Cocks, MS and Wagenmakers, AJM

The effect of different training modes on skeletal muscle microvascular density and endothelial enzymes controlling NO availability

Article

The effect of different training modes on skeletal muscle microvascular density and endothelial enzymes controlling NO availability

Matthew Cocks and Anton J.M. Wagenmakers

Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Tom Reilly Building, Byrom Street, Liverpool L3 3AF, United Kingdom

This review was presented at the symposium *Impact of physical activity, ageing, obesity and metabolic syndrome on muscle microvascular perfusion and endothelial metabolism,* which took place at Physiology 2014, London on 1st July 2014.

Corresponding Author:
Dr Matthew Cocks
School of Sport and Exercise Sciences
Liverpool John Moores University
Tom Reilly Building
Byrom Street
Liverpool L3 3AF
United Kingdom
E-mail: m.s.cocks@ljmu.ac.uk

Abstract

It is becoming increasingly apparent that a high vasodilator response of the skeletal muscle microvasculature to insulin and exercise is of critical importance for adequate muscle perfusion and long-term microvascular and muscle metabolic health. Previous research has shown that a sedentary lifestyle, obesity, and ageing lead to impairments in the vasodilator response, while a physically active lifestyle keeps both microvascular density and vasodilator response high. To investigate the molecular mechanisms behind these impairments and the benefits of exercise training interventions, our laboratory has recently developed quantitative immunofluorescence microscopy methods to measure protein content of eNOS and NAD(P)Hoxidase specifically in the endothelial layer of capillaries and arterioles of human skeletal muscle. As eNOS produces NO and NAD(P)Hoxidase superoxide anions (quenching NO) we propose that the eNOS/NAD(P)Hoxidase protein ratio is a marker of vasodilator capacity. The novel methods show that endurance training (ET) and high intensity interval training (HIT) generally regarded as a time efficient alternative to ET, increase eNOS protein content and the eNOS/NADP(H) oxidase protein ratio in previously sedentary lean and obese young men. Resistance exercise training had smaller but qualitatively similar effects. Western blot data of other laboratories suggest that endurance exercise training leads to similar changes in sedentary elderly men. Future research will be required to investigate the relative importance of other sources and tissues in the balance between NO and O_2^- production seen by the vascular smooth muscle layer of terminal arterioles.

Introduction

The aim of this review is to discuss how exercise training influences the balance between eNOS and NAD(P)Hoxidase (NAD(P)Hox). It will also investigate whether a change in this balance, together with the well-known increase in capillary density, may play a significant role in the increases in insulin-mediated perfusion of skeletal muscle and, therefore, insulin sensitivity that is seen following training. The review will start with a brief outline of the role played by the skeletal muscle microvasculature in the mechanisms that lead to glucose uptake after meal ingestion, and then discuss the roles that eNOS, NAD(P)Hox and capillary density play in these mechanisms. The reader should be aware that this review starts from two premises. The first is that increases in insulin, resulting from meal ingestion and achieved during a hyperinsulinemic euglycemic clamp, lead to insulin-mediated activation of eNOS and subsequent increases in nitric oxide (NO) production in the terminal arterioles of human skeletal muscle. This increase in NO production leads to vasodilatation of the common terminal arterioles, in previously unperfused microvascular units (MVUs) (Fig. 2 in Wagenmakers *et al.* (2015b)), and a coordinated increase in the blood supply to the capillaries served by this terminal arteriole. The second premise is that this mechanism is impaired in obesity, sedentary elderly individuals, insulin resistant states and type 2 diabetes. For a complete and nuanced discussion of the experimental evidence in support of these premises the reader is referred to two of the other reviews from this symposium (Keske *et al.*, 2015; Wagenmakers *et al.*, 2015a), published in this issue of the Journal of Physiology, and earlier reviews of Barrett et al. (2009; 2011; 2014). However, to present a balanced argument the reader is also referred to the confounding model proposed by Professor Poole and colleagues, in which all capillaries in skeletal muscle are simultaneously perfused in resting and active muscle, but only over a fraction of their length, with longitudinal recruitment occurring following stimulation (Poole *et al.*, 2013; Poole, 2014). In writing this review the authors have chosen to prioritise research conducted in humans, with conclusions drawn from rodent or in vitro cell research used to fill in information that is currently absent from the published human literature.

Vasodilator response of muscle microcirculation and metabolic health

The skeletal muscle microcirculation is emerging as an important regulator of health and disease, because of its critical role in matching perfusion with metabolic demands of skeletal

muscle. Like skeletal muscle itself the microcirculation is highly responsive to changes in lifestyle with substantial improvements in microvascular structure and function occurring. Recent work has established the critical role that muscle perfusion plays in health and disease (Murdolo *et al.*, 2008), with an inability of perfusion to meet local metabolic demands emerging as a key factor in the development of skeletal muscle insulin resistance (Barrett *et al.*, 2011; Keske *et al.*, 2015), anabolic resistance leading to sarcopenia and frailty (Wagenmakers *et al.*, 2006; Mitchell *et al.*, 2013), and exercise intolerance (Frisbee *et al.*, 2011; Frisbee *et al.*, 2014).

Balance between NO production and NO quenching

A defining feature of these impairments is an altered balance between the formation of nitric oxide (NO) and the quenching of NO by superoxide anions and other reactive oxygen species (ROS) (Fig. 1). NO is primarily synthesised through the conversion of L-Arginine to L-Citrulline by NO synthase (NOS) (Alderton *et al.*, 2001). There are 3 isoforms of NOS (neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), with all 3 isoforms being expressed in skeletal muscle (Kobzik *et al.*, 1995). Experiments in isolated arteries and arterioles and cultured endothelial cells have shown that the primary source of endothelial NO synthesis is eNOS (McAllister & Laughlin, 2006). NO produced by the endothelium diffuses to the smooth muscle causing vasodilation, however, recent technical advances, allowing the sites of NO synthesis to be visualised, have suggested that as well as endothelial NO production NO synthesised in the vicinity of microvessels also influences smooth muscle NO exposure (Kavdia & Popel, 2004). Therefore, NO generated by nNOS in the muscle sarcolemma has taken on a more significant role in the regulation of skeletal muscle perfusion, in particular during exercise where specific inhibition of nNOS has been shown to reduce rat spinotrapezius blood flow during contraction (Copp *et al.*, 2011). Unlike skeletal muscle perfusion during exercise the significance of nNOS to insulin-mediated increases in microvascular blood flow are unknown, although insulin has been shown to activate nNOS in C2C12 myotubes and mouse skeletal muscle (Hinchee-Rodriguez *et al.*, 2013).

eNOS protein content and activity will together determine the total endothelial NO production. Control of eNOS activation is complex with a number of posttranslational

modifications regulating its activation. As increases in insulin concentration and fluid shear stress lead to eNOS phosphorylation at ser^{1177} (human enzyme), both *in vitro* in cultured endothelial cells (Rafikov *et al.*, 2011) and *in vivo* in the endothelium of human skeletal muscle (Cocks *et al.*, 2012), this is now regarded to be an important site for NO regulation. As well as NO formed by NOS there are a number of non-enzymatic sources of NO production that may contribute to smooth muscle NO exposure. These sources include NO generated from reduction of plasma nitrite (Lundberg *et al.*, 2011) and NO released from red blood cells, where it binds reversibly to haemoglobin (Baskurt *et al.*, 2011).

There are several sources of superoxide anions and ROS in skeletal muscle and its microvasculature that may influence NO availability through the scavenging of NO (Powers *et al.*, 2011; Li *et al.*, 2013). The primary location of ROS production affecting smooth muscle NO exposure is unknown, but it is likely that vascular and perivascular sources interact. There are a number of potential sources of ROS in the skeletal muscle and its vasculature (Jackson *et al.*, 2007), however NAD(P)Hox is assumed to be the major source of superoxide anion production and NO scavenging in skeletal muscle and vascular cells. For more detailed information on alternative sources see Gliemann et al. (2014).

Mechanism leading to impaired insulin-mediated vasodilation of the skeletal muscle microvascuature in sedentary and obese state

Impaired insulin-mediated glucose uptake is the condition where the role of microvascular NO bioavailability in disease progression is best defined (Barrett *et al.*, 2009; Keske *et al.*, 2015). Using the non-specific NOS inhibitor L-NAME Vincent et al. (2004) showed that the microvascular action of insulin is NO mediated in rat skeletal muscle. This study showed that the increase in microvascular blood volume observed following insulin administration was blocked by NOS inhibition and resulted in a reduced muscle glucose uptake. Subsequent studies have shown that increases in microvascular blood volume are impaired in obese men and women following a liquid meal, containing carbohydrate (320kcal), fat (72kcal) and protein (80kcal)(Keske *et al.*, 2009). The proposed mechanism was that reductions in in NO production in response to increases in plasma insulin and elevated superoxide (O_2^-) production leading to NO scavenging together reduce NO production in terminal arterioles of skeletal muscle (Wagenmakers *et al.*, 2006). This mechanism would not only explain the

impaired insulin-mediated increases in microvascular blood volume in skeletal muscle of sedentary and obese adults, but also make a major contribution to the insulin resistance seen in obesity (Sjöstrand *et al.*, 2002; Keske *et al.*, 2009) and type II diabetes (Gudbjornsdottir *et al.*, 2005; Clerk *et al.*, 2007).

Additional mechanisms regulating insulin-mediated skeletal muscle capillary recruitment

Although the studies mentioned above suggest that impairments in insulin-mediated NO production and quenching of NO by superoxide anions contribute to the reduction in insulin-induced vasodilation and capillary recruitment, there, is evidence that several other mechanisms contribute to the regulation of vasodilation in response to insulin. Although a detailed discussion of all of these mechanisms is beyond the scope of this review, the most important ones are described here. In addition to its ability to stimulate NO production insulin also stimulates expression of the potent vasoconstrictor endothelin 1 (ET1). ET1 expression opposes the vasodilator action of NO, and the balance between NO production and ET1 expression is key to insulin-mediated capillary recruitment (Ergul, 2011). ET1 mediates it's effects through two receptors (ET_A and ET_B), with the ratio and location of these receptors determining the net response to ET1 expression. ET_A and ET_B receptors found on smooth muscle are responsible for the vasoconstrictor and proliferative response to ET1, while ET_B receptors located on endothelial cells mediate vasodilation via NO and prostacyclin (Ergul, 2011). Angiotensin II can interact with the insulin signalling pathway to regulate insulin sensitivity (Chai *et al.*, 2011). Angiotensin II has 2 receptors in the skeletal muscle microvasculature (type I (AT1R) and type II (AT2R)). Chai et al. (2011) have shown that AT_2R blockade in rats, with a specific antagonist, abolished insulin-mediated increases in microvascular blood volume and decreased insulin-stimulated glucose disposal, while AT1R blockade increased muscle microvascular blood volume 2 to 3-fold. Finally, the arachidonic acid metabolite thromboxane A_2 is a vasoconstrictor and has also been proposed to contribute to impaired functional vasodilation in insulin resistance (Xiang *et al.*, 2008).

Role of capillary density in meal induced muscle perfusion and nutrient uptake

Apart from differences in NO bioavailability there are also substantial differences in capillary density between different populations. The latter will also have a significant impact on

insulin-mediated increases in perfusion of the skeletal muscle microvascular bed, as both variables contribute to the capillary surface area that is available for transport of glucose and insulin. Bonner et al (2013) have recently shown that capillary rarefaction directly contributes to skeletal muscle insulin resistance using a genetic reduction of capillary density in mice (skeletal and cardiac muscle VEGF deletion). Interestingly, it appears that reduced NO bioavailability also plays a critical role in capillary rarefaction, with Frisbee *et al*. (2005) showing that increasing NO bioavailability could significantly attenuate the capillary rarefaction seen in the obese insulin resistant state. In this study chronic administration of the antioxidant tempol increased microvascular density in obese Zucker rats, however, when tempol ingestion was combined with the NO synthase inhibitor L-NAME the beneficial effect was prevented, therefore suggesting that chronic reductions in NO bioavailability not oxidative stress per se contributed to microvascular rarefaction.

Exercise training to improve insulin sensitivity

Exercise training is known to be beneficial in the treatment and prevention of insulin resistance, due primarily to its effect on skeletal muscle insulin sensitivity (Hawley & Lessard, 2008). Traditional early research focussing on the skeletal muscle fibres has generated convincing evidence that this effect is at least partially mediated by mechanisms that operate in the skeletal muscle fibres (Hawley & Lessard, 2008). Briefly, studies have shown that training results in increased skeletal muscle GLUT4 content, elevation in the content and insulin-mediated activation of key intermediates in the insulin signalling cascade, up-regulation of AMP-activated protein kinase (AMPK) and improvements in lipid turnover and utilisation, which, likely combine to cause the improvements in skeletal muscle insulin sensitivity seen following training (for a thorough review of this topic see Hawley and Lessard (2008)).

Given the important role that the skeletal muscle microvasculature plays in glucose uptake it would seem likely that training induced adaptations within the microvasculature will also contribute to the improvements in skeletal muscle insulin sensitivity seen with training. The remainder of this review will therefore focus on how exercise training can influence skeletal muscle microvascular NO bioavailability and capillary density, and how these changes may contribute to improved skeletal muscle insulin sensitivity following training. We will

therefore examine recent work from our own group and other laboratories into the effect of exercise training interventions on 1) microvascular vasodilator function and NO bioavailability, 2) the enzymes responsible for NO synthesis and quenching, and 3) capillary density and how the observed exercise training adaptations may influence insulin sensitivity and the future development of metabolic disease.

Exercise training to improve microvascular function and NO bioavailability

Due to ease of access and the availability of non-invasive techniques to study cutaneous (skin) microvascular perfusion, the skin has been used as a surrogate for skeletal muscle in humans (Meijer *et al.*, 2012). As such, much of the knowledge on impairments in obesity and insulin resistance and also on exercise training effects has been obtained in the microcirculation of the skin.

Studies using laser Doppler flowmetry combined with heating and intradermal microdialysis of the NO synthase inhibitor L-NMMA have shown that endurance training is an effective method to improve cutaneous NO-mediated vasodilator function in high-risk prediabetic patients (polycystic ovary syndrome and nonalcoholic fatty liver disease) (Pugh *et al.*, 2013; Sprung *et al.*, 2013). However, there are no studies which directly measure insulin-mediated NO bioavailability or insulin-mediated increases in cutaneous capillary blood flow following training. Meijer et al. (2012) have shown that insulin-mediated capillary recruitment in skin parallels the insulin-mediated response of the skeletal muscle microvasculature, however, this conclusion has received criticism from Poole et al. (Poole *et al.*, 2013). In particular the authors of this review express concern as to whether the effect of training on the skin and skeletal muscle microvasculature may be different given the different roles that these microvascular beds play in the exercise response (temperature regulation versus delivery of oxygen and nutrients and removal of waste products).

Despite its clear importance only one study (Rattigan *et al.*, 2001) has investigated the effect of training on skeletal muscle capillary recruitment and glucose uptake. This was a short term (14 days) endurance training study in previously sedentary rats. The exercise training led to parallel increases, during a hyperinsulinemic euglycemic clamp, in skeletal muscle microvascular perfusion (+62%; measured as hindleg 1-methylxanthine (1-MX) clearance)

and hindleg glucose uptake (+93%) (Rattigan *et al.*, 2001). This observation suggests that endurance exercise training improved delivery of insulin and glucose to skeletal muscle during the clamp, via a mechanism involving an increase in insulin-mediated dilation of terminal arterioles and increased NO bioavailability. However, studies using physiological measures of capillary recruitment do not provide information on whether increases in capillary density or NO production by eNOS or decreases in ROS production, leading to reduced NO quenching, are behind the increase in microvascular perfusion and hindleg glucose uptake.

Effect of endurance training on capillary density

The potential of endurance training to increase skeletal muscle capillarization has been known for a long time with the first study in humans conducted by Andersen and Henriksson (1977). The study showed that capillary density was increased by 8wk of endurance training in previously sedentary males. This finding has been consistently replicated (Ingjer, 1979; Cocks *et al.*, 2013) and the mechanisms responsible for increases in capillary density have been reviewed in depth in a recent review (Hoier & Hellsten, 2014). However, much less is known about the efficiency of exercise training to increase capillary density in groups with an impaired muscle microvascular vasodilator response. As the ability of capillary shear stress to increase angiogenesis depends on its ability to activate eNOS and increase endothelial NO bioavailability (Hudlicka *et al.*, 2006), the authors of this review hypothesized that due to the elevated ROS production in sedentary obese, elderly individuals and metabolic syndrome patients (all pre-diabetic conditions) the exercise-induced mechanisms leading to angiogenesis would be impaired. However, a recent study from the author's laboratory showed that 4 weeks of endurance training was enough to increase capillary density in young obese males (Cocks *et al.*, 2015). These increases in capillary density will increase the surface area available for transport of insulin and glucose, and therefore are likely to contribute to the improvements in insulin sensitivity that were seen following training. Akerstrom *et al. (2014)* have recently confirmed the importance of increased capillary density as a mechanism that contributes to increases in insulin sensitivity. In this study, conducted in rats, skeletal muscle specific increases in capillary density were induced by prolonged (21 days) ingestion of the α1-adrenergic receptor agonist Prazosin. The resultant elevation in skeletal muscle capillary density was associated

with a 30% increase in insulin-mediated skeletal muscle glucose disposal, without concomitant improvements in skeletal muscle insulin signalling.

Methods to measure the response of microvascular enzymes in skeletal muscle to training

As discussed above a number of cellular locations contribute to the production of NO and quenching of NO by ROS and, therefore, have an impact on the amount of NO seen by the vascular smooth muscle. Therefore, the authors feel that it is essential that the methods used to measure the protein content of the responsible enzymes are able to investigate the localisation of these enzymes. To gain a full understanding of the effect of training on NO bioavailability the training induced changes in the relevant enzymes should be studied in the skeletal muscle sarcolemma, arterioles and capillaries. Although useful to provide an overview of the changes brought about by training traditional methods using tissue homogenization (followed by Western blots) do not allow for this type of analysis, and cannot provide information on the different vascular sources of NO and ROS production which will affect smooth muscle NO availability. In addition, as exercise training interventions lead to increases in capillary density (Andersen & Henriksson, 1977; Ingjer, 1979; Cocks *et al.*, 2013), analytical methods using tissue homogenates cannot differentiate between an increase in eNOS content of the endothelial layer and an increase in total eNOS content of the homogenate brought about by an increase in microvascular density. Due to these technical limitations of Western blots when applied to whole tissue homogenates, the authors laboratory has developed quantitative immunofluorescence microscopy methods to specifically measure enzyme content and phosphorylation state of enzymes present in the endothelial layer of skeletal muscle microvessels (Cocks *et al.*, 2012) (Fig. 2). This technique has recently been advanced to allow for the differentiation of terminal arterioles and capillaries (Cocks, 2013b; Cocks *et al.*, 2015).

Effect of endurance training on endothelial enzymes controlling NO bioavailability

Using quantitative immunofluorescence the authors have shown that endurance training increases mixed microvascular (terminal arterioles and capillaries) eNOS content in lean sedentary individuals (Cocks *et al.*, 2013) (Fig. 3). Work from the authors also shows that endurance training increases eNOS content specifically in the terminal arterioles (identified with anti-alpha smooth muscle actin) and capillaries of obese males (Cocks *et al.*, 2015). The

increase in eNOS content in both of these studies is associated with improved insulin sensitivity following training. It has recently been observed that an experimental increase in endothelial eNOS content (in vivo administration of bera-prost sodium, a prostaglandin I2 analogue that stimulates eNOS mRNA expression and protein synthesis rate) led to significant insulin-mediated increases in *in vivo* skeletal muscle capillary recruitment, insulin transport into skeletal muscle interstitium and skeletal muscle glucose uptake in several insulin resistant mouse models (Kubota *et al.*, 2011). As such, it is hypothesised that the increase in eNOS content following training contributes to the improved insulin sensitivity following training, through elevated NO production in response to insulin improving capillary recruitment and therefore the delivery of insulin and glucose to the skeletal muscle. Using western blotting in whole muscle homogenates McConnel et al. (2007) have also shown that nNOS protein content is increased by 10 days of intensive endurance training. Although insulin sensitivity was not measured in this study, elevated skeletal muscle nNOS content may contribute to the increase in insulin-mediated smooth muscle NO availability and therefore improve insulin sensitivity.

The authors have previously also shown that 6 weeks of endurance training significantly reduces basal and exercise stimulated eNOS ser^{1177} phosphorylation in the endothelium of the muscle microvasculature in previously sedentary men (Cocks *et al.*, 2013) (Fig. 3). This finding is supported by the work of Gliemann *et al*. (2013) in aged men, where eNOS ser^{1177} phosphorylation was also reduced following 8 weeks of endurance training, measured in whole muscle homogenates via western blotting. This decrease in eNOS phosphorylation has previously been attributed to a reduction in the shear stress stimulus by Cocks et al. (2013), as an increase in capillary density with training could distribute the blood flow over more capillaries both at rest and during 1 h of endurance exercise at 65% of the pre-training VO_{2peak}. The authors have more recently shown that eNOS ser^{1177} phosphorylation is increased under resting conditions in young obese men following training (Cocks *et al.*, 2015), although this increase was abolished when normalised to the increase in eNOS content. Although the programme was shorter (4 weeks versus 6 weeks) and the increase in capillary density was lower (19% versus 32%) the increase in capillary density was still significant. All the training studies discussed have only measured basal eNOS ser^{1177}, implying that future studies should measure the effect of training on insulin-mediated eNOS

phosphorylation to investigate whether increases in the ability of insulin to activate eNOS via ser^{1177} contribute to the improved insulin sensitivity following training. The regulation of eNOS activation is complex involving a number of molecular events (for a complete review see Fleming (2010)), and multiple phosphorylation sites (not just ser^{1177}). However, to the author's knowledge none of these additional mechanisms have been investigated in human muscle in response to a meal, exercise or before and after training. To fully elucidate the effects of training on NO production effort should be made to investigate these other mechanisms underlying eNOS activation.

The authors and other groups have also begun to investigate the effect of training on NAD(P)Hox, in particular NOX2 which is the catalytic subunit of NAD(P)Hox. In lean sedentary individuals the authors have shown that endothelial specific NOX2 content was unchanged by training (Fig. 3), although there was a trend for increased sarcolemma-associated NOX2 content. A significant increase in NOX2 content has however been observed in an aged group following training, using western blotting in whole muscle homogenates (Gliemann *et al.*, 2013). As such, NOX2 may represent an enzyme for which quantitative immunofluorescence microscopy methods that quantify enzymes in the endothelial layer of the muscle microvasculature and the sarcolemma are particularly important, given that the endothelial cell volume is only a fraction of the skeletal muscle fibre volume (Barrett & Liu, 2013). Unlike training in young sedentary or aged individuals endurance training has been shown to reduce endothelial specific NOX2 content in obese individuals, without changing sarcolemma-associated NOX2 content (Cocks et al 2015). As there was an increase in eNOS content in this group this led to an improved balance in the eNOS/NOX2 protein ratio which should increase NO bioavailability due to increased NO production and reduced NO scavenging by O_2^--anions. The above discussion focuses on the content of NOX2. However, in addition to the content of the subunits that form NAD(P)Hox, activation of the complex should also be considered if the effect of training on O_2^- production is to be fully understood.

In addition to reducing ROS production training may also upregulate endogenous antioxidant systems increasing the removal of ROS. Pierce et al. (2011) showed that

endothelial cell manganese SOD and endothelium bound extracellular SOD were both higher in older exercising men than sedentary controls.

Other vasoactive mechanisms that operate in the muscle microvasculature

As discussed above a number of other enzymes may also contribute to the exercise training induced improvements in skeletal muscle endothelial function and capillary recruitment. However, the effect of training on these systems has received far less attention. A recent study using Western blot methodology applied to muscle extracts has shown that training does not alter the protein content of thromboxane synthase and ET 1, but does increase ET receptor A in aged men (Gliemann *et al.*, 2013).

High Intensity Interval Training a time-efficient alternative mode of exercise training

Although a number of studies have shown beneficial effects of supervised endurance based training programmes, the long term feasibility of such programmes, particularly in obesity and type II diabetes, has been questioned (Marwick *et al.*, 2009). It is for this reason that the development of alternative practical models of training are essential. Recently sprint interval training (SIT), which is the most intense mode of high intensity interval training (HIT), has received much attention as an alternative training strategy, due to its ability to mirror or even surpass the effects of traditional endurance training in a fraction of the time (Gibala *et al.*, 2012). Given that the most commonly cited barrier to physical activity is lack of time, it is thought that SIT may represent an exercise training mode that is more attractive to exercise naive individuals and, therefore, may increase sport and exercise participation. Most of the current research into SIT has focussed on the molecular and metabolic adaptations in skeletal muscle fibres. However, two recent studies by the authors (Cocks *et al.*, 2013; Cocks *et al.*, 2015) have shown that SIT also represents an effective training method to improve skeletal muscle microvascular density and the endothelial eNOS/NAD(P)Hox protein ratio. Cocks *et al*. (2013) showed that 6 weeks of SIT led to a significantly greater increase in eNOS content than endurance training in young sedentary males, and Cocks *et al*. (2015) observed similar increases in eNOS content in the endothelial layer of terminal arterioles and capillaries after 4 weeks of constant workload SIT compared to endurance training in young sedentary obese males. Hoier et al. (2013) have also shown eNOS content to be increased following 4 weeks of HIT in healthy young males, using tissue

homogenates and western blotting. Cocks et al. (2015) have also shown that endothelial NOX2 content is reduced by SIT to a similar extent as endurance training in young obese males. In conjunction with these enzyme changes SIT was also as effective as endurance training at increasing capillary density (Cocks et al., 2013; Cocks *et al*. 2015). Jensen et al. (2004) have also demonstrated that HIT leads to increases in capillary density, with a similar increase occurring around type I and type II muscle fibres. Unlike these studies Hoier et al. (2013) did not observe an increase in capillary density in healthy young men following 4 weeks of HIT. However, this HIT training period was preceded by a 4 week endurance training preconditioning period (resulting in a 17% increase in capillary density), and the authors hypothesised that the lack of continued capillary growth may result from phasic growth of capillaries in response to training. Interestingly, this study also investigated the angiogenic response to an acute bout of HIT and endurance training following the preconditioning period. It was observed that HIT led to lower interstitial VEGF-A levels and less stimulus for endothelial cell proliferation than endurance training, leading to the conclusion that HIT leads to a weaker angiogenic stimulus than endurance training.

These studies add to the growing body of literature, showing that SIT and other forms of HIT are a time efficient alternative to endurance training, as advised by the WHO, to achieve many of the beneficial molecular and metabolic adaptations in skeletal muscle (similar increases in insulin sensitivity (Cocks *et al.*, 2013), aerobic capacity (Rakobowchuk *et al.*, 2008), mitochondrial biogenesis and mitochondrial enzyme activity (Burgomaster *et al.*, 2008), fat oxidation during exercise (Burgomaster *et al.*, 2008) and intramuscular triglyceride storage and utilisation during exercise (Shepherd *et al.*, 2013)(for a comprehensive review see Gibala *et al.* (2012)) and its microvasculature seen following endurance training. However, future studies need to focus on the development of practical forms of HIT that can feasibly be adopted by a wide range of populations.

Additional benefits of resistance training

Resistance training is encouraged in elderly individuals and those with obesity, metabolic syndrome and type II diabetes because of its unique ability to maintain/ increase muscle mass and strength, variables that are strongly related to the promotion and maintenance of independence and health (Haskell *et al.*, 2007). Resistance training (RT) is also known to

improve insulin sensitivity (Miller *et al.*, 1994; Ibanez *et al.*, 2005), and as such forms a fundamental component of the American College of Sports Medicine guidelines for physical activity and public health (Haskell *et al.*, 2007). The benefits of RT on insulin sensitivity have previously been attributed to the increases in muscle mass (Szczypaczewska *et al.*, 1989; Miller *et al.*, 1994) and improvements in elements of the insulin signalling cascade within the skeletal muscle (Holten *et al.*, 2004), with little known about the potential contributions of the vasculature. A number of studies have investigated angiogenesis following RT (Hather *et al.*, 1991; McCall *et al.*, 1996; Green *et al.*, 1999; Cocks *et al.*, 2014). The results of these studies suggest that RT does not increase capillary density, but can increase capillary to fibre ratio in proportion to fibre growth. Therefore, RT does stimulate angiogenesis, but this is to prevent an increase in the oxygen diffusion distance resulting from the increase in fibre size caused by RT. However, it is unlikely that this will affect insulin sensitivity as capillary density and capillary to fibre perimeter exchange are not changed by RT (Cocks *et al.*, 2014). The latter is a valuable measure of microvascular density which provides more information on the capacity for transport of substances that rely on receptor or transporter-mediated processes (i.e. insulin and glucose). A recent study by the authors has also shown that, unlike endurance training and SIT, RT does not induce significant increases in endothelial specific eNOS content (although a trend for an increase was present $P = 0.091$), in previously sedentary men (Cocks *et al.*, 2014). Putting these microvascular effects together with the lack of change in endothelial dependent dilation of conduit arteries seen following RT (Rakobowchuk *et al.*, 2005) it would appear that, in previously sedentary individuals, RT's positive effects on insulin sensitivity are not mediated by haemodynamic adaptations. Future work should focus on the effect of RT in at risk groups, where microvascular adaptations may become apparent. However, given the debilitating effects of sarcopenia on independence in both elderly and obese individuals the unique benefits of resistance training on muscle mass and strength should not be overlooked. As such, the authors suggest that the combination of endurance training or SIT with RT may lead to optimal metabolic, performance and health benefits, with ET or SIT providing a powerful means to increase capillary density, microvascular NO bioavailability and aerobic exercise capacity (endurance) and RT being the most efficient means to increase muscle mass and strength. The complimentary effects of these training modes would then maximise the effect of

training on insulin sensitivity and glucose uptake, while promoting mobility and independence through increases in muscle mass.

Conclusion

Analytical methods developed in our laboratory together with Western blot data generated by other laboratories suggest that endurance training and SIT increase capillary density and the eNOS/NAD(P)Hoxidase protein ratio. Our assumption is that the eNOS/NAD(P)Hoxidase protein ratio within the endothelium of terminal arterioles is a marker of the vasodilator capacity of these vessels within skeletal muscle and that this molecular adaptation is likely to improve skeletal muscle microvascular perfusion in response to insulin. The latter mechanism then is also likely to contribute to the well-known benefits of training on insulin sensitivity. To validate the assumption that an increase in eNOS/NAD(P)Hoxidase protein ratio is a marker of the vasodilator capacity of the skeletal muscle terminal arterioles, future training studies in healthy individuals and insulin resistant states should 1) make parallel measurements of the insulin induced vasodilator response, eNOS and NADPHox protein content and activation status, and muscle glucose uptake and 2) measure the protein and activity status of eNOS, nNOS, NOX isomers and other sources of ROS production not only in the endothelial layer of terminal arteries and capillaries, but also in vascular smooth muscle and the muscle fibres.

References

Akerstrom T, Laub L, Vedel K, Brand CL, Pedersen BK, Lindqvist AK, Wojtaszewski JF & Hellsten Y. (2014). Increased skeletal muscle capillarization enhances insulin sensitivity. *American Journal of Physiology-Endocrinology and Metabolism***,** ajpendo. 00020.02014.

Alderton W, Cooper C & Knowles R. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochem J* **357,** 593-615.

Andersen P & Henriksson J. (1977). Capillary supply of quadriceps femoris muscle of man- Adapative response to exercise. *Journal of Physiology* **270,** 677-690.

Barrett EJ, Eggleston EM, Inyard AC, Wang H, Li G, Chai W & Liu Z. (2009). The vascular actions of insulin control its delivery to muscle and regulate the rate-limiting step in skeletal muscle insulin action. *Diabetologia* **52,** 752-764.

Barrett EJ, Keske MA, Rattigan S & Eringa EC. (2014). CrossTalk proposal: De novo capillary recruitment in healthy muscle is necessary. *The Journal of physiology* **592,** 5129-5131.

Barrett EJ & Liu ZQ. (2013). The endothelial cell: An "early responder" in the development of insulin resistance. *Reviews in Endocrine & Metabolic Disorders* **14,** 21-27.

Barrett EJ, Wang H, Upchurch CT & Liu Z. (2011). Insulin regulates its own delivery to skeletal muscle by feed-forward actions on the vasculature. *American Journal of Physiology-Endocrinology and Metabolism* **301,** E252-E263.

Baskurt OK, Ulker P & Meiselman HJ. (2011). Nitric oxide, erythrocytes and exercise. *Clinical hemorheology and microcirculation* **49,** 175-181.

Bonner JS, Lantier L, Hasenour CM, James FD, Bracy DP & Wasserman DH. (2013). Muscle-specific vascular endothelial growth factor deletion induces muscle capillary rarefaction creating muscle insulin resistance. *Diabetes* **62,** 572-580.

Burgomaster KA, Howarth KR, Phillips SM, Rakobowchuk M, MacDonald MJ, McGee SL & Gibala MJ. (2008). Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. *Journal of Physiology-London* **586,** 151-160.

Chai W, Wang W, Dong Z, Cao W & Liu Z. (2011). Angiotensin II receptors modulate muscle microvascular and metabolic responses to insulin in vivo. *Diabetes* **60,** 2939-2946.

Clerk LH, Vincent MA, Barrett EJ, Lankford MF & Lindner JR. (2007). Skeletal muscle capillary responses to insulin are abnormal in late-stage diabetes and are restored by angiogensin-converting enzyme inhibition. *American Journal of Physiology-Endocrinology and Metabolism* **293,** E1804-E1809.

Cocks M, Shaw CS, Shepherd SO, Fisher JP, Ranasinghe A, Barker TA, Tipton KD & Wagenmakers AJ. (2014). Effect of resistance training on microvascular density and eNOS content in skeletal muscle of sedentary men. *Microcirculation* **DOI: 10.1111/micc.12155**.

Cocks M, Shaw CS, Shepherd SO, Fisher JP, Ranasinghe A, Barker TA & Wagenmakers AJ. (2015). Sprint interval and moderate-intensity continuous training have equal benefits on aerobic capacity, insulin sensitivity, muscle capillarisation and endothelial eNOS/NAD (P) Hoxidase protein ratio in obese men. *The Journal of Physiology* **DOI: 10.1113/jphysiol.2014.285254**.

Cocks M, Shaw CS, Shepherd SO, Fisher JP, Ranasinghe AM, Barker TA, Tipton KD & Wagenmakers AJM. (2013). Sprint interval and endurance training are equally effective in increasing muscle microvascular density and eNOS content in sedentary males. *The Journal of Physiology* **591,** 641-656.

Cocks M, Shepherd SO, Shaw CS, Achten J, Costa ML & Wagenmakers AJM. (2012). Immunofluorescence Microscopy to Assess Enzymes Controlling Nitric Oxide Availability and Microvascular Blood Flow in Muscle. *Microcirculation* **19,** 642-651.

Cocks M, Stride, A. Macdonald, R., Shaw, C S., Marshall, J.M., Poucher, A.M., Wagenmakers, A.J.M. (2013b). Impaired Insulin-Mediated Ser1176 Phosphorylation of eNOS in Skeletal Muscle Arterioles of Obese Zucker Rats. *Obesity Facts* **6**.

Copp SW, Hirai DM, Ferguson SK, Musch TI & Poole DC. (2011). Role of neuronal nitric oxide synthase in modulating microvascular and contractile function in rat skeletal muscle. *Microcirculation* **18,** 501-511.

Ergul A. (2011). Endothelin-1 and diabetic complications: focus on the vasculature. *Pharmacological Research* **63,** 477-482.

Fleming I. (2010). Molecular mechanisms underlying the activation of eNOS. *Pflügers Archiv-European Journal of Physiology* **459,** 793-806.

Frisbee JC. (2005). Reduced nitric oxide bioavailability contributes to skeletal muscle microvessel rarefaction in the metabolic syndrome. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **289,** R307-R316.

Frisbee JC, Goodwill AG, Butcher JT & Olfert IM. (2011). Divergence between arterial perfusion and fatigue resistance in skeletal muscle in the metabolic syndrome. *Experimental Physiology* **96,** 369-383.

Frisbee JC, Goodwill AG, Frisbee SJ, Butcher JT, Wu F & Chantler PD. (2014). Microvascular perfusion heterogeneity contributes to peripheral vascular disease in metabolic syndrome. *The Journal of physiology* **DOI: 10.1113/jphysiol.2014.285247**.

Gibala MJ, Little JP, Macdonald MJ & Hawley JA. (2012). Physiological adaptations to low-volume, high-intensity interval training in health and disease. *The Journal of physiology* **590,** 1077-1084.

Gliemann L, Nyberg M & Hellsten Y. (2014). Nitric oxide and reactive oxygen species in limb vascular function: what is the effect of physical activity? *Free radical research* **48,** 71-83.

Gliemann L, Schmidt JF, Olesen J, Biensø RS, Peronard SL, Grandjean SU, Mortensen SP, Nyberg M, Bangsbo J & Pilegaard H. (2013). Resveratrol blunts the positive effects of exercise training on cardiovascular health in aged men. *The Journal of physiology* **591,** 5047-5059.

Green H, Goreham C, Ouyang J, Ball-Burnett M & Ranney D. (1999). Regulation of fiber size, oxidative potential, and capillarization in human muscle by resistance exercise. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **276,** R591-R596.

Gudbjornsdottir S, Sjostrand M, Strindberg L & Lonnroth P. (2005). Decreased muscle capillary permeability surface area in type 2 diabetic subjects. *Journal of Clinical Endocrinology & Metabolism* **90,** 1078-1082.

Haskell WL, Lee IM, Pate RR, Powell KE, Blair SN, Franklin BA, Macera CA, Heath GW, Thompson PD & Bauman A. (2007). Physical activity and public health - Updated recommendation for adults from the American college of sports medicine and the American heart association. *Circulation* **116,** 1081-1093.

Hather BM, Tesch PA, Buchanan P & Dudley GA. (1991). Influence of eccentric actions on skeletal-muscle adaptations to resistance training. *Acta Physiologica Scandinavica* **143,** 177-185.

Hawley J & Lessard S. (2008). Exercise training-induced improvements in insulin action. *Acta physiologica* **192,** 127-135.

Hinchee-Rodriguez K, Garg N, Venkatakrishnan P, Roman MG, Adamo ML, Masters BS & Roman LJ. (2013). Neuronal nitric oxide synthase is phosphorylated in response to insulin stimulation in skeletal muscle. *Biochemical and biophysical research communications* **435,** 501-505.

Hoier B & Hellsten Y. (2014). Exercise-Induced Capillary Growth in Human Skeletal Muscle and the Dynamics of VEGF. *Microcirculation* **21,** 301-314.

Hoier B, Passos M, Bangsbo J & Hellsten Y. (2013). Intense intermittent exercise provides weak stimulus for vascular endothelial growth factor secretion and capillary growth in skeletal muscle. *Experimental physiology* **98,** 585-597.

Holten MK, Zacho M, Gaster M, Juel C, Wojtaszewski JFP & Dela F. (2004). Strength training increases insulin-mediated glucose uptake, GLUT4 content, and insulin signaling in skeletal muscle in patients with type 2 diabetes. *Diabetes* **53,** 294-305.

Hudlicka O, Brown MD, May S, Zakrzewicz A & Pries AR. (2006). Changes in capillary shear stress in skeletal muscles exposed to long-term activity: Role of nitric oxide. *Microcirculation* **13,** 249-259.

Ibanez J, Izquierdo M, Arguelles N, Forga L, Larrion JL, Garcia-Unciti M, Idoate F & Gorostiaga EM. (2005). Twice-weekly progressive resistance training decreases abdominal fat and improves insulin sensitivity in older men with type 2 diabetes. *Diabetes Care* **28,** 662-667.

Ingjer F. (1979). Effects of endurance training on muscle-fibre ATPase activity, capillary supplu and mitochondrial content in man. *Journal of Physiology-London* **294,** 419-432.

Jackson MJ, Pye D & Palomero J. (2007). The production of reactive oxygen and nitrogen species by skeletal muscle. *Journal of Applied Physiology* **102,** 1664-1670.

Jensen L, Bangsbo J & Hellsten Y. (2004). Effect of high intensity training on capillarization and presence of angiogenic factors in human skeletal muscle. *Journal of Physiology-London* **557,** 571-582.

Kavdia M & Popel AS. (2004). Contribution of nNOS-and eNOS-derived NO to microvascular smooth muscle NO exposure. *Journal of Applied Physiology* **97,** 293-301.

Keske MA, Clerk LH, Price WJ, Jahn LA & Barrett EJ. (2009). Obesity Blunts Microvascular Recruitment in Human Forearm Muscle After a Mixed Meal. *Diabetes Care* **32,** 1672-1677.

Keske MA, Premilovac D, Bradley EA, Dwyer RM, Richards SM & Rattigan S. (2015). Muscle microvascular blood flow responses in insulin resistance and ageing. *The Journal of Physiology* **DOI: 10.1113/jphysiol.2014.283549**.

Kobzik L, Stringer B, Balligand J-L, Reid MB & Stamler JS. (1995). Endothelial-type nitric oxide synthase (ec-NOS) in skeletal muscle fibers: mitochondrial relationships. *Biochemical and biophysical research communications* **211,** 375-381.

Kubota T, Kubota N, Kumagai H, Yamaguchi S, Kozono H, Takahashi T, Inoue M, Itoh S, Takamoto I, Sasako T, Kumagai K, Kawai T, Hashimoto S, Kobayashi T, Sato M, Tokuyama K, Nishimura S, Tsunoda M, Ide T, Murakami K, Yamazaki T, Ezaki O, Kawamura K, Masuda H, Moroi M, Sugi K, Oike Y, Shimokawa H, Yanagihara N, Tsutsui M, Terauchi Y, Tobe K, Nagai R, Kamata K, Inoue K, Kodama T, Ueki K & Kadowaki T. (2011). Impaired Insulin Signaling in Endothelial Cells Reduces Insulin-Induced Glucose Uptake by Skeletal Muscle. *Cell Metabolism* **13,** 294-307.

Li H, Horke S & Förstermann U. (2013). Oxidative stress in vascular disease and its pharmacological prevention. *Trends in pharmacological sciences* **34,** 313-319.

Lundberg JO, Weitzberg E, Shiva S & Gladwin MT. (2011). The nitrate–nitrite–nitric oxide pathway in mammals. In *Nitrite and Nitrate in Human Health and Disease*, pp. 21-48. Springer.

Marwick TH, Hordern MD, Miller T, Chyun DA, Bertoni AG, Blumenthal RS, Philippides G & Rocchini A. (2009). Exercise training for type 2 diabetes mellitus impact on cardiovascular risk: a scientific statement from the American Heart Association. *Circulation* **119,** 3244-3262.

McAllister RM & Laughlin MH. (2006). Vascular nitric oxide: effects of physical activity, importance for health. *Essays in Biochemistry* **42,** 119-131.

McCall GE, Byrnes WC, Dickinson A, Pattany PM & Fleck SJ. (1996). Muscle fiber hypertrophy: Hyperplasia, and capillary density in college men after resistance training. *Journal of Applied Physiology* **81,** 2004-2012.

McConell GK, Bradley SJ, Stephens TJ, Canny BJ, Kingwell BA & Lee-Young RS. (2007). Skeletal muscle nNOSμ protein content is increased by exercise training in humans. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **293,** R821-R828.

Meijer RI, de Boer MP, Groen MR, Eringa EC, Rattigan S, Barrett EJ, Smulders YM & Serne EH. (2012). Insulin-Induced Microvascular Recruitment in Skin and Muscle are Related and Both are Associated with Whole-Body Glucose Uptake. *Microcirculation* **19,** 494-500.

Miller JP, Pratley RE, Goldberg AP, Gordon P, Rubin M, Treuth MS, Ryan AS & Hurley BF. (1994). Strength training increases insulin action in healthy 50-yr-old to 65-yer-old men. *Journal of Applied Physiology* **77,** 1122-1127.

Mitchell WK, Phillips BE, Williams JP, Rankin D, Smith K, Lund JN & Atherton PJ. (2013). Development of a new Sonovue contrast-enhanced ultrasound approach reveals temporal and age-related features of muscle microvascular responses to feeding. *Physiological reports* **1,** e00119-e00119.

Murdolo G, Sjostrand M, Strindberg L, Gudbjornsdottir S, Lind L, Lonnroth P & Jansson P-A. (2008). Effects of intrabrachial metacholine infusion on muscle capillary recruitment and forearm glucose uptake during physiological hyperinsulinemia in obese, insulin-resistant individuals. *The Journal of Clinical Endocrinology & Metabolism* **93,** 2764-2773.

Pierce GL, Donato AJ, LaRocca TJ, Eskurza I, Silver AE & Seals DR. (2011). Habitually exercising older men do not demonstrate age-associated vascular endothelial oxidative stress. *Aging cell* **10,** 1032-1037.

Poole DC. (2014). CrossTalk opposing view: De novo capillary recruitment in healthy muscle is not necessary to explain physiological outcomes. *The Journal of physiology* **592,** 5133-5135.

Poole DC, Copp SW, Ferguson SK & Musch TI. (2013). Skeletal muscle capillary function: contemporary observations and novel hypotheses. *Experimental physiology* **98,** 1645-1658.

Powers SK, Talbert EE & Adhihetty PJ. (2011). Reactive oxygen and nitrogen species as intracellular signals in skeletal muscle. *The Journal of physiology* **589,** 2129-2138.

Pugh CJ, Cuthbertson DJ, Sprung VS, Kemp GJ, Richardson P, Umpleby AM, Green DJ, Cable NT & Jones H. (2013). Exercise training improves cutaneous microvascular function in nonalcoholic fatty liver disease. *American Journal of Physiology-Endocrinology and Metabolism* **305,** E50-E58.

Rafikov R, Fonseca FV, Kumar S, Pardo D, Darragh C, Elms S, Fulton D & Black SM. (2011). eNOS activation and NO function: structural motifs responsible for the posttranslational control of endothelial nitric oxide synthase activity. *Journal of Endocrinology* **210,** 271-284.

Rakobowchuk M, McGowan CL, de Groot PC, Hartman JW, Phillips SM & MacDonald MJ. (2005). Endothelial function of young healthy males following whole body resistance training. *Journal of Applied Physiology* **98,** 2185-2190.

Rakobowchuk M, Tanguay S, Burgomaster KA, Howarth KR, Gibala MJ & MacDonald MJ. (2008). Sprint interval and traditional endurance training induce similar improvements in peripheral arterial stiffness and flow-mediated dilation in healthy humans. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **295,** R236-R242.

Rattigan S, Wallis MG, Youd JM & Clark MG. (2001). Exercise training improves insulin-mediated capillary recruitment in association with glucose uptake in rat hindlimb. *Diabetes* **50,** 2659-2665.

Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJM & Shaw CS. (2013). Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5. *Journal of Physiology-London* **591,** 657-675.

Sjöstrand M, Gudbjörnsdottir S, Holmäng A, Lönn L, Strindberg L & Lönnroth P. (2002). Delayed transcapillary transport of insulin to muscle interstitial fluid in obese subjects. *Diabetes* **51,** 2742-2748.

Sprung VS, Cuthbertson DJ, Pugh CJ, Daousi C, Atkinson G, Aziz N, Kemp GJ, Green DJ, Cable N & Jones H. (2013). Nitric oxide-mediated cutaneous microvascular function is impaired in

polycystic ovary sydrome but can be improved by exercise training. *The Journal of physiology* **591,** 1475-1487.

Szczypaczewska M, Nazar K & Kaciubauscilko H. (1989). Glucose-tolerance and insulin-response to glucose-load in body builders. *International Journal of Sports Medicine* **10,** 34-37.

Vincent MA, Clerk LH, Lindner JR, Klibanov AL, Clark MG, Rattigan S & Barrett EJ. (2004). Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake in vivo. *Diabetes* **53,** 1418-1423.

Wagenmakers AJ, Strauss JA, Shepherd SO, Keske MA & Cocks M. (2015a). Increased muscle blood supply and transendothelial nutrient and insulin transport induced by food intake and exercise: Effect of obesity and ageing. *The Journal of physiology* **DOI: 10.1113/jphysiol.2014.284513**.

Wagenmakers AJM, Strauss JA, Shepherd SO, Keske MA & Cocks M. (2015b). Increased muscle blood supply and transendothelial nutrient and insulin transport induced by food intake and exercise: Effect of obesity and ageing. *The Journal of Physiology* **DOI: 10.1113/jphysiol.2014.284513**.

Wagenmakers AJM, van Riel NAW, Frenneaux MP & Stewart PM. (2006). Integration of the metabolic and cardiovascular effects of exercise. *Essays in Biochemistry* **42,** 193-210.

Xiang L, Dearman J, Abram SR, Carter C & Hester RL. (2008). Insulin resistance and impaired functional vasodilation in obese Zucker rats. *American Journal of Physiology-Heart and Circulatory Physiology* **294,** H1658-H1666.

Additional Information

Competing Interests

The authors confirm that there are no conflicts of interest.

Funding

Funding for the referenced work of the authors was gratefully received from the Insulin Dependent Diabetes Trust.

Figure Legends

Figure 1. The balance between nitric oxide (NO) production and NO scavenging in the skeletal muscle microvasculature impacts human metabolic health.

Highly trained individuals have an optimal balance between eNOS and NAD(P)Hox protein content and activity in the skeletal muscle microvasculature, and combine high physical activity levels with a high exercise tolerance, high anabolic response to resistance exercise, and the highest reported levels of insulin sensitivity and glucose tolerance. In sedentary individuals this balance is less favourable and we hypothesize that it is because of this adaptation that the sedentary state comes with a high risk for the development of obesity, metabolic syndrome and type 2 diabetes, conditions in which a low NO bioavailability leads to severe impairments in insulin sensitivity, glucose tolerance, anabolic resistance, and exercise tolerance. Exercise training interventions of 4-6 weeks in sedentary individuals with and without obesity have led to an improved balance between eNOS and NAD(P)H ox protein content.

Figure 2. Image analysis for quantitative immunofluorescence.

The skeletal muscle microvascular endothelium is identified using the endothelial marker UEA-I Lectin FITC conjugated (A). Using this endothelial image an endothelial mask is created using image analysis software (Image Pro Plus 5.1) (B). The endothelial enzyme being investigated is then identified using an appropriate validated antibody (in this case eNOS) (C). The endothelial outline is then transferred to the corresponding endothelial enzyme image. The fluorescence intensity of the endothelial image is then quantified within the endothelial outline. E and F. Terminal arterioles are differentiated from capillaries; an arteriole is marked with an arrow. E. Anti-alpha smooth muscle actin is used as an identifier of terminal arterioles; F. The endothelium of capillaries and terminal arterioles is marked with UEA-I Lectin FITC conjugated.

Figure 3. Effects of endurance training on eNOS and NOX2 content and eNOS ser^{1177} phosphorylation in lean sedentary individuals.

A, C, E. Representative widefield microscopy images of skeletal muscle pre (a, b) and post (c, d) endurance training. a, c The skeletal muscle microvascular endothelium was revealed using Ulex Europaeus-FITC conjugated lectin (green). A; b, d Skeletal muscle eNOS

expression (red). C; a, b Skeletal muscle eNOS ser^{1177} phosphorylation (red). E; a, b Skeletal muscle NOX2 expression (red). Bar represents 20µm. B, D, F. Mean fluorescence intensity is summarized. The mean level of eNOS, eNOS ser^{1177} or NOX2 pre training was assigned a value of 1, and the relative intensity post training was calculated. Data are means ± SEM for 8 (eNOS) or 7 (eNOS ser^{1177} and NOX2) participants * $P < 0.05$, significant main effect of training. Adapted with permission from Cocks *et al*. (2013a).

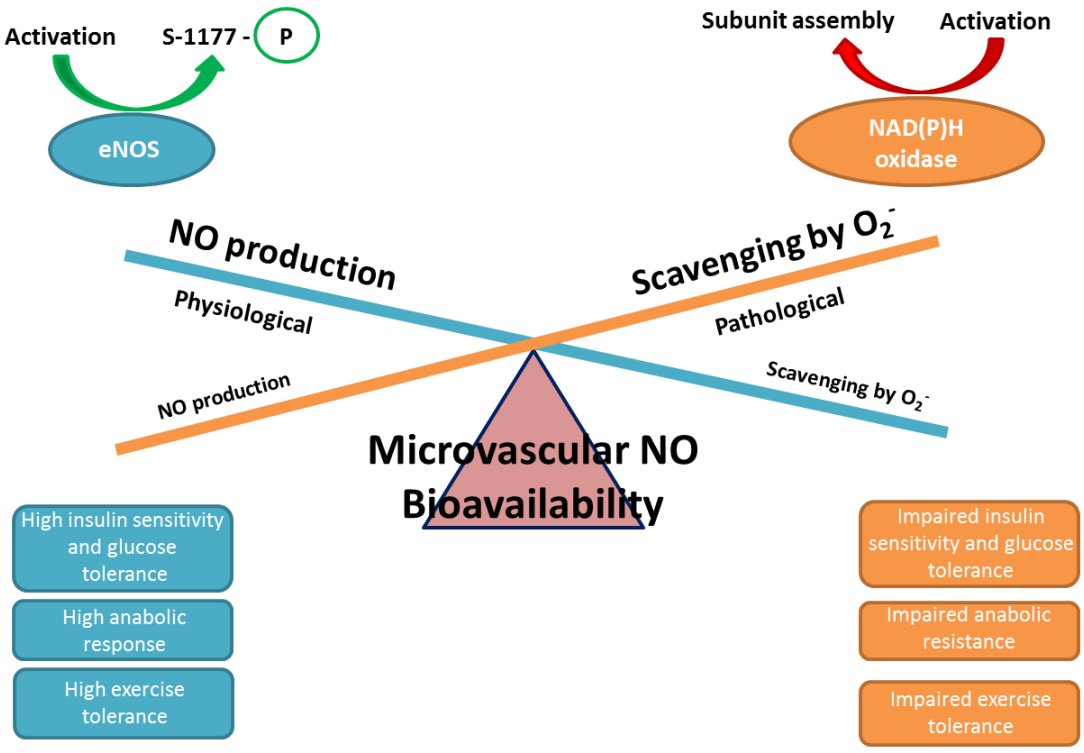

Figure 1.

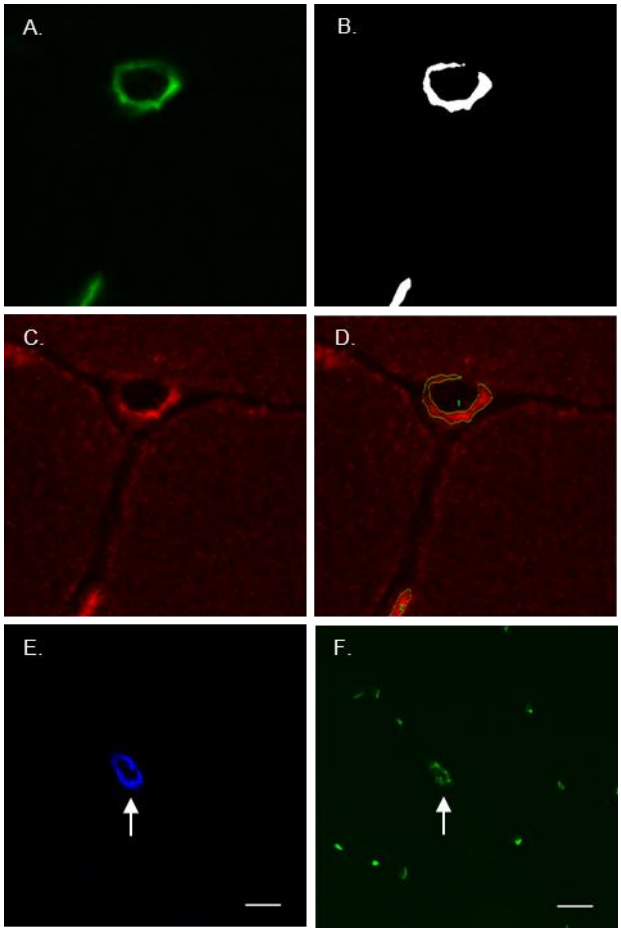

Figure 2.

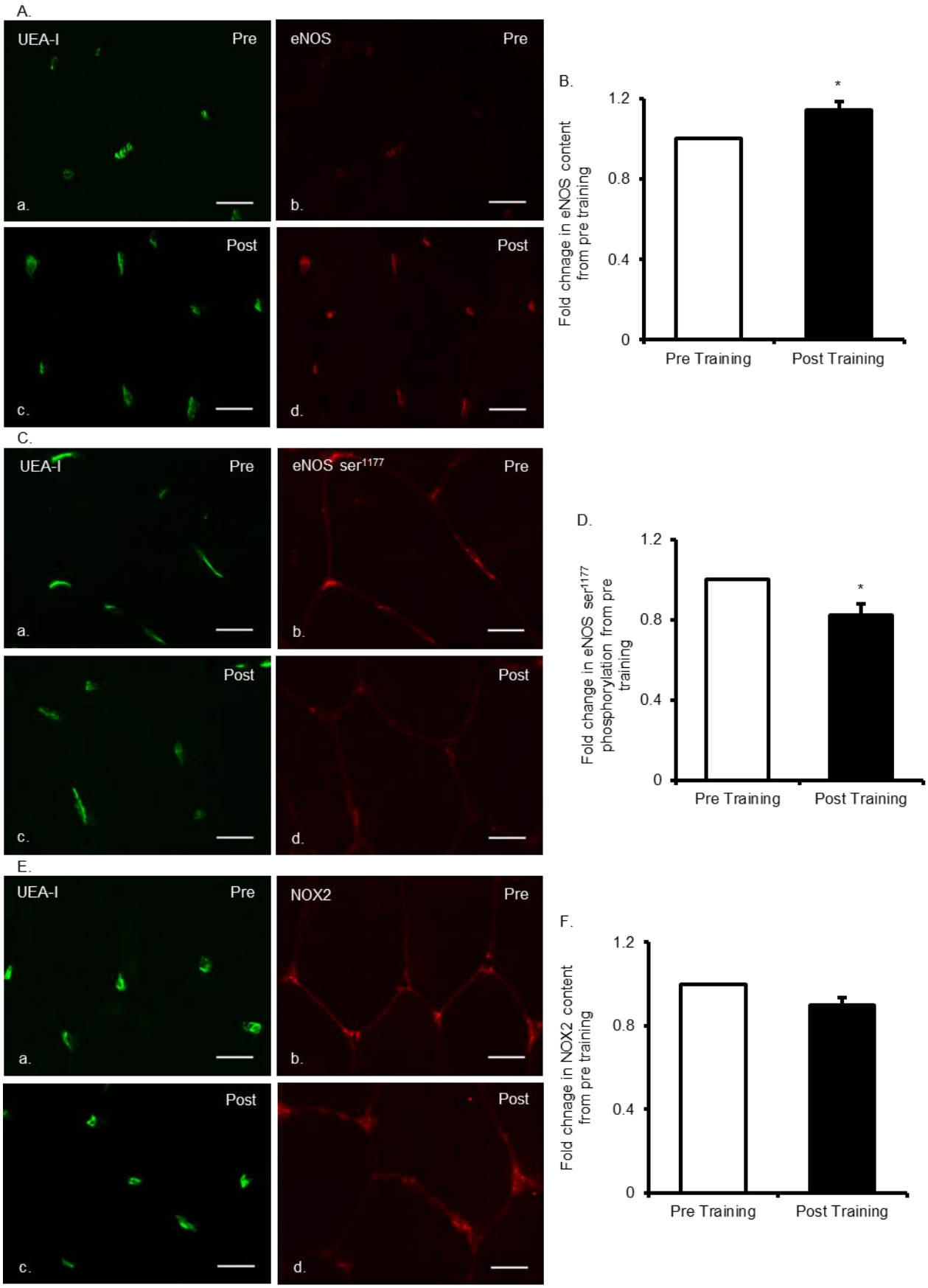
A.
UEA-I
Pre
eNOS
Pre
a.
b.
Post
Post
c.
d.
B.
Fold chnage in eNOS content from pre training
1.2
0.8
0.4
0
*
Pre Training
Post Training
C.
UEA-I
Pre
eNOS ser1177
Pre
a.
b.
Post
Post
c.
d.
D.
Fold change in eNOS ser1177 phosphorylation from pre training
1.2
0.8
0.4
0
*
Pre Training
Post Training
E.
UEA-I
Pre
NOX2
Pre
a.
b.
Post
Post
c.
d.
F.
Fold chnage in NOX2 content from pre training
1.2
0.8
0.4
0
Pre Training
Post Training

Figure 3.

Figure 3.