Plus 5.1 software.

Capillarization

Muscle sections were incubated with anti-myosin type I (developed by Dr Blau DSHB) followed by goat anti-mouse IgM 350 (Invitrogen, Paisley, UK) to identify type I muscle fibres. This was performed in combination with UEA-I-FITC (Sigma-Aldrich, UK) and wheat germ agglutinin-350 (WGA-350; Invitrogen, UK) as markers of the endothelium and plasma membrane, respectively.

For analysis, slides were viewed using a Nikon E600 microscope using a 40x 0.75 numerical aperture objective. Images were captured using a SPOT RT KE colour three shot camera (Diagnostic Instrument Inc., MI, USA).

Capillaries were quantified in a fibre type specific manner manually using the UEA-I, WGA-350 and myosin heavy chain images. The following indexes were measured (30): 1) number of capillaries around a fibre (capillary contacts (CC)), 2) capillary-to-fibre ratio on an individual-fibre basis (C/F_I), the number of capillaries sharing a fibre (sharing factor (SF)), 4) capillary density (CD) and 5) capillary-fibre-perimeter exchange (CFPE) index. Fibre cross sectional area and perimeter were measured using ImagePro Plus 5.1 software.

Statistics

Capillary contacts, capillary-to-fibre ratio on an individual-fibre basis, capillary-fibre-perimeter exchange, fibre cross sectional area and perimeter were analyzed using a factorial ANOVA, with the factors 'training' (pre versus post) and 'fiber type' (type I versus type II). eNOS content and eNOS ser¹¹⁷⁷ phosphorylation in capillaries and arterioles were also analyzed using a factorial ANOVA, with the factors 'training' (pre versus post) and 'vessel type' (capillary versus arteriole). All other analysis was done using a paired samples *t*-test. All analyses were performed using statistical analysis software (SPSS for windows version 16.0 (SPSS, Chicago, IL). Significance was set at $P \leq 0.05$. Data is presented as means \pm S.E.M.

6.4 Results

Training effect

$\text{VO}_{2\text{peak}}$ and W_{max} increased by 9% and 12%, respectively following training ($P < 0.05$), but there was no change in body weight ($P = 0.846$), BMI ($P = 0.811$) or percentage body fat ($P = 0.190$) following training. Resting heart rate was unchanged by training ($P = 0.242$), as were brachial artery MAP ($P = 0.656$), systolic blood pressure ($P = 0.427$) and diastolic blood pressure ($P = 0.290$; Table 1).

Insulin sensitivity

Both glucose and insulin AUC were reduced by training ($P < 0.05$; Table 1), resulting in a significant improvement in ISI Matsuda by 18 % ($P < 0.05$; Table 1).

Cardiovascular risk profile

Fasting NEFA concentration was significantly reduced following training by 38% ($P < 0.05$; Table 1). However, fasting plasma total triglyceride concentration and fasting cholesterol were not significantly reduced following SIT (plasma triglyceride $P = 0.174$, cholesterol $P = 0.173$; Table 1).

Basal eNOS content and phosphorylation

4 weeks of SIT significantly ($P < 0.05$) increased eNOS content in arterioles (10%) and capillaries (13%), resulting in a significant main effect of training on skeletal muscle microvascular eNOS content ($P < 0.05$) (Fig. 1). eNOS content was significantly higher in skeletal muscle arterioles than capillaries ($P < 0.05$) both pre- and post-SIT. eNOS ser¹¹⁷⁷ phosphorylation measured in the basal state was increased in arterioles and capillaries by 15%

and 7%, respectively following training, resulting in a main effect of training on eNOS ser¹¹⁷⁷ phosphorylation ($P < 0.05$) (Fig. 2). Skeletal muscle eNOS ser¹¹⁷⁷ phosphorylation was significantly higher in skeletal muscle arterioles than capillaries ($P < 0.05$). Mean arteriole diameter for assessment of eNOS and eNOS ser¹¹⁷⁷ phosphorylation pre and post training was $9.6 \pm 0.8 \mu\text{m}$ and 8 ± 1 arterioles were analysed for each condition.

NOX2

Skeletal muscle total microvascular (capillaries and arterioles) endothelial NOX2 content was reduced by 15% following training ($P < 0.05$), but skeletal muscle membrane NOX2 was unchanged by training ($P = 0.413$) (Fig. 3).

Microvascular filtration capacity and capillarization

Microvascular filtration capacity was reduced following training ($P < 0.05$) (Fig. 4). Fibre cross sectional area and perimeter were not different in type I and II fibres (Fibre cross sectional area $P = 0.436$, perimeter $P = 0.188$) and were unaltered by training in either type I or II fibres (Fibre cross sectional area $P = 0.513$, perimeter $P = 0.974$). Capillary density was unchanged by training ($P = 0.722$). Capillary-fibre-perimeter exchange index was higher in type I than type II fibre ($P < 0.05$), but was unaffected by training ($P = 0.454$). Neither capillary contacts nor capillary-to-fibre ratio were different depending on fibre type (capillary contact $P = 0.082$, capillary-to-fibre ratio $P = 0.083$) and both were unaffected by training in either fibre type (capillary contact $P = 0.329$, capillary-to-fibre ratio $P = 0.342$) (table 2, Fig. 5).

Arterial stiffness

AIx @75bpm was significantly reduced following training ($P < 0.05$) (Fig. 6a). Aortic PWV was also significantly reduced by 11% following training ($P < 0.05$) (Fig. 6b) and peripheral PWV tended to decrease following training ($P = 0.066$) (Fig. 6c).

Table 6.1. Subject characteristics, resting hemodynamics and peak oxygen uptake pre and post training.

Variable	Pre training	Post training
Age (yr)	23±3	
Height (cm)	176.6±3.3	
Weight (Kg)	111.1±6.1	111.3±5.7
BMI (kg.m ⁻²)	35.5±1.1	35.6±1.1
% body fat (%)	31.8±2.3	31.4±2.5
VO _{2peak} (ml.Kg ⁻¹ .min ⁻¹)	35.9±2.8	39.3±2.3*
Wmax (W)	239.7±11.4	267.7±13.8*
ISI Matsuda	1.8±0.1	2.1±0.2*
Glucose AUC (mmol.L ⁻¹ .120min ⁻¹)	988±68	891±50*
Insulin AUC (mmol.L ⁻¹ .120min ⁻¹)	13837±1453	11789±1601*
Resting heart rate (bpm)	66±4	61±3
Mean arterial pressure (mmHg)	86±4	88±4
Systolic blood pressure (mmHg)	128±5	126±7
Diastolic blood pressure (mmHg)	66±5	69±4
Triglycerides (mmol.L ⁻¹)	2.05±0.15	1.75±0.23
Total cholesterol (mmol.L ⁻¹)	6.4±0.4	5.5±0.5
NEFA (mmol.L ⁻¹)	0.52±0.06	0.32±0.03*

Values are means ± S.E.M. * Significant change from pre training, $P < 0.05$

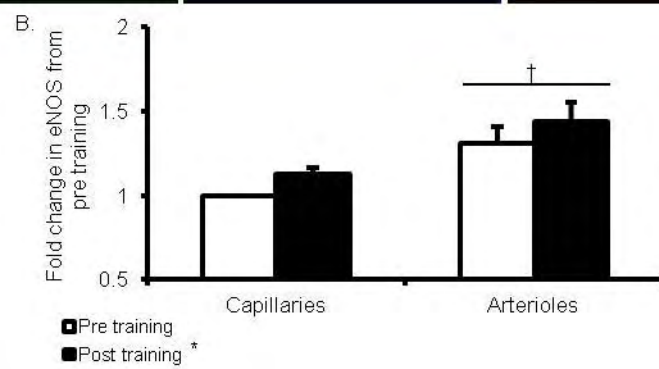
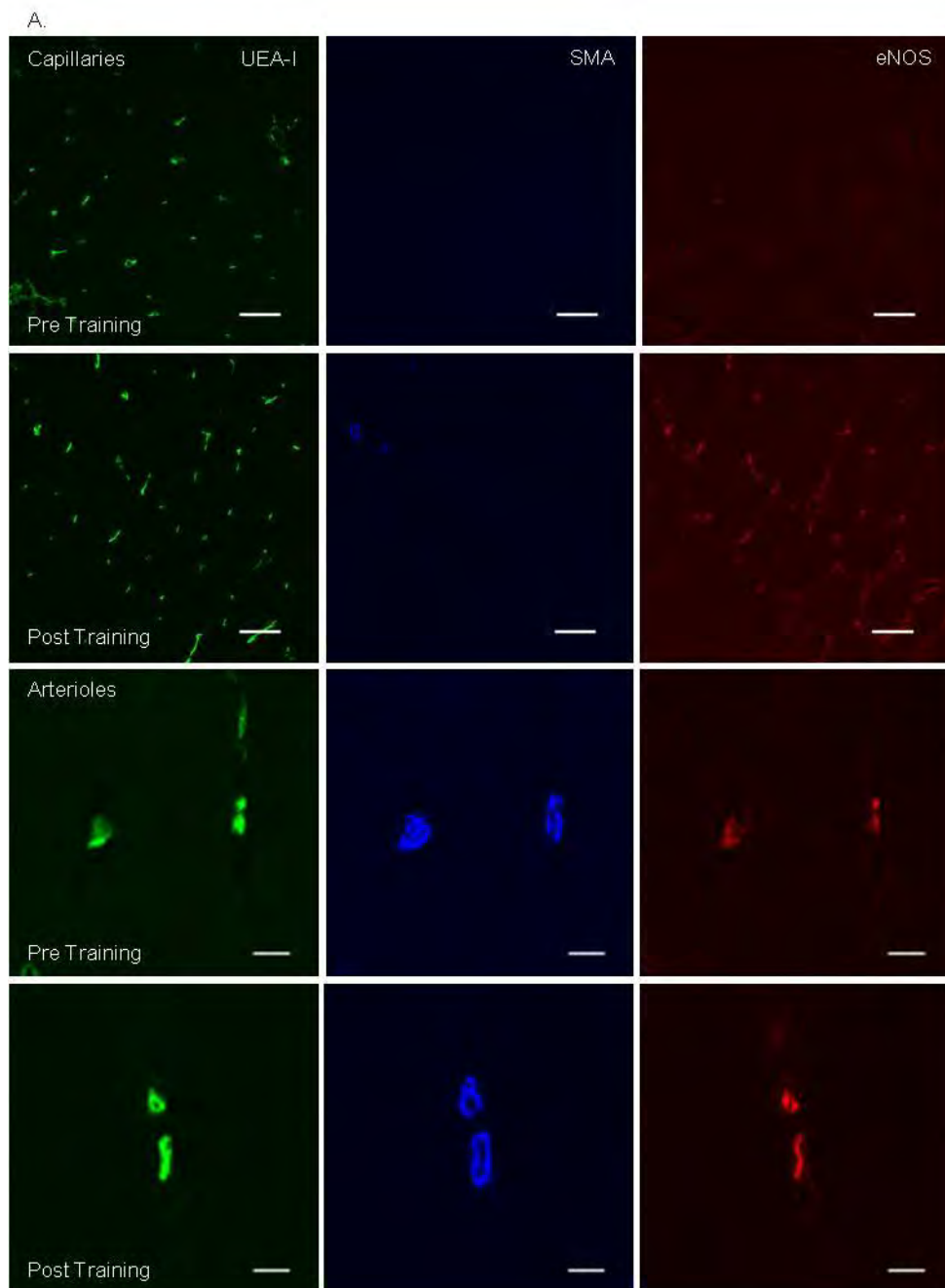


Figure 6.1. Effect of SIT on eNOS content in capillaries and arterioles.

A. Confocal images of skeletal muscle pre (left) and post (right) SIT in capillaries and arterioles. The skeletal muscle microvascular endothelium was revealed using Ulex Europaeus-FITC conjugated lectin (green). Arterioles and capillaries were differentiated using anti smooth muscle actin in combination with Alexa-Fluor 633 conjugated secondary antibody (blue). Skeletal muscle eNOS expression was revealed using Alexa-Fluor 546 conjugated secondary antibody (red). Bar = 50µm. B Mean fluorescence intensity of eNOS is summarized. The mean level of eNOS pre training in capillaries was assigned a value of 1, and the relative intensity of eNOS in arterioles and post training was calculated. * Main effect of training, $P < 0.05$. † Main effect of vessel type, $P < 0.05$.

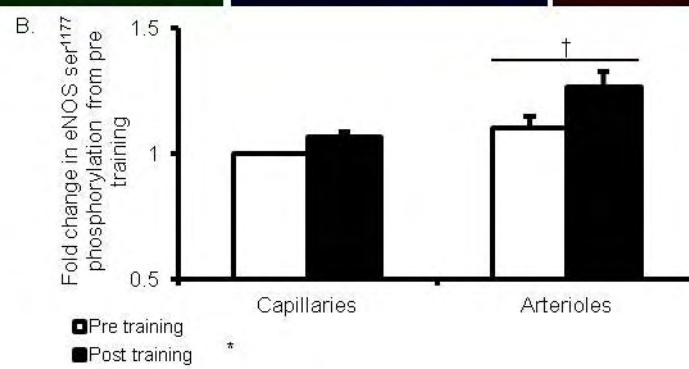
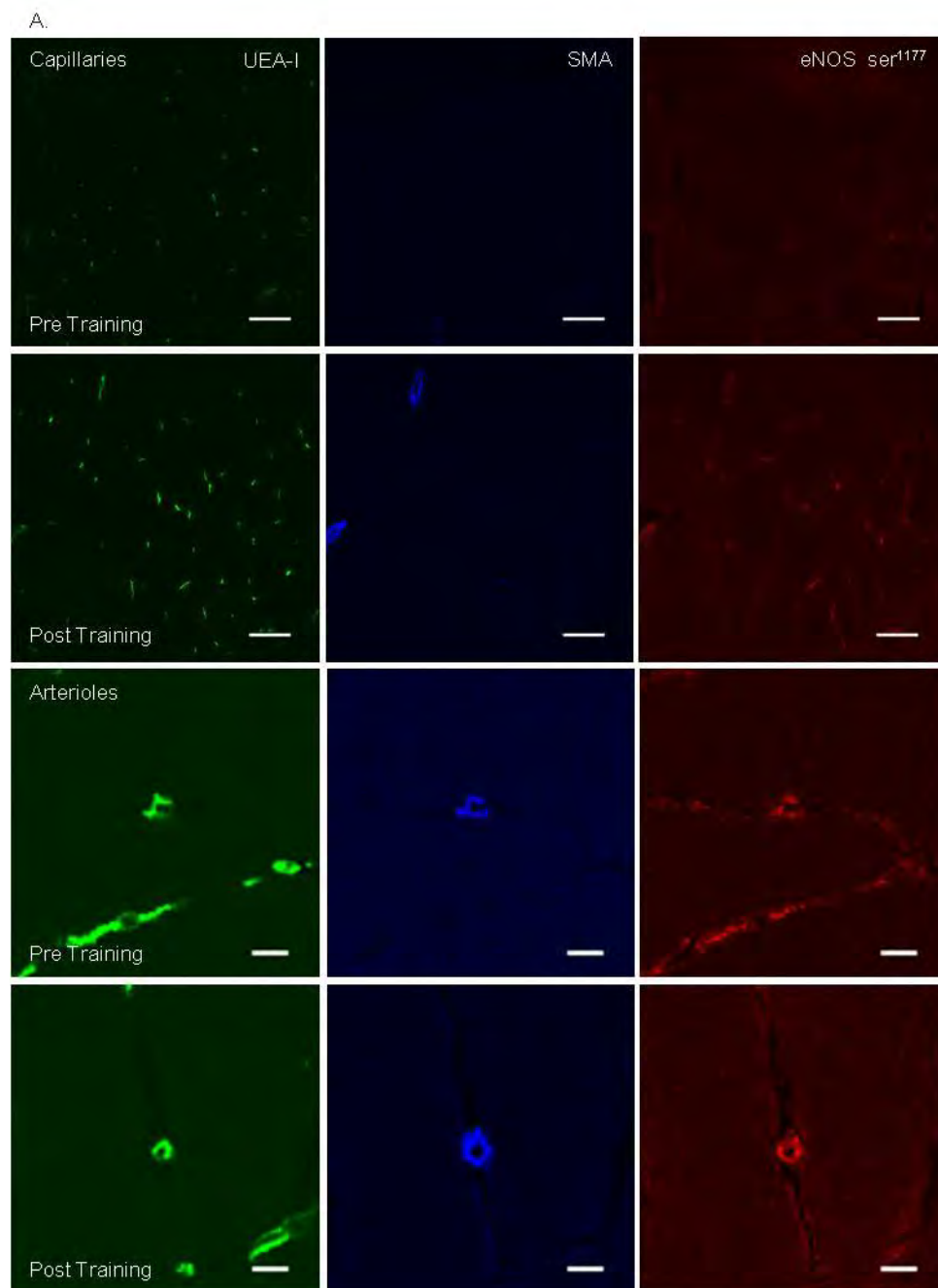


Figure 6.2. Effect of SIT on eNOS ser¹¹⁷⁷ phosphorylation in capillaries and arterioles.

A. Confocal images of skeletal muscle pre (left) and post (right) SIT in capillaries and arterioles. The skeletal muscle microvascular endothelium was revealed using Ulex Europaeus-FITC conjugated lectin (green). Arterioles and capillaries were differentiated using anti smooth muscle actin in combination with Alexa-Fluor 633 conjugated secondary antibody (blue). Skeletal muscle eNOS ser¹¹⁷⁷ phosphorylation was revealed using Alexa-Fluor 546 conjugated secondary antibody (red). Bar = 50µm. B Mean fluorescence intensity of eNOS ser¹¹⁷⁷ is summarized. The mean level of eNOS ser¹¹⁷⁷ pre training in capillaries was assigned a value of 1, and the relative intensity of eNOS in arterioles and post training was calculated. * Main effect of training, $P < 0.05$. † Main effect of vessel type, $P < 0.05$.

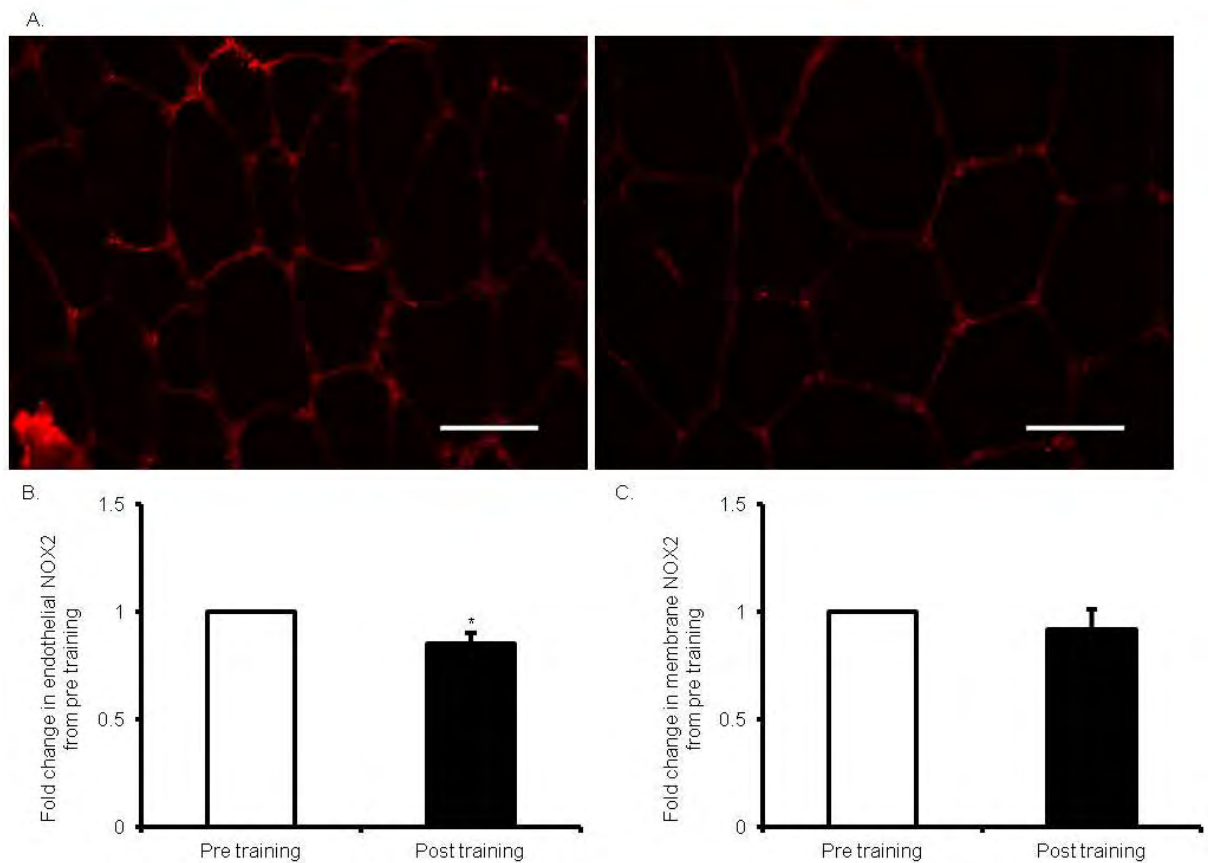


Figure 6.3. Effect of SIT on NOX2 content in capillaries and arterioles.

Widefield microscopy images of skeletal muscle pre (left) and post (right) SIT. The skeletal muscle microvascular endothelium was revealed using Ulex Europaeus-FITC conjugated lectin (green), and skeletal muscle membrane was revealed using wheat germ agglutinin-350 (WGA-350, blue). Skeletal muscle NOX2 content was revealed using Alexa-Fluor 594 conjugated secondary antibody (red). Bar = 50 μ m. B Mean fluorescence intensity of NOX2 within the endothelium is summarized. C Mean fluorescence intensity of sub-sarcolemma NOX2 is summarized. The mean level of NOX2 pre training was assigned a value of 1, and the relative intensity of NOX2 post training was calculated. * Significant change from pre training, $P < 0.05$.

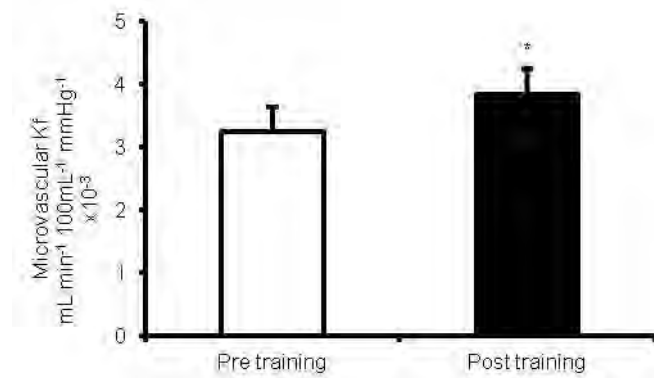


Figure 6.4. Calf microvascular filtration capacity pre and post training.

Calf microvascular K_f measured using venous occlusion plethysmography. Fluid filtration (J_v) was measured over the final 2 minutes of five 5 minute cumulative pressure steps (10mmHg). J_v was then plotted against pressure and the resulting slope of the line gave a measure of K_f . K_f is a functional measure proportional to the capillary surface area available for transendothelial transport of insulin and glucose. * Significant change from pre training, $P < 0.05$.

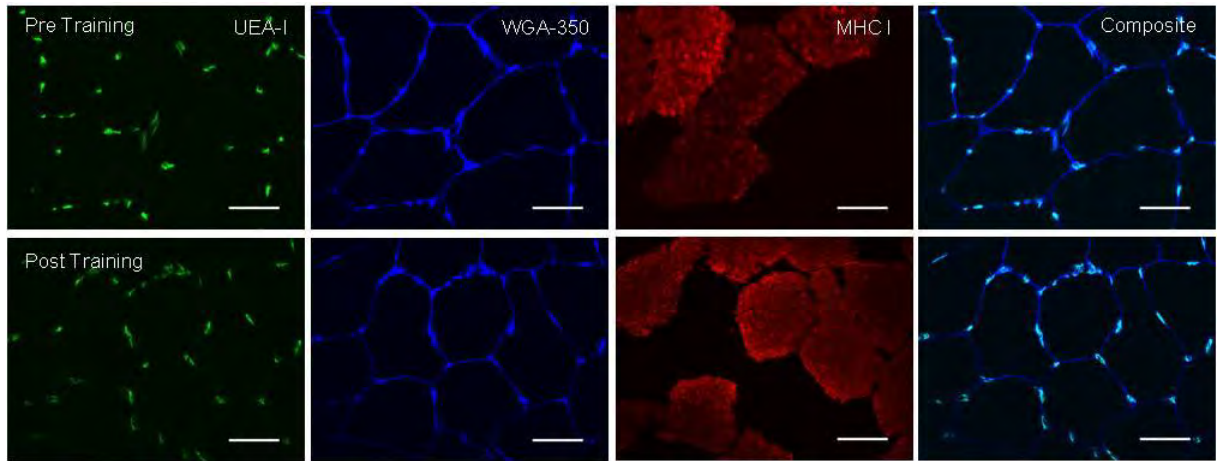


Figure 6.5. Effect of training on skeletal muscle capillarization.

Widefield microscopy images of skeletal muscle pre (left) and post (right) SIT. Skeletal muscle capillarization was revealed using Ulex Europaeus-FITC conjugated lectin (UEA-I, green), the skeletal muscle membrane was revealed using wheat germ agglutinin-350 (WGA-350, blue) and fibre type was revealed using anti-myosin type I (red). Composite image shows a combination of the UEA-I and WGA-350 images. Bar = 50 μ m.

Table 6.2. Capillarization pre and post training.

Variable	Pre training	Post training
Overall FA (mm ²)	3761±385	4134±300
Type I FA (mm ²)	3493±376	4142±419
Type II FA (mm ²)	4037±499	4207±309
Overall Perimeter (mm ²)	267.3±23.0	267.6±9.8
Type I Perimeter (mm ²)	242.4±13.7	264.5±13.2
Type II Perimeter (mm ²)	293.8±39.6	273.8±10.5
Overall CC	5.03±0.45	5.56±0.32
Type I CC	5.29±0.59	5.88±0.39
Type II CC	4.85±0.38	5.31±0.27
Overall C/F _I	1.90±0.19	2.12±0.12
Type I C/F _I	2.01±0.25	2.26±0.18
Type II C/F _I	1.82±0.16	2.00±0.12
Overall CFPE	7.43±0.58	8.01±0.47
Type I CFPE	8.14±0.75	8.62±0.45
Type II CFPE	6.88±0.56	7.37±0.47
SF	2.71±0.04	2.69±0.02
CD (caps/ mm ²)	845.0±76.9	868.5±42.5

Values are means ± S.E.M. *P* <0.05. FA, fibre cross sectional area, SF, sharing factor, CD, capillary density, CC, capillary contacts, C/F_I, capillary-to-fibre ratio on an individual-fibre basis, CFPE, capillary- fibre-perimeter exchange.

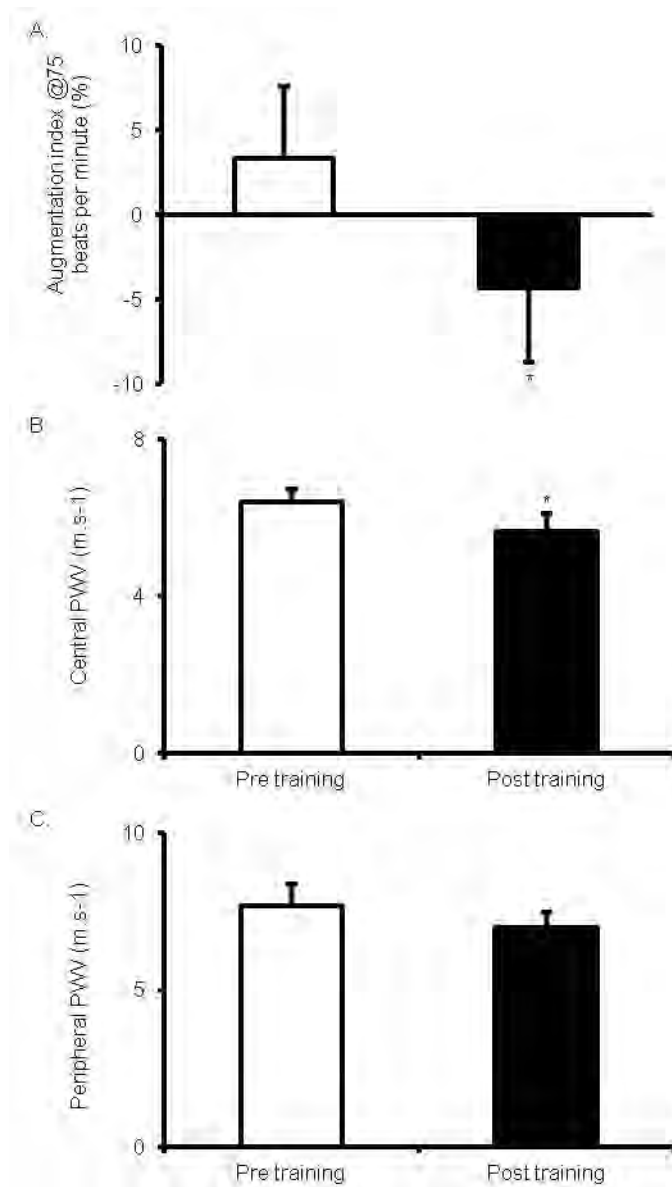


Figure 6.6. Measures of systemic wave reflections and arterial stiffness pre and post training.

A. Augmentation index normalised to 75 beats per minute pre and post SIT. B. Central pulse wave velocity pre and post SIT. C. Peripheral pulse wave velocity pre and post SIT. Values are means \pm S.E.M. * Significant change from pre training, $P < 0.05$.

6.5 Discussion

The most important novel findings of the present study are that 4 weeks of constant workload SIT in young previously sedentary obese males: 1) increased both skeletal muscle arteriole and capillary endothelial eNOS content and ser¹¹⁷⁷ phosphorylation in the basal state; 2) decreased microvascular NOX2 content and 3) increased microvascular K_f a measure of the capillary surface area available for transendothelial transport of insulin and glucose. As hypothesised these microvascular adaptations were paralleled by improvements in maximum aerobic capacity and whole body insulin sensitivity. Finally our results also show that constant workload SIT is effective at decreasing cardiovascular risk factors and arterial stiffness in obese participants. These results suggest that constant workload SIT is an effective, time efficient training mode for changing most of the measured variables in a direction consistent with health benefits in young obese males.

Time efficient training stimulus

We and others have previously shown that “all out” SIT based on repeated Wingate’s is an effective way of improving aerobic exercise capacity and insulin sensitivity in previously sedentary individuals (3, 11, 17). However these “all out sprint” protocols have been criticised for the demanding nature and high levels of motivation required to complete these interventions. Also the specialised equipment required to perform Wingate’s prevent it from being implemented in community interventions (25). These criticisms have led to the suggestion that SIT may not be a suitable method of training in obese individuals and those with exercise limitations (19). We therefore developed a method of SIT which would maintain the anaerobic nature of “all out” SIT, but would be within the physical abilities of obese individuals. The current study has shown that 4 wk of this new constant workload SIT

method was effective at increasing $\text{VO}_{2\text{peak}}$ in obese individuals with similar improvements observed as after 6 weeks of 'all out' SIT in lean sedentary individuals (9% current study versus 8% lean sedentary; (17). As aerobic capacity has been shown to be a more powerful predictor of mortality than established clinical risk factors such as hypertension and type II diabetes (43), this improvement in $\text{VO}_{2\text{peak}}$ may have long-term health benefits if maintained over the rest of the lifespan. The new method of training was also effective at increasing insulin sensitivity in the obese group studied. As the insulin resistance in obesity is strongly associated with the development of type II diabetes (26), the improvement in insulin sensitivity may ultimately result in reduced progression to type II diabetes. . The increase in $\text{VO}_{2\text{peak}}$ and insulin sensitivity observed following training are probably the consequence of increases in mitochondrial density, improved lipid metabolism (28) and the increase in microvascular NO bioavailability and function, that is likely to result from the observed changes in eNOS and NAD(P)Hox.

Microvascular function

The technique used in the current study to investigate eNOS content and ser¹¹⁷⁷ phosphorylation is an adaptation of the previous technique outlined by Cocks et al. (18) allowing differentiation between arterioles and capillaries. As mean arteriole diameter measured $9.6 \pm 0.8 \mu\text{M}$ we suggest that mainly 2nd order and terminal arterioles (TA) are analysed by the technique (38). We believe that this is a significant improvement on the previously described method (18) as it specifically provides information on terminal arterioles. Insulin and exercise induced vasodilation of terminal arterioles has been proposed to control capillary recruitment, microvascular blood volume, capillary surface available for transport of insulin and glucose and microvascular blood flow. Therefore, knowledge of

arteriole specific enzyme content and eNOS ser¹¹⁷⁷ phosphorylation will help to provide mechanistic information on the control of microvascular perfusion in response to a number of important stimuli such as, exercise, insulin and VEGF and on the blunting of these signals in sedentary and obese individuals and patients with type II diabetes and cardiovascular disease.

Both eNOS protein content and ser¹¹⁷⁷ phosphorylation were increased following SIT in skeletal muscle 2A and TA arterioles. Neither skeletal muscle eNOS content or phosphorylation have been previously investigated in arterioles and capillaries specifically following any form of training, in man or animal models, in the healthy nor in the obese state. However the finding of increased eNOS content following SIT is similar to previous work in young sedentary males where mixed skeletal muscle microvascular (arterioles, capillaries and venules) eNOS content was increased following 6wk of SIT (17).

The findings do however suggest that eNOS ser¹¹⁷⁷ phosphorylation may respond differently to SIT in the obese than in lean sedentary individuals, with an increase in both capillary and arteriole specific eNOS ser¹¹⁷⁷ phosphorylation in obesity after 4 weeks of SIT and a reduction in the sedentary young men following 6wk of SIT (mixed skeletal muscle microvasculature). It is difficult to draw comparisons between the two studies due to the different exercise mode and duration of training however; as the increases in VO₂peak are comparable the training stimuli appear to induce similar adaptations. However, the difference in response of eNOS ser¹¹⁷⁷ could be due to a number of reasons. Firstly, the reduced eNOS ser¹¹⁷⁷ phosphorylation in sedentary lean individuals was proposed to be due to a reduction in basal shear stress as a result of the increase in capillary density seen after 6wk SIT (17); while in the current study no change in capillary density was observed. However, such differences in the angiogenic

response are unlikely to explain the increase in eNOS ser¹¹⁷⁷ phosphorylation post-training shown in the present study. Basal eNOS ser¹¹⁷⁷ phosphorylation has been shown to be impaired following high fat feeding in rats (8), as such, it is possible that basal eNOS¹¹⁷⁷ phosphorylation is inhibited in obesity. Therefore, the increase in eNOS ser¹¹⁷⁷ phosphorylation in the present study may be the result of reduced inhibition of basal eNOS ser¹¹⁷⁷ phosphorylation following SIT.

Skeletal muscle microvascular NAD(P)Hox content was reduced following SIT in obese participants. This decrease in NADP(H)ox following 4 wk of SIT is therefore important as it will reduce NO quenching and increase NO bioavailability, thus better supporting the insulin, exercise and VEGF induced activation of eNOS and the angiogenic effect of exercise. The findings of the current study suggest that adaptations to skeletal muscle microvascular NAD(P)Hox content may differ between lean sedentary and obese sedentary men as skeletal muscle microvascular NOX2 content was not reduced after 6 weeks of SIT in sedentary males (17). We previously suggested that this might be the consequence of the amount of NAD(P)Hox expression being minimal in the lean sedentary young males before training, who despite of being sedentary are in a healthy state probably without significant microvascular pathology (17).

The increase in arteriolar eNOS content and ser¹¹⁷⁷ phosphorylation and reduction in microvascular NOX2 content, both enzymes responsible for NO bioavailability, may contribute to the improved insulin sensitivity observed in the current study. Insulin mediated increases in skeletal muscle microvascular blood flow have been reported to be inhibited in obesity (15) and contribute to the impaired glucose disposal. Insulin dependent elevations in

microvascular blood flow are thought to be mediated by dilation of terminal arterioles (5), through insulin induced eNOS ser¹¹⁷⁷ phosphorylation (45, 51). In addition to eNOS mediated production of NO, quenching of NO by O²⁻ generated by NAD(P)Hox may further reduce NO bioavailability impairing insulin dependent increases in microvascular blood flow (52). Therefore the improved protein content of eNOS and NAD(P)Hox and increased ser¹¹⁷⁷ phosphorylation of eNOS following SIT could have beneficial effects on insulin mediated vasodilatation in obesity contributing to the observed improved insulin sensitivity.

Previous studies have shown that an adequate NO bioavailability is required to support the angiogenic effect of exercise training (simulated by electrical stimulation in rats), as treatments of rats with L-NMMA prevented angiogenesis in skeletal muscle (40). However, despite the improvement in enzymes responsible for NO bioavailability no change was observed in any of the measures of angiogenesis used in the current study. We suggest the absence of an increase in angiogenesis may be the result of high NAD(P)Hox protein expression in the obese leading to substantial quenching of the produced NO and therefore to too low an NO bioavailability to support the angiogenic affect of VEGF. While the young sedentary men investigated previously following SIT (17) had lower NAD(P)Hox at the start of the training and therefore achieved significant increases in capillary density.

Although there was no change in capillarization following SIT there was an increase in skeletal muscle K_f. The increase in K_f is of functional relevance as K_f is associated with insulin sensitivity (16). The primary determinant of K_f has been suggested to be microvascular surface area for exchange mediated by an increase in capillary density (10, 13). The increase in K_f without increases in capillary density was therefore not expected, but

changes in K_f have previously been reported using an electrical stimulation protocol designed to induce minimal capillary growth (10). The increases in K_f in the present study may therefore, be due to other factors, such as, an increase in permeability of the microvasculature increasing fluid exchange (23), and/ or an increase in the perfused surface area of the microvasculature. As discussed above it may be possible that not all the microvasculature is perfused at rest (14) and that the elevation in K_f is the result of an increased ability to dilate terminal arterioles, increasing perfusion of skeletal muscle. If true the changes in enzymes responsible for NO bioavailability discussed above may have an important role in this functional change.

Cardiovascular risk factors

The improvements in microvascular function were paralleled by a significant reduction in fasting plasma fatty acid (FA) concentration. In cultured endothelial cells incubation with a high concentration of FA inhibits NO production in a dose dependent fashion (47) and insulin mediated elevations in skeletal muscle microvascular volume are severely blunted following acute intralipid/heparin exposure (37). High levels of plasma FA are assumed to contribute to the insulin resistance in obese individuals (7). As a result this reduction in plasma FA concentrations after 4 weeks of SIT may contribute to both the improved microvascular function and increase in whole body insulin sensitivity. In addition, there was also a trend for a reduced fasting plasma TG and cholesterol. As plasma TG and plasma cholesterol are recognised as strong risk factors for cardiovascular disease and these decreases are seen after only 4 weeks, constant workload SIT in future long-term interventions in larger groups may well prove to be a powerful means to lower these cardiovascular risk factors.

Arterial stiffness

In the present study 4 weeks of constant workload SIT significantly reduced central artery stiffness and produced a strong trend for reduced peripheral artery stiffness in young healthy obese males. To the authors knowledge this is the first study to investigate arterial stiffness following SIT in an obese group and only the second to study training of any form on arterial stiffness in obesity. In line with the current study, Arena et al. (2) showed that 10 wks of aerobic training reduced aortic pulse wave velocity in obese individuals. The reduced central artery stiffness observed in the present study is of clinical relevance as obesity is related to increased central artery stiffness even in young individuals (54) and is associated with negative cardiovascular outcomes (12). Therefore, the finding that SIT can reduce central artery stiffness is of significant importance in this young otherwise healthy obese group.

Previous studies using SIT (17) or endurance training (29) have shown no change in peripheral artery stiffness in sedentary young non-obese individuals. However peripheral artery stiffness in response to training has not been investigated in an obese group, who are known to exhibit, elevated peripheral artery stiffness (41, 54). This elevation in peripheral artery stiffness observed in obesity may explain the strong trend for reduced peripheral artery stiffness following SIT in the obese group studied. Future studies in larger groups will be required to investigate the clinical relevance of this observation.

As well as decreasing arterial stiffness SIT caused a reduction in systemic wave reflections measured through AIx. AIx has been shown to be of independent predictive value for all cause mortality (35), and provides additional information than that of PWV alone, as AIx is determined by changes in small artery tone and structure as well as central artery stiffness

(31). The reduction in AIx following SIT in obesity is therefore of potential clinical interest as well.

Conclusion

In conclusion this study provides novel information that constant workload SIT is an effective training mode to increase skeletal muscle eNOS content and basal ser¹¹⁷⁷ phosphorylation while reducing NOX2 content in young obese males. In addition SIT also increased microvascular K_f. The study shows that these changes in microvascular function and enzymes involved in NO bioavailability were paralleled by improvements in maximal aerobic capacity and insulin sensitivity suggesting that microvascular changes may contribute to the functional improvements observed. The SIT intervention used in this study involved a maximum time commitment of 1 h per week leading to the conclusion that constant workload SIT is a time efficient alternative to achieve metabolic effects that are likely to lead to long-term health benefits in young previously sedentary obese males.

3.6 Acknowledgments The antibody against myosin (human slow twitch fibres, A4.840) used in the study was developed by Dr. Blau, and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

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Chapter 7

General discussion

7.1 Overview

Dramatic changes in lifestyle over the latter half of the 20th century have led many to develop a sedentary lifestyle which does not fit the genetic makeup of *Homo sapiens* (11). In combination with an abundant supply of food and unhealthy food choices and eating habits this has led to the current global obesity epidemic, which is threatening the world. Both inactivity and obesity elevate the risk for development of cardiovascular and metabolic disease (7, 47). In addition to direct health implications and reductions in quality of life, the economic cost of a sedentary lifestyle and obesity warrants urgent action (1, 2). Reductions in aerobic exercise capacity and impairments in glycaemic control are among the key maladaptation's from the optimally trained healthy phenotype and play important roles in the development of the chronic diseases that are associated with inactivity, obesity and ageing. An inability to match perfusion of skeletal muscle to metabolic demand, likely contributes to both the reduced aerobic exercise capacity and impaired glycaemic control often progressing in skeletal muscle insulin resistance in many sedentary and obese individuals (**chapter 1**). Capillary rarefaction leading to a reduction in the surface area available for exchange of insulin, nutrients and oxygen and an impaired ability to recruit capillaries in response to insulin and exercise are the primary reasons for this skeletal muscle underperfusion (**chapter 1**), although suggestions have been made that a reduced bulk blood flow to the skeletal muscle due to impaired feed artery dilation may contribute as well. As insulin and exercise (shear-stress) induced vasodilatation and the exercise-induced increase in angiogenesis are all nitric oxide (NO)-dependent, often in synergy with other mechanisms, reductions in NO bioavailability at the start of this PhD project were hypothesised to be responsible for the impaired perfusion of skeletal muscle in inactivity and obesity (**chapter 1**).

Endurance training (ET) has long been known to increase aerobic capacity (31) and insulin sensitivity (26) and therefore is part of the ACSM guidelines for physical activity and health promotion (25). One of the established effects of ET is that it increases eNOS expression and protein content at all levels of the arterial tree and improves NO dependent vasodilatory functions such as flow mediated dilation (FMD) (**chapter 1**). As such, improved perfusion of skeletal muscle likely contributes to the improvements in aerobic exercise capacity and insulin sensitivity observed in sedentary and obese individuals following ET (**chapter 1**). However many people do not meet current activity guidelines and as the most common cited reason for this is a lack of time (49), there is a need to develop alternative time efficient training strategies. High intensity interval training (HIT) and sprint interval training (SIT) has received great interest due to its ability to mirror a number of the metabolic adaptations of ET, but with a massive reduction in time commitment (20). In addition to ET and SIT, resistance training (RT) is an important training method due to its effects on muscle mass, strength and insulin sensitivity (25, 30). However its effect on the vasculature is understudied. The few studies to investigate RT's effect on macrovascular health suggest that it has either no or a negative effect (**chapter 1**). Despite the important role that all three modes of exercise training may have on cardiovascular and metabolic health, their effect on the skeletal muscle microvasculature and in particular the enzymes that control NO bioavailability has not been investigated. Therefore, the aims of this thesis were 1) develop a reliable method to measure the protein content and phosphorylation state of enzymes that determine NO production (eNOS) and NO quenching (NADP(H)oxidase) specifically in the microvasculature of human skeletal muscle, 2) investigate the effect of ET, SIT and RT in lean sedentary individuals on these endothelial microvascular enzymes and on capillary density, 3) investigate the effect of an adapted SIT protocol on muscle capillary density and these endothelial microvascular

enzymes in terminal arterioles and capillaries in obese males , and 4) use the indicated immunofluorescence methods in obese Zucker rats (OZR) to investigate the molecular mechanisms that lead to impaired microvascular dilation and insulin resistance during a hyperinsulinemic euglycemic clamp.

7.2 The use of immunofluorescence microscopy to investigate skeletal muscle microvascular enzymes

Previous studies investigating vascular enzymes responsible for NO bioavailability in human skeletal muscle have either used Western blots or sandwich enzyme immunoassay techniques applied to extracts of whole muscle homogenates (27, 28). Techniques using whole muscle homogenates have obvious disadvantages if the aim is to measure their content in the endothelial layer of specific blood vessels. These are discussed at length in **chapter 2**. In animal models enzymes responsible for NO bioavailability have also been investigated using Western blots applied to whole muscle homogenates (18) and through isolation of first to fifth (terminal) order skeletal muscle arterioles (34, 37, 38) followed by homogenisation and Western blots. However, apart from being technically challenging requiring skilled hands and large muscle samples this technique does not allow for the measurement of the protein content of enzymes as they occur in situ in the endothelial layer of terminal arterioles and capillaries, which as discussed in **chapter 1** play key roles in the upregulation of skeletal muscle perfusion in response to insulin and exercise. The underlying mechanism has been proposed to involve dilation of terminal arterioles leading to recruitment of previously underperfused capillaries and enlargement of the capillary surface area available for transendothelial transport of insulin, glucose, other nutrients and oxygen. Therefore, in **chapter 2** a description is given of the development of an immunofluorescence microscopy technique which allows

the visualisation and subsequent quantitation of skeletal muscle microvascular eNOS content and ser¹¹⁷⁷ phosphorylation and NAD(P)H oxidase subunit NOX2 content, without the confounding factors associated with techniques using whole muscle homogenates. The primary objective of **chapter 2** was to establish if the adapted immunofluorescence microscopy techniques could be used reliably. The secondary objective was to use the techniques developed to investigate the effect of 1 h of moderate intensity endurance exercise on eNOS ser¹¹⁷⁷ phosphorylation to investigate whether these newly developed methods were able to measure changes in the physiological range. The final objective (method development and applications described in **chapters 3 and 6**) was to adapt the technique to allow for independent analysis of capillaries and arterioles in human and rat skeletal muscle.

Evidence is provided in **chapter 2** that quantitative immunofluorescence microscopy can be used reliably to investigate skeletal muscle endothelial enzymes specifically in the endothelial layer of skeletal muscle microvessels (assessed as a mixture of capillaries and 4th and 5th order arterioles and venules). Subsequently, quantitative immunofluorescence microscopy showed that eNOS ser¹¹⁷⁷ phosphorylation is elevated in the human skeletal muscle microvasculature following acute exercise. This has led to the hypothesis that the observed activation of eNOS suggests that increases in skeletal muscle microvascular NO production may play a role in the microvascular dilation and functional hyperaemia during endurance exercise, although the full extent of its involvement is difficult to speculate on due to the large number of vasodilatory mechanisms and the previously observed redundancy of the often complementary systems, with some being major regulation systems and other playing a role in fine-tuning (15).

In addition in **chapters 3 and 6** the technique was successfully adapted to allow for investigation of arterioles and capillaries independently of each other. As explained in chapter 1 both insulin and exercise have been hypothesized to lead to the recruitment of previously underperfused capillaries via their vasodilatory effect on the terminal arterioles. It, therefore, is crucially important that eNOS ser¹¹⁷⁷ phosphorylation is measured specifically in the terminal arterioles. This was achieved with the addition of a vascular smooth muscle stain in **chapter 3 and 6**. This will allow us in future studies to investigate if there are reductions in eNOS content and/ or impairments in eNOS ser¹¹⁷⁷ phosphorylation in skeletal muscle arterioles, which explain the blunting of the vasodilatory response to insulin and exercise in sedentary compared to trained individuals, in obese compared to lean, and in old compared to young. If these differences exist then insulin and exercise stimulated eNOS ser¹¹⁷⁷ phosphorylation will also be used as future outcome measure in exercise training interventions.

7.3 Sprint interval training: a time efficient training mode to increase vascular function in sedentary and obese individuals

Previous studies into the effectiveness of low volume sprint interval training (SIT) have focused on SIT's ability to improve markers of skeletal muscle adaptation and potential molecular mechanisms behind these adaptations (20). However, only one previous study has compared SIT's effect on the vasculature to that of ET (44) and this study did not look at the skeletal muscle microvasculature and the enzymes in its endothelial layer that control NO bioavailability. Therefore in **chapter 4** the immunofluorescence techniques developed in **chapter 2** were used to investigate the effect of ET and SIT in young sedentary males on skeletal muscle microvascular density, eNOS and NOX2 contents and eNOS ser¹¹⁷⁷

phosphorylation in the basal state and following acute exercise pre and post training. In addition the effect of ET and SIT on arterial stiffness was investigated.

The results showed that: 1) 6 weeks of ET and SIT in young previously sedentary adult males increased skeletal muscle capillarization to a similar extent, 2) SIT induced greater increases in skeletal muscle microvascular eNOS content than ET, 3) Acute exercise caused an increase in skeletal muscle eNOS ser¹¹⁷⁷ phosphorylation irrespective of training status with a reduced post training eNOS ser¹¹⁷⁷ phosphorylation in both the basal and post exercise state, 4) Neither ET nor SIT had an effect on NOX2 content in the young sedentary healthy males in this study. Finally ET and SIT produced similar changes in central (measured using aortic pulse wave velocity) and systemic (systemic wave reflections measured through augmentation index) indexes of vascular stiffness.

As previously observed for aerobic capacity and fat use during exercise, SIT was as effective as ET in this study at increasing microvascular density and more effective at increasing eNOS content in the skeletal muscle microvascular endothelium. Numerous exercise related signals may increase eNOS gene expression and protein content, but two stimuli may be particularly important in the difference observed between ET and SIT (16). Firstly, studies in cultured endothelial cells and intact arteries have shown that the elevation in shear stress is an important signal for the upregulation of eNOS mRNA and eNOS protein expression (4). As exercise intensity during the 30 second bouts of SIT are much higher than during cycling at 65% $\text{VO}_{2\text{max}}$ the blood shear stress seen by the endothelial layer of the muscle microvasculature is most likely to be much higher as well. This increased shear stress may be a potential reason for the elevated increase in eNOS content after SIT. Secondly, small

increases in vascular oxidative stress in a range which does not lead to NO quenching or oxidative damage are known to accompany exercise and have been shown to also stimulate eNOS expression (4). In particular hydrogen peroxide (H_2O_2) has been shown to elevate eNOS expression, and in transgenic rats over expressing catalase (responsible for decomposition of H_2O_2 to water and oxygen) increases in eNOS expression were not evident following exercise training (33). As oxidative stress is elevated more following high intensity than moderate intensity exercise (22) this could be a second mechanism leading to a larger increase in eNOS protein expression following SIT.

A limitation of the study conducted in **chapter 4** and other chapters within this thesis is that no measure of microvascular dilation in response to insulin or exercise has been made. This makes it difficult to speculate on the physiological relevance of the changes in eNOS content and ser¹¹⁷⁷ phosphorylation. As such, discussion of potential methods that could be used in future studies to make parallel microvascular function measurements will be discussed at length in the subsection future research of this chapter.

Following the success of the SIT protocol used in **chapter 4**, **chapter 6** aimed to investigate the effect of SIT on microvascular function in obesity. However following the experience from **chapter 4**, and suggestions from others regarding the feasibility of ‘all out’ SIT using repeated Wingate’s (14, 20) in individuals and populations with exercise limitations, we chose to use an adapted SIT protocol involving constant load cycling, as a more realistic alternative in the obese group that was investigated in this study. Using the data collected from **chapter 4** which suggested the mean workload during repeated Wingate’s to be approximately 200% W_{max} , we designed a protocol using a constant load of 200% W_{max} for 30s with 2 minutes

rest between the exercise bouts. Using this protocol a pilot study was performed to investigate the feasibility (adherence) and effectiveness of 4 weeks of this new constant workload SIT protocol on microvascular structure and the enzymes controlling NO bioavailability in obese sedentary males. It also investigated whether the 4 week constant workload SIT led to measurable increases in aerobic capacity and insulin sensitivity. As the clinical effects of exercise training are important for the obese population measurements of variables that determine cardiovascular risk were also made.

The results showed that the constant workload SIT protocol was effective at increasing both skeletal muscle arteriole and capillary endothelial eNOS content and ser¹¹⁷⁷ phosphorylation in young previously sedentary obese males. SIT also led to decreased microvascular NOX2 content. All these enzyme changes are expected to increase NO production and bioavailability. SIT increased microvascular K_f (filtration capacity), which is a recognised functional measure of the capillary surface area in skeletal muscle available for transport of insulin, glucose and oxygen and for net transport of plasma water at increased venous pressures. The principle of the K_f measurement is based on the latter variable (19). These microvascular adaptations were paralleled by improvements in maximum aerobic capacity and whole body insulin sensitivity. However, surprisingly microvascular density was not improved by 4 weeks constant workload SIT. These improvements in microvascular function and structure coincided with improvements in aerobic capacity, insulin sensitivity and a better cardiovascular risk profile. Finally our results also indicated that constant workload SIT was effective at reducing arterial stiffness in obesity. As such the SIT protocol used proved to be an effective and extremely time efficient method of training to improve aerobic capacity, insulin sensitivity and vascular function. The adapted SIT protocol was more time efficient

than the repeated Wingate based SIT used in **chapter 4** with a maximum session length of only 19.5 min (3.5 min exercise, 12 min recovery, 2 min warm-up and 2 min cool down) compared to 30 minutes for the Wingate based SIT in **chapter 4**. Although it would be useful to compare the effect of the new SIT method to repeated Wingate's, this would require a comparison in the same group with exactly the same training duration. Future research in larger groups will be required to answer the question which of the two methods achieves the largest changes and is most acceptable to achieve a chronic change in lifestyle and physical activity levels.

Following this successful pilot the study should be expanded to include more participants, reducing possible statistically errors, and compared with an ET group as was conducted in **chapter 4**. As in **Chapter 4**, this ET group would provide a control allowing for comparison of the different training modes to fully elucidate the effectiveness and efficiency of constant workload SIT in obesity.

7.4 Resistance training (RT) to improve microvascular function

Although ET is the primary mode of exercise recommended to achieve improvements in fitness and health by authorities, recently RT has been included in American College of Sports Medicine (ACSM) exercise guidelines due to its effects on muscle mass, strength and insulin sensitivity (25). As previously discussed microvascular adaptations play a role in the improvement in insulin sensitivity observed following ET and SIT in previously sedentary men. However, there is a paucity of data regarding the effect of RT on the microvasculature, and if such adaptations could contribute to the elevation in insulin sensitivity which is known to occur following RT. As such, **chapter 5** aimed to investigate the effect of RT on muscle

microvascular density and microvascular enzyme contents to establish whether such changes occur following RT and could be part of the mechanism that leads to increases in insulin sensitivity following RT in previously sedentary men.

As observed in many earlier studies, the study showed that RT significantly improved insulin sensitivity and muscle strength. However the increase in insulin sensitivity was not accompanied by an increase in capillary density, eNOS content or ser¹¹⁷⁷ phosphorylation or decreased NOX2 content. These data provide evidence that the increases in insulin sensitivity following RT are not mediated by improvements in microvascular density and/ or eNOS content. The results of this thesis therefore suggest that the mechanisms leading to improvements in insulin sensitivity are different for RT than for ET or SIT. It is my suggestion, therefore, that a combination of ET (or SIT) with RT may prove to be the most effective training approach as it may increase muscle insulin sensitivity via multiple mechanisms. In addition to the potential benefits on insulin sensitivity combined training would maintain the unique beneficial effects of RT on muscle mass and strength, which are critical to maintaining a lifelong mobility and independency in the growing elderly population.

Finally our results also indicated that unlike previous suggestions (39) RT did not increase central artery stiffness. Although this adds to a number of publications suggesting that RT performed according to the recommendations of the ACSM (2-3 set of 12 repetitions at 80% 1 repetition maximum) does not cause elevations in arterial stiffness (10, 42, 43), future studies should investigate the discrepancy between effects and other studies using different RT

protocols (concentric lifting protocols, high volume RT or high intensity super sets) (3, 13, 41, 42) and reports of increased arterial stiffness in strength trained athletes (40).

7.5 The effect of metabolic syndrome on eNOS signalling following insulin stimulation

Previous work using contrast enhanced ultrasound (CEU) in rat and man and leg 1-methylxanthine (1-MX) extraction in rats has led to the hypothesis that insulin-induced NO dependent dilatation of terminal arterioles leads to an increase in microvascular blood volume and capillary recruitment in healthy rats and humans, which is instrumental to the insulin-induced increase in skeletal muscle glucose uptake during an hyperinsulinemic-euglycemic clamp, oral glucose tolerance test or ingestion of a mixed carbohydrate containing meal (5, 6, 12, 45). However it is suggested that impairments in this mechanism are partly responsible for the reduced glucose uptake in response to insulin observed in obesity and the metabolic syndrome (5, 6, 12, 45). No studies so far have investigated the *in vivo* eNOS signalling mechanisms behind the insulin induced increase in microvascular blood volume/capillary recruitment in lean individuals and potential mechanisms of impairment in the metabolic syndrome. As such, the aim of the study in **Chapter 3** was to investigate the effect of the metabolic syndrome on eNOS content and ser¹¹⁷⁶ phosphorylation in terminal arterioles and capillaries using the obese Zucker rat (OZR) as a model of the metabolic syndrome.

The results show that there was no difference between the eNOS content and fasted eNOS ser¹¹⁷⁶ phosphorylation in the endothelium of terminal arterioles of lean Zucker rats (LZR) and OZR, but during a hyperinsulinemic-euglycemic (HE-) clamp insulin stimulated eNOS ser¹¹⁷⁶ phosphorylation was significantly impaired in the OZR, even resulting in a reduction in

eNOS ser¹¹⁷⁶ phosphorylation during the HE-clamp. In addition the results showed that eNOS content and fasted ser¹¹⁷⁶ phosphorylation were also not different between LZR and OZR in the endothelium of capillaries. Unlike terminal arterioles, insulin stimulation during the HE-clamp did not increase eNOS ser¹¹⁷⁶ phosphorylation in LZR, but did result in a significant reduction in eNOS ser¹¹⁷⁶ phosphorylation in OZR. These impairments in insulin stimulated eNOS ser¹¹⁷⁶ phosphorylation were accompanied by a substantial reduction in whole body glucose clearance during the first 21 minutes of HE-clamp and reduced whole insulin sensitivity. This study, therefore, suggests that impairments in insulin mediated eNOS ser¹¹⁷⁶ phosphorylation lead to the previously observed blunting of insulin mediated increases in muscle microvascular volume (54) and that this molecular mechanism is instrumental to the reduced insulin sensitivity in OZR.

7.6 Future research

7.6.1 Microvascular function

As discussed earlier a limitation of the work in this thesis is that we were not able to relate the observed changes in endothelial enzymes to measurements of the vasodilation of terminal arteries, recruitment of previously underperfused capillaries and increases in muscle microvascular blood volume and muscle microvascular blood flow. This would have provided more insight into the functional relevance of the observed changes in these enzymes on the impairments seen in obesity and metabolic syndrome, and in the beneficial training adaptations seen in sedentary and obese individuals.

A number of techniques are purported to measure relevant aspects of microvascular perfusion or function. However many of these techniques have a number of limitations (discussed in

detail by Clark et al.), or do not measure microvascular perfusion within the skeletal muscle, which is an essential condition for the coupling of the enzyme data obtained from the skeletal muscle microvascular endothelium with functional measures.

Intramuscular microdialysis in combination with the forearm model and blood flow measurements provides a direct measurement of capillary permeability-surface area product (24) and could therefore be regarded as the “gold standard” method for simultaneously assessing changes in transendothelial transport of insulin and glucose and the capillary surface area available for transport of insulin, glucose, other nutrients and oxygen. The method has previously shown that increases in capillary permeability-surface area product in healthy individuals occur during an oral glucose tolerance test and an HE- clamp at very low physiological blood insulin concentrations (24). Further increases in insulin infusion during a clamp to levels traditionally used in clinical research aiming to measure insulin resistance, did not further increase the capillary surface area product. A second publication has shown that increases in capillary permeability surface area during a HE- clamp are even smaller in type 2 diabetics than in obese individuals (23). Despite the analytical power of this method it is complex, technically challenging and the invasive nature of the method have prevented a widespread use so far.

Contrast enhanced ultrasound (CEU) is an indirect method which can be used to measure microvascular blood volume (MBV) and microvascular flow velocity (MFV) in skeletal muscle with the product of these variables giving muscle microvascular blood flow (MBF) (50-53, 55). This implies that all functional consequences of dilation of terminal arterioles leading to increases in muscle microvascular blood volume and the local muscle

microvascular blood flow can be measured. The disadvantage of the method is that it requires the infusion of microspheres and is therefore invasive, a high level of ultrasound knowledge and skills, expensive ultrasound equipment able to use linear probes are required, and finally, CEU cannot be used on moving or contracting muscles. Adaptations to the original CEU technique to allow for continuous real-time imaging have shown that physiological levels of insulin (75 μ U/ml) lead to an increase in MBV resulting in elevated glucose uptake without increases in MFV (46). The same is true for measurements taken immediately after light exercise, while higher exercise intensities lead to increases in both MBV and MFV.

CEU involves the infusion of microspheres (the size of red blood cells and filled with contrast agent) visualised by ultrasound in the tissue of interest (most publication in human skeletal muscle and heart). The acoustic intensity of the image obtained is proportional to the concentration of microspheres within the volume of tissue being imaged. A single pulse of high-energy ultrasound is administered to destroy all the microspheres within the ultrasound beam. Subsequently, the rate of microsphere replenishment can be measured and reflects MFV while the plateau level of microspheres reached after destruction reflects MBV. As larger vessels fill more quickly, background subtraction of the average acoustic intensity generated within 1 second after the high-energy pulse is applied, thus eliminating larger vessels from the analysis, providing assessment of MBV and MFV in terminal arterioles, capillaries (major contribution), and 4th and 5th order venules. As a result I believe this technique would allow us to relate changes in microvascular eNOS content and insulin and exercise induced increases in ser¹¹⁷⁷ phosphorylation to increases in muscle microvascular perfusion and muscle microvascular blood volume (MBV) as a measure of increases in capillary surface area in future studies.

Following introduction of these methods, it would be of great interest to combine these techniques with similar methods to investigate macrovascular function, as this would allow for integrated assessment of vascular adaptations at multiple vascular levels. As such, flow mediated dilation (FMD) and/or total limb blood flow assessed with plethysmography and/or Doppler ultrasound in response to insulin or exercise could be used in conjunction with the quantitative immunofluorescence from endothelial scrapings of arteries to provide assessment of the endothelial enzymes determining NO production and NO quenching in feed arteries. Such an integrated approach simultaneously examining the muscle microvasculature and the macrovasculature would allow us to investigate differences in time course of response to inactivity or training interventions within these discrete vascular beds, to thus identify the most important early targets for future interventions involving diet, exercise and pharmaca. This is important as it has been suggested that microvascular and macrovascular adaptations interact to elevate cardiovascular and metabolic disease risk and that muscle microvascular adaptations in fact precede macrovascular impairments (32). In addition, it has been suggested that the time course of adaptations to inactivity and exercise training may differ between vascular beds (48). For example within conduit arteries it is suggested that functional changes following inactivity may precede structural changes, however this is not the case within the resistance vasculature (48). It is also highly likely that there are differences between arms and legs due to the inherent difference in usage of these limbs in day to day life in sedentary individuals. As such investigation of both the muscle microvasculature and the macrovasculature taking into account functional adaptations and changes in signalling mechanisms and endothelial enzyme changes would provide more information on the effect that inactivity and training interventions have on the potential mechanisms behind any

differences between vascular beds. This information may have the potential to inform and impact therapeutic interventions in that it indicates the best possible targets and best possible timing for implementation of such interventions.

A second area that could be investigated with the use of this integrated approach is the contribution that impaired limb blood flow and microvascular perfusion, respectively, play in the reduced aerobic capacity of older adults. This is a heavily investigated but poorly understood research topic with a large clinical relevance as reductions in leg and muscle microvascular blood flow have large implications for exercise capacity (determining mobility and independence and therefore quality of life) and the development of chronic metabolic diseases as explained in this thesis. Using this integrated approach it would be possible to examine whether reductions in NO bioavailability through impaired NO production or increased NO quenching lead to impairments in the response to exercise both at the muscle microvascular and the macrovascular level and to provide evidence that distinct mechanisms are responsible for the impaired exercise hyperemia of elderly adults within different vascular beds as suggested by Frisbee et al. in OZR (17).

Finally, the observation in chapter 6 that capillary density does not change following 4 weeks of SIT, may suggest the possibility that functional adaptations such as increases in angiogenesis are delayed in obesity. This raises the question of whether the efficiency of vascular adaptations to training in an obese group may be impaired. As NO bioavailability is important for exercise induced angiogenesis and many other vascular adaptations (for example, increases in FMD and insulin-mediated increases in MBV), it is possible that high levels of NO quenching by O_2^- produced by NAD(P)Hox may be responsible for a blunting of

the adaptive response to training in the first weeks. It may therefore be that a second block of training following the increases in eNOS content and reduction in NOX2 content may be more efficient at inducing angiogenesis and other functional adaptations, due to gradual increases in NO production and reductions in NO quenching increasing NO bioavailability. As such, it would be interesting to investigate a longer training intervention (3 months) with measurements of vascular enzymes responsible for NO bioavailability and functional outcomes at regular intervals (2, 4, 6, 8, and 12 weeks), to investigate whether elevated oxidative stress through increased NAD(P)Hox needs to be cleared, and increased NO production through increased eNOS content and phosphorylation occur to maximise the benefits of training interventions.

7.6.2 High intensity interval training

The work contained within this thesis (**chapter 4 and 6**) and previously by others (8, 44) suggests that low volume SIT is an effective method of training which appears to result in similar adaptations to ET despite substantial time savings. However it is my belief that much more research is needed into all forms of SIT and/or HIT as exercise training modes.

Current SIT protocols use either the original ‘all out’ repeated Wingate SIT method developed by Gibala et al. (9), **Chapter 4**), or various constant workload protocols based on power outputs obtained from these original Wingate studies (constant load SIT and HIT) (**Chapter 4**, (29, 35, 36)). These constant workload HIT protocols have been developed due to the highly demanding nature of “all out” SIT, which requires high levels of motivation and specialised equipment (**chapter 6**) (14, 20, 21). However this has resulted in a large range of workloads and time interval encompassed by the HIT title. It is my belief that the wide range of

workloads and interval durations likely result in different adaptations. As such it would seem essential to perform a study or series of studies investigating various different HIT and SIT protocols, evaluating their effectiveness against each other based on physiological and clinical variables such as aerobic capacity, insulin sensitivity and cardiovascular risk factors, but also time efficiency and psychological aspects such as enjoyment and motivation which are extremely important aspects of an effective training programme, especially for its implementation in the community. It is the current researcher's belief that such studies would show substantial differences in the adaptations and mechanisms of adaptation between different HIT and SIT protocols and therefore would inform expert consensus reports and future authoritative public health statements and guidelines on the efficiency and feasibility of HIT and SIT.

In addition to work optimising HIT and SIT protocols, future studies should begin to evaluate the long term health benefits of HIT and SIT as we know very little about it in comparison with what is known about the health benefits of endurance training. Current studies vary from only 2-6 weeks (20, 21). As such the similar effects of SIT compared to ET which manifest after short term training may not be observed over a period of many months. Gibala et al. (21) have speculated that the time course for physiological changes may differ between ET and SIT and the very high intensity nature of SIT may result in rapid adaptations whereas adaptations induced by lower intensity ET may be slower to occur but progress further. Therefore it would seem essential to investigate the effects of ET and SIT over longer time periods to see if the comparable adaptations are maintained before definitive conclusions about SIT's efficiency can be drawn. Finally, long term studies are also necessary to establish the potential effects of SIT on health, the risk for development of chronic disease and

premature mortality. If these effects are as impressive as the results in this thesis suggest then successful implementation of SIT into the lifestyle of sedentary, obese and elderly population in the long-term would also reduce the prevalence of chronic disease in these at risk populations and limit the use of the relevant treatments, thus, leading to massive savings in health care costs.

7.7 References

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