

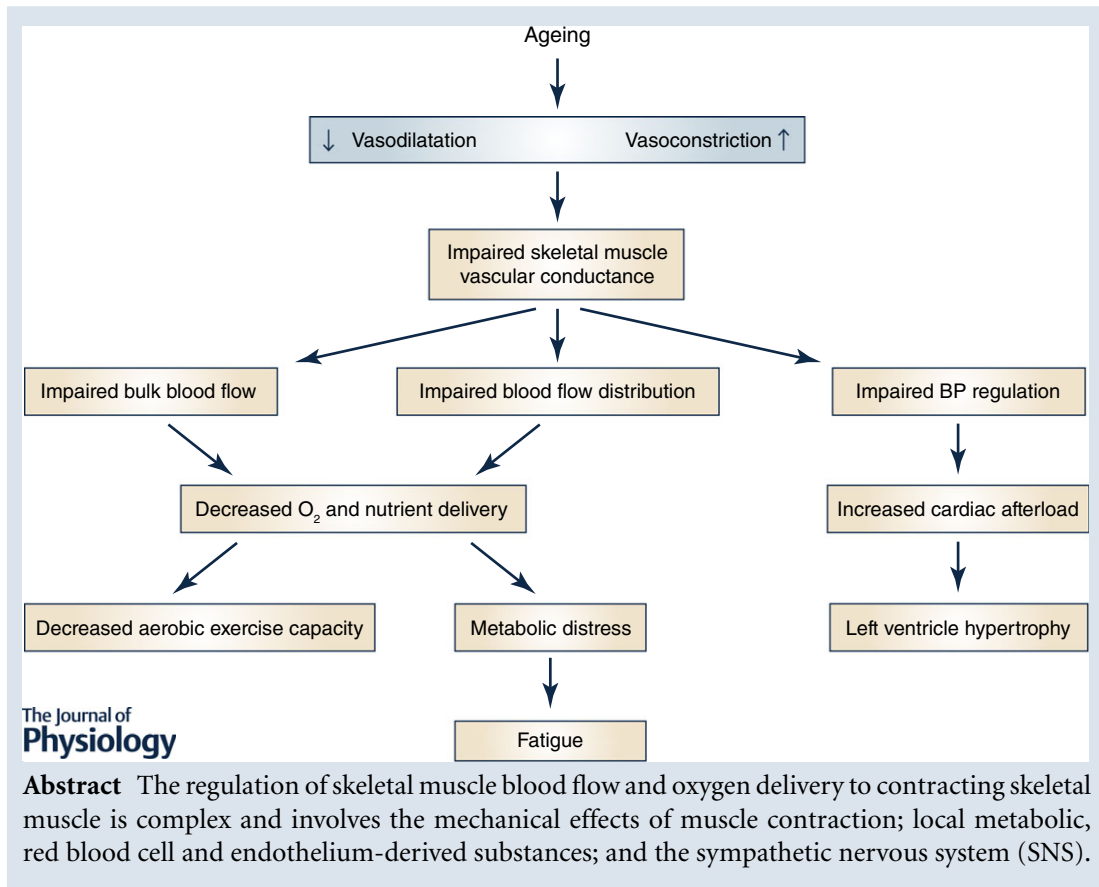
SYMPOSIUM REVIEW

Regulation of skeletal muscle blood flow during exercise in ageing humans

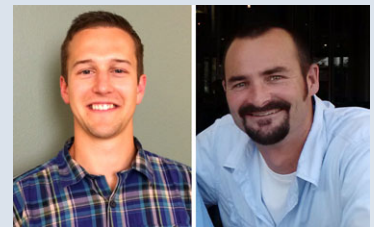
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With advancing age in humans, skeletal muscle blood flow is typically reduced during dynamic exercise and this is due to a lower vascular conductance, which could ultimately contribute to age-associated reductions in aerobic exercise capacity, a primary predictor of mortality in both healthy and diseased ageing populations. Recent findings have highlighted the contribution of endothelium-derived substances to blood flow control in contracting muscle of older adults. With advancing age, impaired nitric oxide availability due to scavenging by reactive oxygen species, in conjunction with elevated vasoconstrictor signalling via endothelin-1, reduces the local vasodilatory response to muscle contraction. Additionally, ageing impairs the ability of contracting skeletal muscle to blunt sympathetic vasoconstriction (i.e. 'functional sympatholysis'), which is critical for the proper regulation of tissue blood flow distribution and oxygen delivery, and could further reduce skeletal muscle perfusion during high intensity and/or large muscle mass exercise in older adults. We propose that initiation of endothelium-dependent hyperpolarization is the underlying signalling event necessary to properly modulate sympathetic vasoconstriction in contracting muscle, and that age-associated impairments in red blood cell adenosine triphosphate release and stimulation of endothelium-dependent vasodilatation may explain impairments in both local vasodilatation and functional sympatholysis with advancing age in humans.

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Abstract figure legend Simplified schematic diagram depicting the effects of impaired skeletal muscle vascular control with age in humans.

Abbreviations ACh, acetylcholine; ATP, adenosine triphosphate; EDH, endothelium-derived hyperpolarization; EET, eicosatrenoic acid; ET-1, endothelin-1; FVC, forearm vascular conductance; LBNP, lower body negative pressure; MVC, maximum voluntary contraction; NO, nitric oxide; NOS, nitric oxide synthase; PE, phenylephrine; PG, prostaglandin; SNS, sympathetic nervous system.

The onset of dynamic exercise constitutes a major haemodynamic stress that is met with highly coordinated cardiovascular adjustments in order to ensure adequate oxygen delivery to contracting skeletal muscle. Within skeletal muscle, regulation of blood flow and oxygen delivery results from the integration of a number of stimuli including the mechanical effects of contraction, local metabolic and endothelium-derived substances, vasoactive factors associated with erythrocytes (red blood cells), and the sympathetic nervous system (SNS). During dynamic exercise, contracting skeletal muscle has the vasodilatory capacity to increase blood flow by nearly 100-fold (Richardson *et al.* 1993), which when extrapolated to whole-body exercise, greatly exceeds the pumping capacity of the heart. Therefore, increased sympathetic nervous system activity, resulting in elevated cardiac output and peripheral vasoconstrictor signalling mediated primarily via release of noradrenaline (NA) from sympathetic nerve endings, which binds to α_1 - and α_2 -adrenoceptors on resistance vessel smooth muscle cells, is necessary to achieve appropriate regulation of mean arterial pressure in the face of profound metabolic vasodilatation. The elevation in peripheral vasoconstriction during high intensity, whole-body exercise is essential to redistribute cardiac output away from inactive and

splanchnic tissues and towards active skeletal muscle. However, within the vasculature of contracting skeletal muscle, specific signalling results in blunting of the vasoconstrictor response in order to ensure adequate tissue oxygen delivery. This unique ability of contracting skeletal muscle to blunt sympathetically mediated vasoconstriction is commonly referred to as 'functional sympatholysis' (Remensnyder *et al.* 1962), and proper integration of these local vasodilator and neural vasoconstrictor signals within contracting skeletal muscle is imperative for normal blood flow control during exercise.

Skeletal muscle blood flow and oxygen delivery are strong predictors of aerobic exercise capacity. Primary (healthy) ageing is associated with a progressive decline in aerobic exercise capacity, and disease states that increase in prevalence with advancing age, such as congestive heart failure and diabetes, often are associated with a further reduction in exercise capacity and tolerance (Holloszy & Kohrt, 1995). This reduction in aerobic capacity is an independent predictor of cardiovascular disease morbidity and mortality, as well as reductions in physical functional capacity and overall quality of life. Thus, understanding the age-associated changes that occur in the control of skeletal muscle blood flow and tissue oxygen delivery is of great clinical significance. Although the authors recognize

and appreciate studies conducted in various experimental animal models, the focus of this review will be on our understanding of vascular control in contracting skeletal muscle of ageing humans.

Skeletal muscle blood flow during exercise in healthy older adults

Healthy human ageing is associated with a number of maladaptive changes within the cardiovascular system that result in impaired aerobic exercise capacity, eventually leading to reductions in functional independence and overall quality of life. Among these changes is impaired regulation of skeletal muscle blood flow. In young healthy humans, increases in blood flow are observed immediately upon the release of a single contraction. This rapid and transient increase in blood flow peaks within approximately five cardiac cycles and is graded with contraction intensity (Tschakovsky *et al.* 2004). While initially attributed to a muscle pump effect, it is now well established that the transient increase in blood flow is primarily due to a local vasodilatory response that serves as a feedforward mechanism for exercise hyperaemia (Crecelius *et al.* 2013a). In older adults, impairments in rapid onset vasodilatation can be observed as early as the first cardiac cycle after initiation of a single muscle contraction and persist throughout the response across mild and moderate contraction intensities (Carlson *et al.* 2008; Casey & Joyner, 2012). Impairments in local blood flow control are also thought to contribute to slowed oxygen uptake kinetics during the onset of dynamic exercise in older individuals (DeLorey *et al.* 2004). To date, there have been relatively few studies investigating the mechanisms that contribute to impaired rapid onset vasodilatation and transient blood flow responses in older humans (Kirby *et al.* 2009; Casey & Joyner, 2012). The major part of our understanding regarding the mechanistic age-associated changes in vascular control is derived from studies utilizing steady-state submaximal exercise and therefore will be the primary focus of this review (Abstract figure).

In 1974, Wahren *et al.* were the first group to report age-associated impairments in blood flow during submaximal graded cycle exercise in humans. In the decades following, findings from this initial study have been corroborated by most (Proctor *et al.* 1998, 2003a; Poole *et al.* 2003; Lawrenson *et al.* 2003; Donato *et al.* 2006; Kirby *et al.* 2009) but not all (Proctor *et al.* 2003b; Donato *et al.* 2006; Parker *et al.* 2008) investigations on the topic. In subsequent investigations utilizing graded cycle exercise, reduced leg blood flow was observed during steady-state submaximal exercise in both sedentary and endurance trained older men (Proctor *et al.* 1998; Poole *et al.* 2003), as well as in healthy, non-endurance trained

women (Proctor *et al.* 2003a). Additionally, leg blood flow is impaired at maximal exercise in both men and women; however, this could be partially due to age related declines in maximal cardiac output (Proctor *et al.* 2004). In order to minimize the potential confounding influence of central limitations associated with ageing, many studies have utilized small muscle mass exercise such as isolated (single) knee extensor and forearm exercise to investigate age-related changes in local blood flow regulation. Within the forearm and single knee extensor models, most (Poole *et al.* 2003; Donato *et al.* 2006; Kirby *et al.* 2009; Mortensen *et al.* 2012) but not all (Donato *et al.* 2006; Parker *et al.* 2008) studies also report attenuated steady-state exercise blood flow with age. While not exclusively, these impairments can exist at both absolute and relative work rates (Lawrenson *et al.* 2003; Donato *et al.* 2006), and independent of age-associated declines in muscle mass (Proctor *et al.* 1998; Kirby *et al.* 2009). However, studies from various groups have identified both training status and sex as important modifiers of the age associated decline in blood flow during small muscle mass exercise (Beere *et al.* 1999; Parker *et al.* 2008; Mortensen *et al.* 2012).

The vast majority of experimental data indicate that during submaximal dynamic exercise in both arm and leg, there is an age-associated attenuation in skeletal muscle blood flow that is typically due to impaired vascular conductance. Even in studies reporting maintained exercise blood flow in older subjects (Magnusson *et al.* 1994; Proctor *et al.* 2003b; Donato *et al.* 2006; Parker *et al.* 2008), the presence of elevated mean arterial pressure points to a state of increased peripheral resistance during exercise in the majority of studies to date. Elevated resistance within contracting skeletal muscle of older individuals may arise from increased vascular stiffness (i.e. reduced compliance), impaired local vasodilatory or vasoconstrictor signalling, and/or elevated sympathetic vasoconstrictor tone. Understanding age-associated changes in vascular architecture, as well as both vasodilatory and vasoconstrictor signalling, and further how these signals interact to control blood flow, is critical to identify potential therapeutic strategies to improve blood flow regulation and oxygen delivery in aged individuals. Along these lines, the vascular endothelium has been identified as an important site for the integration of both vasodilatory and vasoconstrictor signalling (Kerr *et al.* 2012) and may underlie many of the changes in local blood flow control associated with age.

Local control of muscle blood flow with age: role of endothelium-derived substances

The endothelium produces a number of vasodilatory substances including nitric oxide (NO), prostaglandins

(PGs), and eicosatrenoic acids (EETs) that may contribute to exercise hyperaemia (Clifford & Hellsten, 2004). It is important to note that findings from specific studies attempting to elucidate the contributions of these pathways may differ due to the timing of pharmacological inhibitor infusions (prior to exercise onset *versus* during exercise once steady-state hyperaemia is achieved), local *versus* systemic effects of inhibitors, the exercising muscle studied (forearm *versus* knee extensor), and the exercise modality; a complete summary of these findings is beyond the scope of this review. In humans, the most widely studied of these endothelial vasodilatory pathways is NO. To date, in young healthy subjects, the overwhelming majority of studies identify a non-obligatory role for NO in mediating exercise hyperaemia when quantified as an absolute change from rest to steady-state exercise (Radegran & Saltin, 1999; Bradley *et al.* 1999; Frandsenn *et al.* 2001; Schrage *et al.* 2004; Heinonen *et al.* 2011). However, studies from Schrage *et al.* (2004) demonstrate ~20% decrease in forearm blood flow and vascular conductance when nitric oxide synthase (NOS) inhibition is performed after steady-state exercise hyperaemia is achieved, indicating that NO contributes to vascular tone during dynamic exercise in humans. These data are consistent with those obtained in a more recent study by Wray *et al.* (2011) demonstrating that NO contributes to exercise hyperaemia during handgrip exercise at higher work rates.

While an independent role of NO in exercise hyperaemia remains controversial, it is clear that there is significant cross-talk between NO and PGs in the regulation of vascular tone. In this context, while neither NO nor PGs consistently contribute to exercise hyperaemia independently in either the leg or forearm, muscle blood flow and vascular conductance can be attenuated by ~15–30% when their production is inhibited in combination (Boushel *et al.* 2002; Mortensen *et al.* 2007, 2009b; Heinonen *et al.* 2011). However, it should be noted that other studies have demonstrated relatively normal hyperaemic responses to exercise during combined NO and PG inhibition (Crecelius *et al.* 2011b, 2014, 2015b). Similar interactions have been reported for NO and EET production in humans (Hillig *et al.* 2003) as well as an antagonistic interaction between NO and endothelin-1 (ET-1), a potent endothelium-derived vasoconstrictor (Goligorsky *et al.* 1994; Westby *et al.* 2011). Altogether, in young healthy individuals it appears that the endothelium produces a number of vasodilatory compounds that act in a redundant and potentially synergistic manner to promote exercise hyperaemia, even when the presence of a single vasodilatory pathway is experimentally removed (Joyner & Wilkins, 2007).

The hallmark of cardiovascular ageing is progressive endothelial dysfunction (Taddei *et al.* 1995) typically identified as impaired responsiveness to endothelium-

dependent vasodilatory stimuli such as intra-arterial infusion of acetylcholine (ACh) or shear stress (flow-mediated dilatation). Classically, endothelial dysfunction is characterized by impaired NO bioavailability secondary to elevated reactive oxygen species and inflammation. In older individuals, it has been hypothesized that endothelial dysfunction underlies impaired exercise hyperaemia via reduced local dilatory signalling during exercise. Schrage *et al.* (2007) were the first to test this hypothesis and demonstrated that the contribution of NO to forearm exercise hyperaemia in older adults was ~45% lower than their younger counterparts. In addition, the vasodilatory role for prostaglandins (albeit transient) observed in young individuals was absent in older individuals (Schrage *et al.* 2004, 2007). This was the first study to suggest in humans that the role of local endothelium-derived substances in blood flow control was reduced with age during exercise. Subsequently, the observation that NO has a reduced contribution to exercise hyperaemia in older individuals was confirmed by other groups utilizing handgrip exercise (Crecelius *et al.* 2010; Trinity *et al.* 2013). In accordance with the hypothesis that reactive oxygen species scavenge NO and thus reduce exercise hyperaemia, our group demonstrated that intra-arterial infusion of the anti-oxidant ascorbic acid during exercise in older individuals restores blood flow to levels observed in younger individuals (Kirby *et al.* 2009), and a follow-up study demonstrated that ascorbic acid-mediated improvements in exercise hyperaemia were due to improved NO availability (Crecelius *et al.* 2010). Together, these studies support the notion that with age impaired NO bioavailability, due to increased scavenging by reactive oxygen species or potentially reductions in the NOS cofactor tetrahydrobiopterin, contributes to the age-associated impairment in exercise hyperaemia in the human forearm.

In contrast to observations in the forearm, infusion of an antioxidant (N-acetylcysteine) did not improve exercise blood flow in the leg of older individuals despite improving markers of NO availability (Nyberg *et al.* 2012). These findings were attributed to differences between the leg and forearm in their reliance on NO to mediate exercise hyperaemia. Indeed, the vasodilatory pathways mediating exercise hyperaemia in the arm and leg may be different (Wray & Richardson, 2006). However, it is also worthy to note that in the investigation by Nyberg *et al.* in the leg, N-acetylcysteine administered intravenously, had profound systemic effects such as lowered blood (perfusion) pressure, and the mechanisms of action of this antioxidant may differ from that of ascorbic acid, all of which may impact conclusions regarding local regulation of blood flow. Studies utilizing local intra-arterial infusion of ascorbic acid or the antioxidant cocktail (composed of ascorbic acid, vitamin E, and α -lipoic acid) are needed to more directly understand the role oxidative stress in

age-associated impairments of local blood flow control in the leg.

In addition to the loss of endothelium-derived vasodilatory signalling with age, recent work from Wray *et al.* suggests that the endothelium-derived vasoconstrictor ET-1 may contribute to age-associated impairments in muscle blood flow control during exercise (Barrett-O'Keefe *et al.* 2014). In young individuals, ET-1 release was elevated during exercise and was shown to actively restrain blood flow during graded knee extensor exercise (Barrett-O'Keefe *et al.* 2013). With age, multiple groups have identified elevated ET-1 signalling as a contributor to lower resting skeletal muscle blood flow and vascular tone (Van Guilder *et al.* 2007; Thijssen *et al.* 2007), and Wray *et al.* extended these findings to impairments in exercise hyperaemia with advancing age. Specifically, they observed that ET-1-mediated restraint of exercise blood flow was augmented in older adults and that blockade of endothelin subtype A receptors improved exercise hyperaemia and lowered blood pressure during exercise to levels observed in young adults. Given the interaction between NO and ET-1 in the vasculature, it remains unclear whether impaired NO bioavailability with age influences ET-1-mediated vasoconstriction or production during exercise in older adults and this awaits further study. Taken together, the collective evidence indicates that loss of endothelial vasodilatory substances (NO, PGs) in conjunction with elevated ET-1 vasoconstrictor signalling contributes to impaired local blood flow control with advancing age.

Impaired functional sympatholysis with advancing age

Initial investigations into the mechanisms of impaired blood flow with age demonstrated that ageing is associated with more than twofold greater noradrenaline spillover during cycle exercise (Taylor *et al.* 1992; Proctor *et al.* 1998), and this is superimposed on the elevated basal muscle sympathetic nerve activity observed in older adults (Ng *et al.* 1993; Davy *et al.* 1998; Dinunno *et al.* 1999). This finding implicated age-associated changes in sympathetic regulation of the vasculature as a primary contributor to impaired blood flow in older adults. The first study to directly investigate sympathetic responsiveness in contracting muscle during dynamic exercise in older subjects was undertaken by Koch *et al.* (2003). Utilizing the cold pressor test to elevate sympathetic outflow during moderate intensity cycle ergometer exercise, they observed a greater decrease in leg vascular conductance in older men relative to younger control subjects. This study was the first to indicate that despite an attenuated responsiveness to α -adrenoceptor agonists during resting conditions (Dinunno *et al.* 2002), older subjects may display greater responsiveness to sympathetic

vasoconstriction during exercise. These findings have been replicated experimentally via use of lower body negative pressure (LBNP) to evoke sympathoexcitation, local intra-arterial tyramine infusions to evoke endogenous NA release, as well as infusions of direct α_1 - and α_2 -adrenoceptor agonists, during both handgrip (Fadel *et al.* 2004; Dinunno *et al.* 2005; Kirby *et al.* 2011) and knee extensor exercise (Mortensen *et al.* 2012). To date, studies on the effect of age on the modulation of sympathetic vasoconstriction in active muscle clearly demonstrate impairments in this regulation in both men and women, across multiple muscle beds, at both absolute and relative exercise intensities, utilizing diverse methods of sympathetic/ α -adrenoceptor stimulation (Fig. 1) (Koch *et al.* 2003; Fadel *et al.* 2004; Dinunno *et al.* 2005; Wray *et al.* 2009; Kirby *et al.* 2011; Mortensen *et al.* 2012).

The physiological importance of impaired functional sympatholysis in the regulation of muscle blood flow during exercise with advancing age was highlighted in a recent study by Mortensen *et al.* (2012). In this study, the investigators observed that lifelong physical activity preserves functional sympatholysis in older individuals relative to their sedentary counterparts (Mortensen *et al.* 2012b). More importantly, during dynamic knee extensor exercise at an absolute work rate, older sedentary individuals demonstrating impaired sympatholysis had lower \dot{V}_{O_2} , elevated blood lactate and higher arterial pressure, despite similar levels of bulk blood flow to the active limb when compared to older active individuals with intact sympatholysis. Therefore, despite no difference in the magnitude of the local vasodilatory response to an absolute workload in older sedentary individuals compared with age-matched trained adults, the inability to appropriately redistribute blood flow and oxygen delivery during sympathetic stimulation resulted in significant metabolic dysregulation. This suggests that proper regulation of exercising muscle blood flow entails more than just a balance between vasodilatation and vasoconstriction; rather, the proper vasodilatory and sympatholytic signals must be present to appropriately redistribute blood flow within skeletal muscle. In this context, not all vasodilatory substances and/or pathways are equal in their ability to blunt sympathetic vasoconstriction (more discussion below), and this is an important distinction when considering this basic physiological phenomenon of functional sympatholysis, and moreover, the effect of age on the regulation of blood flow.

While the previous studies clearly demonstrate that ageing is associated with heightened sympathetic activity and vasoconstrictor responsiveness in active muscle during exercise, they were not designed to investigate whether the sympathetic nervous system exhibits greater 'restraint' of skeletal muscle blood flow during exercise in older adults. Richards *et al.* (2014) conducted the

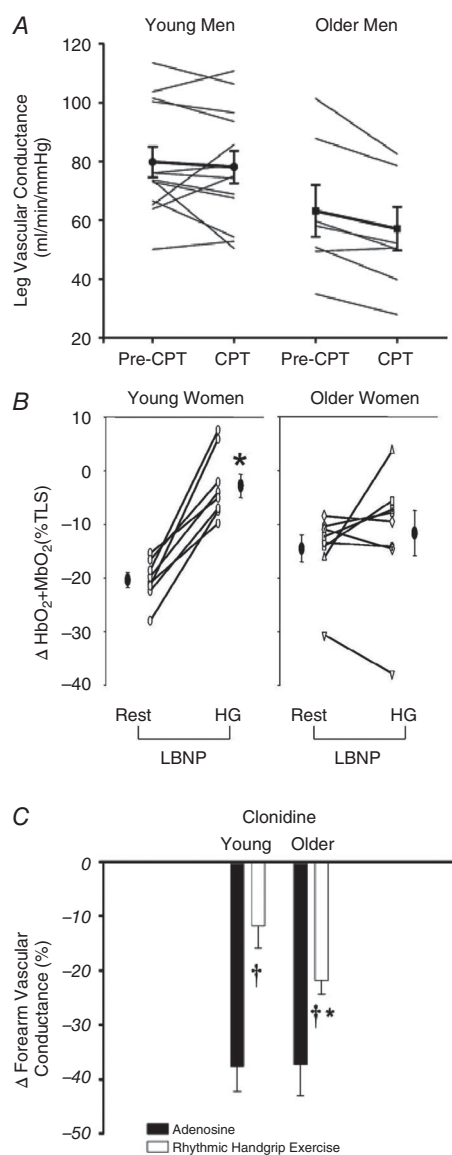


Figure 1. Age-associated impairments in functional sympatholysis

A, sympathetic vasoconstrictor responses to cold pressor test (CPT) during moderate intensity cycle exercise were greater in older relative to young men (% Δ FVC: $-14 \pm 3\%$ vs. $-2 \pm 4\%$, respectively; $P < 0.05$; FVC: forearm vascular conductance). B, similarly, vasoconstrictor responses (assessed as decreases in skeletal muscle oxygenation) during sympathetic stimulation via lower body negative pressure (LBNP) were not blunted during moderate intensity handgrip exercise compared with resting conditions in older women, and were greater during exercise compared with young women ($*P < 0.05$ vs. Rest within condition). C, postjunctional α_2 -adrenoceptor stimulation (via intra-arterial clonidine) reduced vascular conductance similarly in resting skeletal muscle of young and older men during infusion of the vasodilator adenosine (filled bars). In contrast, vasoconstrictor responses during moderate intensity handgrip exercise were greater in older men relative to young men (open bars) ($\dagger P < 0.05$ vs. Adenosine within condition; $*P < 0.05$ vs. Young). The collective data demonstrate impaired modulation of sympathetic α -adrenoceptor vasoconstriction in contracting muscle of older adults. From Koch *et al.* (2003), Fadel *et al.* (2004) and Dinenzo *et al.* (2005).

first investigation of sympathetic restraint during sub-maximal steady-state exercise across a range of handgrip exercise intensities (~ 15 – 70% of maximum work rate) in ageing humans. The primary finding of this study was that sympathetic vasoconstriction does not restrain forearm skeletal muscle blood flow and vascular conductance during exercise in young or older adults, thus suggesting that elevated sympathetic restraint does not contribute mechanistically to age-associated impairments in skeletal muscle haemodynamics during handgrip exercise. It is important to note here that this small muscle mass exercise evoked rather modest increases in heart rate and mean arterial pressure (i.e. minimal sympathetic activation), and thus whether these findings can be replicated during larger muscle mass exercise of the lower extremities, such as knee extensor exercise or cycling that are likely to evoke greater sympathoexcitation, remains to be determined. An additional consideration is the inability of this study to determine the spatial distribution of blood flow within the active forearm muscle, as use of Doppler ultrasound can only provide information regarding bulk flow to the forearm vasculature. Thus, it also remains unclear how α -adrenergic blockade affects distribution between active and inactive tissues within the forearm in young and older adults. In older physically active (i.e. not sedentary) adults, use of positron emission tomography revealed a more homogeneous blood flow pattern within the leg during sustained (non-rhythmic) isometric knee extensor exercise relative to young subjects, suggesting an impaired ability to redistribute blood flow effectively within the exercising muscle (Rudroff *et al.* 2014). However, more studies are needed to determine whether tonic α -adrenoceptor activation impacts muscle blood flow regulation with age, and further, how the distribution of blood flow and oxygen delivery is impacted in young and older adults during sympathetic activation across a wide range of exercise intensities and muscle mass recruited during dynamic exercise.

Current understanding of functional sympatholysis in young healthy humans. Investigations into the potential pathways contributing to sympatholysis in young healthy humans thus far have been equivocal. Considering the well documented role of NO in modulating resting vascular tone in humans (Vallance *et al.* 1989; Haynes *et al.* 1993; Seddon *et al.* 2008), in combination with data obtained in experimental animals (Thomas & Victor, 1998), much focus has centred on a potential role for NO in mediating functional sympatholysis. In healthy humans, Chavoshan *et al.* (2002) utilizing systemic doses of N^G -nitro-L-arginine methyl ester (L-NAME) to block NO production, LBNP to elevate sympathetic outflow during handgrip exercise, and near-infrared spectroscopy as an indirect measure of tissue perfusion, provided evidence to support a role for NO in functional

sympatholysis. In contrast, studies from Dinunno *et al.* (2004) and more recently from Crecelius *et al.* (2015a) demonstrate that local NOS inhibition (via intra-arterial N^G -monomethyl-L-arginine (L-NMMA) or L-NAME) does not impact the ability of contracting muscle to blunt vasoconstrictor responses to tyramine (endogenous NA release) or selective α_1 - and α_2 -adrenoceptor agonists. Further, intra-arterial infusion of sodium nitroprusside, a direct NO donor, fails to blunt vasoconstrictor responses to tyramine, as well as α_1 - and α_2 -adrenoceptor agonists (Tschakovsky *et al.* 2002; Rosenmeier *et al.* 2003). These observations strongly suggest that NO is neither sufficient nor obligatory to observe functional sympatholysis in humans.

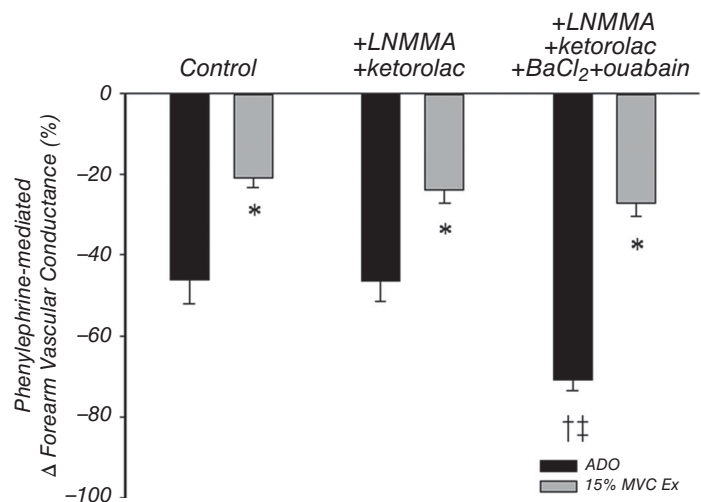
Recently, our laboratory embarked on perhaps the most comprehensive attempt to pharmacologically manipulate functional sympatholysis in humans to date. We assessed vasoconstrictor responses to intra-arterial infusion of phenylephrine (α_1 -agonist) during handgrip exercise and control adenosine infusion. These responses were assessed before and after combined blockade of NO (L-NMMA) and PGs (ketorolac), as well as pathways involved in smooth muscle hyperpolarization: inwardly rectifying potassium (K_{IR}) channels (low dose barium chloride) and Na^+/K^+ -ATPase (ouabain). Contrary to our hypothesis, despite combined inhibition of these vasodilatory pathways clearly augmenting α_1 -mediated vasoconstriction in resting muscle, there was absolutely no effect on α_1 -mediated vasoconstriction in contracting muscle (Crecelius *et al.* 2015b) (Fig. 2). It is important to point out here that the combination of these pharmacological inhibitors has been shown to reduce exercise hyperaemia by approximately ~30% (Crecelius *et al.* 2014) and attenuate reactive hyperaemia by close to 90% (Crecelius *et al.* 2013c). Thus, considering that this complex pharmacological approach substantially reduces

the hyperaemic responses during these stimuli, it is rather impressive that no effect on functional sympatholysis was observed. This is also consistent with our posit that not all vasodilating substances and/or pathways are necessarily sympatholytic. Taken together, the mechanisms that contribute to functional sympatholysis in contracting skeletal muscle of humans still remain to be elucidated.

In addition to NO and PGs, studies conducted primarily in various animal models have identified endothelium-derived hyperpolarization (EDH) as an important modulator of vascular tone in a variety of vascular beds (Behringer & Segal, 2012). In response to endothelium-dependent vasodilators such as ACh, intracellular Ca^{2+} signals evoke potassium efflux through Ca^{2+} activated K^+ channels and subsequent hyperpolarization of the endothelial cells. Because the endothelium and vascular smooth muscle cells are electrically coupled via gap junctions located in myo-endothelial projections, hyperpolarization originating in the endothelium can be transmitted directly to smooth muscle cells resulting in vasodilatation (Dora *et al.* 2003; Griffith, 2004). In addition to direct electrical coupling locally to vascular smooth muscle, EDH can spread to adjacent endothelial cells and conduct vasodilatory signals upstream to more proximal feed arteries, thereby facilitating the redistribution of blood flow within a tissue. This is in contrast to NO donors which have been shown to cause robust vasodilatation locally, without initiating a conducted vasodilatory response (Kurjiaka & Segal, 1995a). Interestingly, conducted dilatation in response to ACh applied to resistance vessels has been shown to blunt α -adrenergic vasoconstriction in hamster skeletal muscle arterioles; conversely, sympathetically induced vasoconstriction can limit conducted vasodilatation (Kurjiaka & Segal, 1995b). Similar to ACh, the endothelium-dependent vasodilator

Figure 2. Lack of an obligatory role for NO, PGs, K_{IR} channels, or Na^+/K^+ -ATPase in mediating functional sympatholysis in healthy humans

In young adults, vasoconstrictor responses to phenylephrine (PE; α_1 -adrenoceptor agonist) in resting tissue during infusion of adenosine (ADO) as a high flow control condition and moderate intensity handgrip exercise (15% maximum voluntary contraction (MVC)). Combined inhibition of nitric oxide (NO; L-NMMA), prostaglandins (PGs; ketorolac), inwardly rectifying potassium channels (K_{IR} channels; $BaCl_2$), and Na^+/K^+ -ATPase (ouabain) had no effect on the exercise-induced attenuation of vasoconstriction in response to PE (* $P < 0.05$ vs. ADO within condition; † $P < 0.05$ vs. Control; ‡ $P < 0.05$ vs. L-NMMA+ketorolac). From Crecelius *et al.* (2015b).



adenosine triphosphate (ATP) has been shown to cause robust EDH and conducted vasodilatation in resistance vessels (McCullough *et al.* 1997; Winter & Dora, 2007). While a physiological role for ACh in mediating the haemodynamic response to exercise in humans remains controversial (Joyner & Halliwill, 2000), ATP is thought to play an important role in the matching of oxygen delivery to demand during physiological stressors such as hypoxia and exercise (Ellsworth, 2004; Crecelius *et al.* 2015a).

To the best of our knowledge, the only vasodilator that has been shown to be independently sympatholytic in humans is ATP. Rosenmeier *et al.* (2008) were the first to demonstrate that local intra-arterial infusion of ATP significantly blunts tyramine-induced vasoconstriction in the leg, similar to what is observed during moderate knee extensor exercise. Our laboratory subsequently demonstrated that this unique ability of ATP to blunt sympathetic vasoconstriction involves both post-junctional α_1 - and α_2 -adrenoceptors, and importantly, that this was graded with ATP concentration such that low levels of ATP did not impact α_1 -mediated vasoconstriction whereas increasing ATP concentration progressively limited α_1 -mediated vasoconstriction (Kirby *et al.* 2008). These observations are quite similar to the intensity-dependent nature of functional sympatholysis in contracting skeletal muscle (Tschakovsky *et al.* 2002). Further connecting ATP and exercise hyperaemia, venous plasma ATP levels draining active muscle increase in proportion to exercise intensity (Gonzalez-Alonso, 2002; Kirby *et al.* 2012) and are likely to arise from red blood cells as they become deoxygenated and mechanically deformed when traversing the microcirculation of contracting skeletal muscle (Crecelius *et al.* 2013b; Kirby *et al.* 2013). In humans, endothelium-dependent ATP-mediated vasodilatation appears to rely modestly on NO and PG synthesis (Mortensen *et al.* 2009a; Crecelius *et al.* 2011a); however, our laboratory has demonstrated that the primary pathway underlying ATP-mediated vasodilatation involves activation of K_{IR} channels (Crecelius *et al.* 2012) and inhibition of these channels significantly reduces skeletal muscle blood flow during exercise (Crecelius *et al.* 2014). Collectively, these findings point to a potentially important role for ATP in coordinating exercise hyperaemia in young healthy humans.

Potential mechanisms underlying impaired sympatholysis with age. In the context of ageing and endothelial dysfunction, it would seem logical that the responsiveness to ATP would decline as is observed with other endothelium-dependent vasodilators such as ACh. However, within the forearm circulation, vasodilatation to exogenous ATP is not attenuated in older individuals (Kirby *et al.* 2011), fitting with a relatively minimal reliance of ATP on NO to produce vasodilatation in the forearm. In contrast, ATP-mediated vasodilatation

in the leg has been shown to be significantly attenuated in older adults (Mortensen *et al.* 2012). Perhaps most intriguing, despite the divergent effect of age on the normal vasodilatory response to ATP in the forearm and the leg, the ability of exogenous ATP to blunt sympathetic vasoconstriction is maintained in both limbs of older adults (Fig. 3). Indeed, in studies originally performed

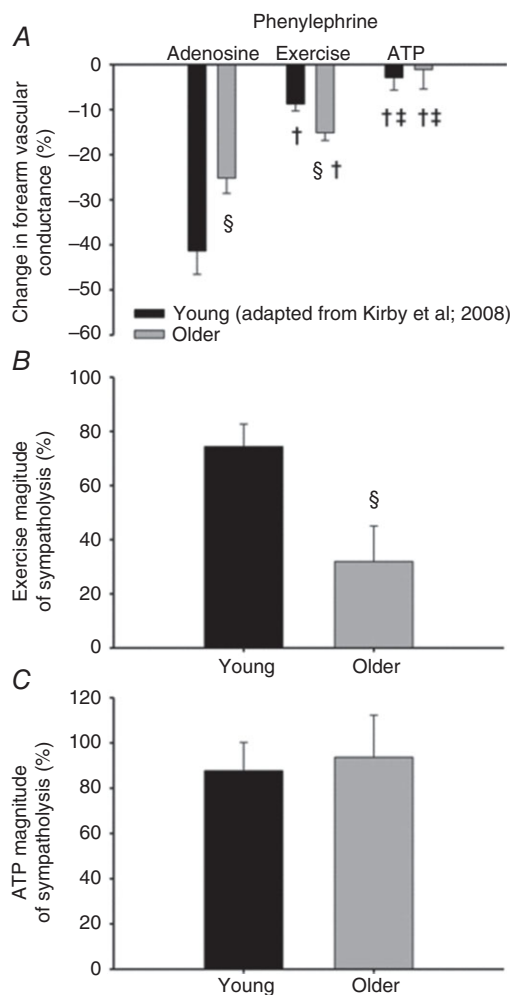
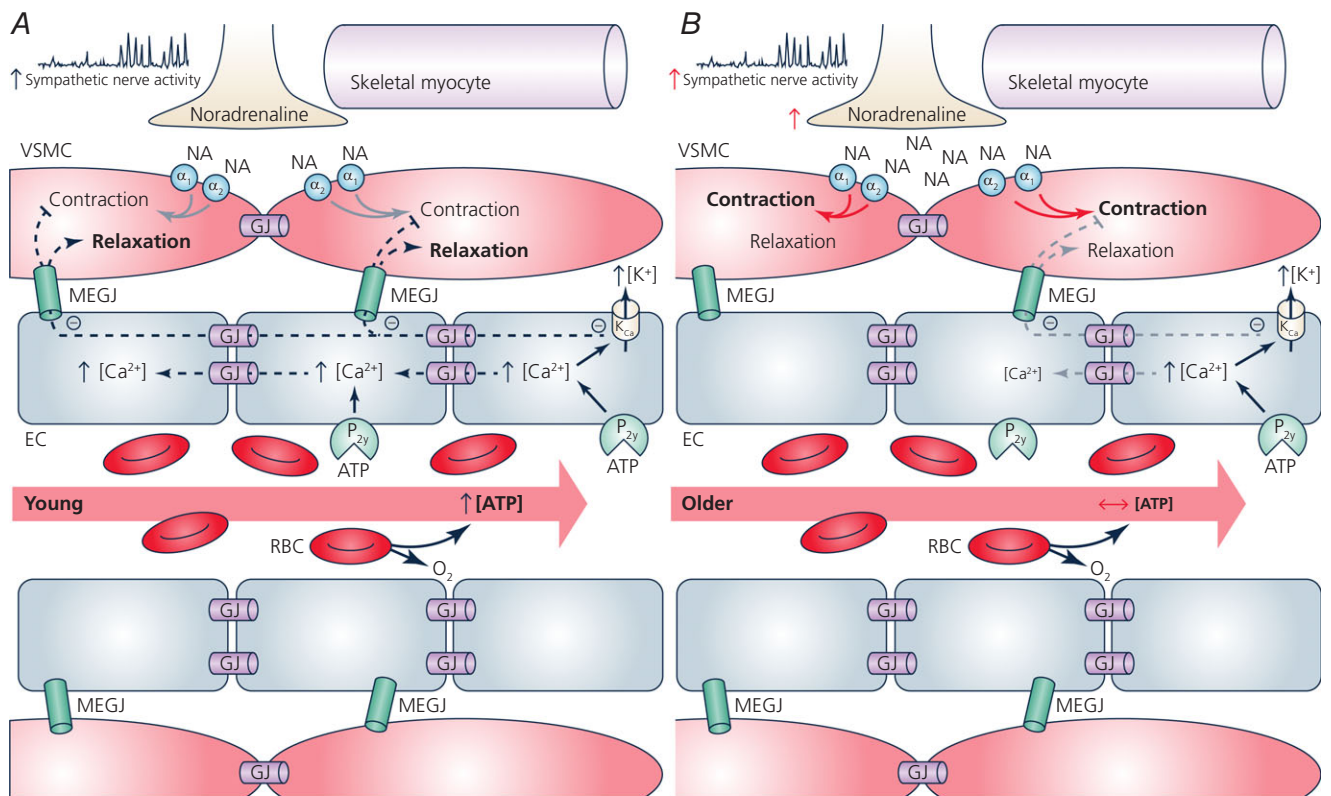


Figure 3. Modulation of α_1 -mediated vasoconstriction during exercise and ATP infusions in young and older adults

Vasoconstrictor responses to phenylephrine (PE; α_1 -adrenoceptor agonist) during passive vasodilatation with adenosine, moderate handgrip exercise (15% MVC), and passive vasodilatation with adenosine triphosphate (ATP). A, vasoconstrictor responsiveness in resting skeletal muscle during infusion of adenosine was attenuated in older adults, whereas vasoconstriction was greater in older adults during handgrip exercise. ATP blunted vasoconstriction similarly in both young and older adults. B and C, magnitude of sympatholysis, describing the ability of contracting skeletal muscle to blunt sympathetic vasoconstriction, is impaired in older adults during exercise (B), but maintained during exogenous ATP infusions (C). The percentage magnitude of sympatholysis was calculated as $((\%FVC \text{ adenosine} - \%FVC \text{ exercise or ATP})/\%FVC \text{ adenosine}) \times 100$. ($\S P < 0.05$ vs. Young within condition; $\dagger P < 0.05$ vs. Adenosine within age group; $\ddagger P < 0.05$ vs. Exercise within age group). From Kirby *et al.* (2011).

in our laboratory (Kirby *et al.* 2011) and subsequently by Mortensen *et al.* (2012), exogenous ATP retained the ability to blunt vasoconstriction despite the presence of impaired NO bioavailability and endothelial dysfunction (impaired ACh-mediated vasodilatation). These observations highlight a few key points. First, these data further demonstrate that sympatholysis occurs independent of NO bioavailability in both young and older adults. Second, and perhaps more importantly, these findings suggest that classic endothelial dysfunction characterized by reduced NO bioavailability may not

be the cause of impaired sympatholysis with advancing age. Rather, the endothelium seems to be capable of responding to sympatholytic stimuli (e.g. intravascular ATP), indicating that the lack of a functional signal may be the primary culprit in the age impairment underlying this phenomenon. To this end, our laboratory has recently demonstrated that intravascular [ATP] draining active skeletal muscle of older adults does not increase during exercise, whereas a progressive increase is observed with graded exercise intensity in young healthy adults. This impairment may be related to attenuated red blood



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Figure 4. Working hypothesis: contribution of ATP to functional sympatholysis in young and older adults

A, young, red blood cells (RBCs) traversing the microcirculation of contracting skeletal muscle encounter areas of high oxygen demand resulting in diffusion of oxygen to the active tissue, desaturation of haemoglobin, and subsequent release of the potent vasodilator adenosine triphosphate (ATP). Locally, ATP binds to purinergic receptors (P_{2y}) located on the endothelial cell (EC), which elevates intracellular calcium, in turn activating iK_{Ca} and sK_{Ca} channels (calcium activated potassium channels, K_{Ca}) resulting in efflux of potassium (K^+) and hyperpolarization of the endothelium. Endothelium-derived hyperpolarization (EDH) conducts bi-directionally, away from the local signal, along the endothelium through gap junctions (GJ) and spreads directly to the vascular smooth muscle cells (VSMC) through myoendothelial gap junctions (MEGJ). The effect of EDH on VSMC tone is twofold, causing vasodilatation as well as blunting sympathetic vasoconstriction, thus facilitating distribution of blood flow and oxygen to areas of high metabolic demand. *B*, older, while *exogenous* ATP is still capable of evoking vasodilatation and can blunt sympathetic vasoconstriction in older adults (denoted as small grey arrows), impaired *endogenous* ATP release from RBCs may result in reduced local and conducted vasodilatation, as well as an impaired ability to modulate sympathetic vasoconstriction in contracting muscle. These impairments may result in attenuated bulk blood flow delivery to, and inefficient distribution within, contracting skeletal muscle of older adults. \ominus Indicates hyperpolarization.

cell-mediated ATP release in response to deoxygenation (Kirby *et al.* 2012). Thus, given the dual nature of ATP eliciting vasodilatation (both local and conducted) as well as blunting sympathetic vasoconstriction, impairments in regulation of intravascular ATP could potentially explain attenuated local vasodilatation and impaired functional sympatholysis with age. Figure 4 depicts our working hypothesis regarding the vascular signalling that underlies functional sympatholysis in young healthy humans and how this is impaired with advancing age.

Summary and future directions

With advancing age, the normal regulation of exercising muscle blood flow is impaired and may lead to metabolic dysregulation and exercise intolerance. The age-associated impairments in vascular conductance are thought to be the result of (1) altered bioavailability of endothelium-derived substances and (2) the impaired ability to blunt sympathetic vasoconstriction within active tissues. The attenuated local vasodilatory response can be explained in part by impaired NO and PG availability due to classic endothelial dysfunction and a shift towards the production of the endothelium-derived vasoconstrictor ET-1. While the ability of exercise to blunt sympathetically mediated vasoconstriction and redistribute bulk flow is also impaired with age, this is likely to reflect more than a simple imbalance between vasodilatory and vasoconstrictor signalling and we hypothesize this is due to a reduction in hyperpolarization of endothelial cells. Despite classic endothelial dysfunction (impaired NO bioavailability), exogenous ATP retains the ability to blunt sympathetic vasoconstriction. This finding suggests that a vasodilatory pathway independent of NO and PGs, such as EDH, is important for the proper regulation of vascular tone within contracting skeletal muscle. Further studies are needed to better understand the basic signalling mechanisms capable of modulating sympathetic tone in contracting muscle in humans, and specifically, how EDH may contribute to functional sympatholysis. Additionally, the finding that the vasculature of older individuals remains capable of responding to sympatholytic stimuli such as ATP suggests the loss of a functional signal as the primary contributor to impaired functional sympatholysis. In this context, the normal graded rise in plasma [ATP] during exercise observed in young subjects is impaired in older adults and may be related to impaired red blood cell ATP release in response to deoxygenation. Loss of ATP signalling during exercise could contribute to both attenuated local vasodilatory signalling and impaired functional sympatholysis. Future studies should be aimed at developing therapeutic strategies to improve red blood cell ATP release during exercise in ageing and disease populations as an approach to improve both

local vasodilatory signalling and regulation of sympathetic tone. Finally, studies investigating blood flow distribution within active skeletal muscle are necessary, not only to more fully understand the age-associated impairments in blood flow distribution, but also to properly assess the efficacy of therapeutic interventions in improving regional blood flow control with age and disease.

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Additional information

Competing interests

No conflicts of interest, financial or otherwise, are declared by the author.

Author contributions

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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