The Skeletal Muscle Pump During Contractile Transitions

by

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Abstract

The aim of this study was to characterize the contribution of skeletal muscle contraction to the immediate hyperemic blood flow response as well as the continued involvement in matching tissue perfusion to elevated metabolic rates. There exists a substantial reserve for increased blood flow within skeletal muscle in response to dynamic exercise however the interaction of neural regulation, vasoactive metabolites, and mechanical characteristics are incompletely understood. To address questions concerning blood flow in response to transitions from rest to various metabolic rates an isolated canine gastrocnemius *in situ* model was employed. Seven canines were used for this investigation with the gastrocnemius muscles isolated for isometric contractions with tetanic stimulation. Measures were made for blood flow, blood pressure, force, and near infrared spectrophotometric analyses under conditions of spontaneous blood flow. The following transitions were investigated: from rest to tetanic contractions of 1/3 s, rest to 2/3 s, rest to 1/1 s and during the transition from 1/3 s to 2/3s all with spontaneous blood flow response intact. Additionally, an estimation for the blood flow response with no mechanical contribution from the muscle pump was made with determination for the kinetics of the estimate. The time constant (tau) for the blood flow response was not significantly different between the measured flow with contraction (Q_{wc}) and the estimate with no contraction (Q_{nc}) for the 1/3 s stimulation rate (12.8 ± 5.5 s vs 11.8 ± 3.2 s respectively), the transition from the high baseline (1/3 s) to a higher rate ($2/3_{HB}$) (21.2 ±

3.4 s vs 21.7 ± 4.8 s respectively), from rest to 2/3 s (25.6 ± 12.0 s vs 22.1 ± 1.3 s respectively), or from rest to 1/1 s (16.7 ± 3.0 s vs 22.1 ± 1.3 s respectively). Initially, for this model, there is a positive contribution to total blood flow provided by the contracting skeletal muscle, however this diminishes within the first few contractions. At higher stimulation rates the net effect of the contracting muscle is to limit local blood flow in the exercising muscle. In conclusion, the muscle pump may contribute to local perfusion at exercise onset with diminishing returns as rhythmic contractions continue. In the steady state the main contributions of the muscle pump is to aid in the maintenance of central hemodynamic

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List of Abbreviations

Ach……Acetylcholine

NO…….Nitric Oxide

NOS…...Nitric Oxide Synthase

SNP……Sodium Nitroprusside

H^+……...Hydrogen Ion

Ca^{2+} …...Calcium Ion

K^+……...Potassium Ion

PKA…...Protein Kinase A

K_{Ca}…….Calcium-activated Potassium Channel

PKG…..Protein Kinase G

PKC…..Protein Kinase C

AA……Arachidonic Acid

L-NMMA.. N^G-monomethyl-L-arginine

SNP…..Sodium nitroprusside

T_{max}…Maximal Tension

ATP……Adenosine Triphosphate

ADP……Adenosine Diphosphate

AMP……Adenosine Monophosphate

ADO……Adenosine

EDHF…..Endothelial Derived Hyperpolarizing Factor

VSMC….Vascular Smooth Muscle Cells

cGMP…..Cyclic Guanosine Monophosphate

PGI_2……Prostacyclin

ET……...Endothelin

H_2O_2…...Hydrogen Peroxide

COX…...Cyclooxygenase Enzymes

L-NAME.. N^G-nitro-L-arginine methyl-ester

Hb……..Hemoglobin

I. REVIEW OF LITERATURE

As eloquently stated by Loring B. Rowell, a historical perspective exposes a chain of ideas that reveals the ability to build on the findings of others and thus provide that strength that is the continuity of science (128). Our understanding of the work of our predecessors guides us down an evolving path of discovery and investigation. The focus of this investigation will be to discuss those factors which contribute to blood flow regulation, particularly in exercising skeletal muscle. Blood flow through a skeletal muscle vascular bed is determined by both the perfusion pressure as well as the vascular tone. A large fraction of the vascular resistance in skeletal muscle is controlled at the level of small muscular "feed" arteries which lie external to the muscle and therefore are not directly exposed to the conditions within the skeletal muscle they perfuse. Upon entering the muscle further blood flow control is mediated by small arteriolar networks and finally terminal arterioles. Herein, many of the key regulators in matching systemic perfusion to metabolic demand will be discussed. The primary aim of this investigation was to determine muscle pump contribution to the rapid hyperemic response at exercise onset as well as the ongoing contribution to total blood flow for various contractile frequencies and transitions which in turn engendered various metabolic rates.

Research physiologists have long sought to define the mechanisms responsible for precisely matching tissue perfusion to metabolic demand during transitions from rest to

dynamic exercise as well as the maintenance of adequate perfusion during steady state activities. The pioneering work of Sir William Harvey first detailed the structure of the heart as well as blood flow through the body in *De Motu Cordis*, 1628 (65). Since his detailed works further investigations have sought to delineate all of the regulating factors in blood flow control. Technological improvements as well sophisticated investigative techniques systematically revealed an increasingly complex system responsible for the regulation of whole body circulation. By the end of the 17th century Jan Swammerdam had recorded in his notebooks microscopic descriptions of red blood cells (107), however the first published descriptions came from the light microscopy work of Anton van Leeuwenhoek (151). During this time blood transfusion was observed to be capable of reviving a previously exsanguinated animal of the same species (98). Other investigators sought to cure human maladies by transfusing blood of docile animals into humans (93). This practice was soon halted after the death of Antoine Mauroy in 1667 following transfusion twice with calf's blood (93). Subsequently, for more than a century there was little progress made in delineating the factors contributing to the components of blood or the factors that regulated perfusion. However, near the turn of the 19th century Scottish physician John Hunter, based on his observations in anatomy and surgery, insightfully stated that "blood goes where it is needed" (73).

Since this simple, discerning statement, over two centuries of investigation have vastly increased our understanding of skeletal muscle hyperemia. Pioneering work by Gaskell examined the mechanical contribution of skeletal muscle, thus laying the groundwork for the muscle pump hypothesis by stimulating motor nerves producing brief contractions while collecting the venous effluent into a graduated cylinder for 5 s periods.

An initial expulsion of blood was observed, followed by a delayed increase in venous blood flow peaking between 10 and 15 s (53). This "muscle pump" action was believed to contribute to maintaining central blood pressure dynamics. This hypothesis would also ignite more than a century of investigation into the mechanisms for matching perfusion as well as the role contracting muscle plays in drawing increased blood flow to itself during exercise.

Exercise Hyperemia

A precise matching of blood flow to tissue metabolism is required in most living tissues. Generally, inadequate perfusion, whether due to insufficiencies in local flow or limitations to whole body vascular dynamics, are more limiting than over perfusion. Early efforts to quantify peak blood flow utilizing plethysmography or xenon washout (59, 105) produced estimates of 50-60 $ml \cdot 100\ g^{-1} \cdot min^{-1}$. Based on these estimates Mellander & Johansson (105) concluded that a healthy young heart could sufficiently supply skeletal muscle with adequate blood flow while maintaining blood pressure. This conclusion was contradicted by Secher et al. (133), when arm exercise was added to ongoing leg exercise. Despite unaltered power output by the legs, lower limb blood flow was reduced with the addition of arm exercise indicating a vasoconstriction of the feeding arteries to the legs and pointing toward a limitation within the cardiovascular system to maintain both adequate blood pressure and blood flow when a large skeletal muscle mass is involved in dynamic exercise. Subsequently, investigations measuring arterial inflow or venous outflow, utilizing a knee extensor model, found peak exercise hyperemic responses to have been previously underestimated by a factor of four or five (5, 129). In trained endurance athletes peak blood flow may reach $400ml \cdot 100g^{-1} \cdot min^{-1}$,

approximately eight times higher than originally posited (126). When examining muscle $\dot{V}O_2$ at either the whole body level (132, 154) or across a single muscle group (5, 125) increases based on extraction capabilities are similarly 2.5 to 3 fold higher than rest. Therefore, the vast increases in $\dot{V}O_2$ are predominantly mediated by increases in conductance facilitated by changes of vessel caliber.

The proportional matching of skeletal muscle blood flow to the metabolic needs of tissue has now been well characterized. Utilizing isolated preparations in rat for both twitch and tetanic stimulations, skeletal muscle blood flow was observed to increase in proportion to contraction frequency (99). Additionally, the aforementioned investigation found heterogeneities in blood flow distribution based on fiber type variations of whole muscle, indicating qualitative differences in the blood flow response to exercise. At rest and at walking speeds blood flow to deep slow-twitch fibers is three to four times greater than that to peripheral fast muscles. Upon initiation of walking exercise, blood flow increases proportionally to the oxidative muscles while it decreases to the glycolytic; however as exercise intensities are increased to moderate running speeds, further blood flow to all muscles increases in proportion to the exercise intensity (90). This proportional increase in blood flow was observed across a wide range of running speeds up to maximal running speed in rats (8). The heterogeneity has further been observed in the fast type muscle of the gracilis versus slow type soleus of the cat; however, both muscles exhibit an active hyperemic response that is linearly related to oxygen consumption (16).

Vascular smooth muscle cells (VSMC) are responsible for integrating and coordinating responses to multiple vascular control signals. The multitude of vasoactive

factors then exert an influence primarily by altering ion channel open/close properties leading to direct depolarization of the VSMC's or by triggering second messenger systems leading to depolarization. In the following sections, a number of putative vasoactive factors will be discussed.

Potassium (K^+)

Potassium (K^+) was recognized in early investigations to cause vasodilation in response to intra-arterial injection, thus altering total vascular conductance (37). That interstitial [K^+] increases following muscular contraction was first observed in stimulated muscle preparations (69, 85). These findings were subsequently reproduced during human exercise utilizing micro dialysis to confirm increased [K^+] in the venous effluent (77, 97). The increase in [K^+] is proportional to the total period of contractile activity (69) as well as proportional to higher rates of metabolic activity (58, 77). During the first minutes of rhythmic stimulations in either the hindlimb or individual gracilis muscle of dogs, the blunting of vasoconstriction was attributed to increases in K^+ release from the muscle, which subsequently interferes with the movement of calcium (Ca^{2+}) ions attenuating the ability of resistance vessels to constrict (12). Hnik *et al*. additionally reported that [K^+] rapidly increased in the interstitial space following contraction. The rapidity of K^+ release following a single contraction (112, 149) makes this the only muscle-derived activator of vasodilation that is likely capable of contributing directly to the immediate hyperemic response. Although causative linkage remains elusive, the time course and magnitude for the increases in venous [K^+] have been correlated in both animal (110) and human (83) models to the initiation of muscle hyperemia following contraction.

Regulation of $[K^+]$ at the vascular smooth muscle cell involves four primary regulatory channels: K_{ATP}, K_{Ca}, K_{IR} and K_V and these are important in the regulation of both $[K^+]$ as well as $[Ca^{2+}]$ and subsequent contractile state (91). Potassium channels play a central role in the maintenance and regulation of the cellular membrane potential in smooth muscle. This in turn controls intracellular $[Ca^{2+}]$, as well as Ca^{2+} flux directly affecting contractile tone of the vascular smooth muscle (115). Within the vascular smooth muscle cells there are K^+ channels that respond to changes in cellular energy metabolism (K_{ATP} channels) as well as those which may stabilize the resting membrane K^+ conductance (K_{IR}) (120, 144). Hyperpolarization of the membrane due to the outward flux of K^+ due to K_{ATP} activation decreases the influx of Ca^{2+} resulting in vasodilation. Conversely closing of the K_{ATP} channels leads to membrane depolarization thereby opening voltage-dependent calcium channels resulting in a Ca^{2+} influx and vasoconstriction. K_{ATP} channel activity is modulated directly by the metabolic conditions surrounding the vascular smooth cell with increased ADP/ATP ratio, acidosis, and hypoxia all representing activators. Additionally, K_{ATP} channels are controlled by prostacyclin (PGI_2), adenosine and β_2-adrenoreceptors that activate via cAMP/protein kinase A (PKA), as well as nitric oxide (NO) activation through cGMP (120).

Calcium-activated potassium (K_{Ca}) channels are highly expressed in vascular smooth muscle cells and when activated lead to cell hyperpolarization and closing of voltage-sensitive calcium channels, resulting in vasodilation (19). This channel type belongs to a family of conductance channels, each with characteristic structural and gate properties. The primary stimulus to K_{Ca} channels is thought to be membrane depolarization, thus these represent a mechanism of negative feedback to

vasoconstriction (19). Common vasodilatory substances: adenosine, endothelium-derived hyperpolarizing factor (EDHF), nitric oxide (NO), norepinephrine and hydrogen ion (H^+) may partially exert their effect through stimulation of PKA and protein kinase G (PKG), which phosphorylate K_{Ca} leading to vasodilation (19, 103, 115). Substances associated with vasoconstriction such as endothelin (ET) and angiotensin-II reduce K_{Ca} channel opening through PKC activation (72, 94, 106). Supplemental to the negative feedback characteristics of the K_{Ca} channels are voltage-dependent K channels (K_V channels). These channels are similarly responsive to membrane potential fluctuations such that depolarization causes their opening and resultant vasodilation (32, 34). Further, K_V channels respond to redox signaling, primarily through the formation of superoxide ($O_2\text{-}^{\cdot}$) which, from a variety of cellular enzymes, inhibits K_v channels directly or by dismutation to hydrogen peroxide (H_20_2) which activates NADPH oxidase to release superoxide and act on vascular smooth muscle cells (61). Because of conserved functions, the redundant nature therein, and the ability to react to multiple vasoactive stimuli it is likely that the effects of a complex group of factors are integrated to mediate K^+ channel contributions to vascular tone at any instant.

Lactate

Lactate is the end product of glycolysis and its accumulation occurs when its production outstrips removal. There are several underlying mechanisms which together promote lactate production, limit lactate removal, and thus lead to lactate accumulation. The accumulation of lactate has been well documented during exercise (21, 54, 79, 80, 131). Classic thought stemming from the work of Gaskell persisted through the work of Hill and viewed the accumulation of lactate as a potential moderator of vascular tone.

However, with regard to exercise hyperemia, the correlation between lactate accumulation and blood flow at various exercise intensities is weak (97, 101) and the time course for elevated lactate does not compare favorably with flow changes (97). Finally, following exercise termination lactate levels remain elevated (131) after flow has returned to near resting levels. In addition to poor correlation for the time course of vascular changes and lactate, it is now recognized that there are exercise intensities that elicit substantial changes in flow while lactate concentration [La^-] remains minimally elevated above resting levels. Lactate has been identified as an independent vasodilator with arteriolar dilation dependent on H_2O_2-mediated activation of vascular smooth muscle guanylate cyclase (30). Taken cumulatively, the evidence suggests that the role of lactate in vasodilation may be as a synergist with other vasoactive substances.

Hydrogen ion (H^+)

Independent of the presence of lactate in the vasculature of contracting skeletal muscle, alterations in H^+ ion concentration; i.e., pH changes, have been implicated in the regulation of vascular tone. Elucidation of a single mechanistic effect associated with [H^+] changes is difficult to identify in the dynamic *in vivo* setting. To explore the effects of lowered pH during hypercapnia as well as normocapnia with acidosis, isolated rat cerebral arteries were examined for smooth muscle (Ca^{2+}) concentration [Ca^{2+}] and membrane potential changes. Under hypercapnic and normocapnic conditions the response to acidosis was similar, causing dilation. This effect was attributed to the indirect effects of lowered pH on decreased [Ca^{2+}] thus causing relaxation (117). However, the mechanism of this response is unclear as hypercapnic acidosis caused

hyperpolarization of the smooth muscle membrane whereas normocapnic acidosis as well as reduced pH in bicarbonate-free solution led to dilation through decreased [Ca^{2+}].

It remains possible that during hypercapnia the CO_2 molecules have a direct effect on membrane potential that is separate from any effects of H^+ on [Ca^{2+}]; however other investigations suggest a role for ATP-sensitive K^+ channels. The effects of acidification on the arteriolar response to a depolarizing extracellular [K^+] showed that cell membrane potential is linearly related to vessel diameter with low pH (~6.8) leading to a hyperpolarization independent of [K^+] (40). Direct effects of pH changes on K_{ATP} channels have been observed both with and without the presence of ATP. Without ATP present the K^+ flux is minimally affected while the presence of ATP provides channel inhibition leading to decreased open probability (P_{open}) times. The changes in the kinetic behavior produced from decreasing pH had minimal effect on P_{open} (36). Taken together, these results likely indicate that changes in pH have a nominal direct effect on vessel diameter but interact synergistically with other vasoactive contributors.

Adenosine Triphosphate (ATP)

ATP functions as more than the energy intermediate for cellular work and its vasoactive properties arise from the intact molecule as well as its metabolites, adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine (ADO). ATP acts as a potent vasodilator by activating P_{2Y} purinergic receptors on vascular endothelial cells triggering subsequent vasodilators such as nitric oxide (NO), prostaglandins, and endothelial hyperpolarizing factor (EDHF) (27, 28, 122). ATP, ADP, AMP, and ADO are found at relatively high concentrations in the venous effluent of exercising muscle. Early investigations into the release of these metabolites found increased ATP in the

effluent of exercising frog muscle (17). Subsequent investigation determined that ATP is added to the blood during its passage through the muscle bed as arterial ATP concentrations remained stable while venous concentrations increased in proportion to exercise intensity (50). Additionally, [ATP] increased in the venous effluent during light forearm exercise with or without cuff occlusion, while no changes in venous [ATP] were observed during occlusion alone, suggesting exercise as the key stimulus for increases in [ATP] (49).

The "adenosine hypothesis" was first introduced by Berne and colleagues who forwarded adenosine as a the primary regulator of vascular tone by showing that the nucleoside can induce coronary vasodilation in the perfused heart (15). In cardiac tissue the release of ADO has been shown to be phasic in nature and its effectiveness in maintaining vasodilation diminishes with time (39). In skeletal muscle, both blood flow and interstitial [ADO] increase proportionally to the intensity of muscle contraction, associating its vasoactive properties with muscular work (66).

ATP may originate from several locations ultimately interacting with the interstitium or at vascular smooth cells; these origins include: 1) endothelial cells (114), 2) sympathetic nerve terminals (18, 82), and 3) release from red blood cells (44, 45, 55, 141). Purinergic signaling is complicated by the variety of receptors and their localization. The purinergic receptors are divided into the P_1 and P_2 families. P_1 receptors with the four subtypes A_1, A_2A, A_2B, and A_3 selectively bind ADO and act through G-protein coupled receptor signaling (52). The P_2 receptor family is further divided into the P_2X receptor type with seven isoforms that act as ionotropic membrane channels in response to ATP stimulus (116) and the P_2Y receptor type with eight

isoforms operating as metabotropic membrane receptors in response to ATP binding (152). Localization of specific subtypes can lead to varied responses with stimulation from ATP.

It is likely that the involvement of ATP in matching tissue perfusion to metabolic demand is tissue specific and arises from both ATP released to the lumen causing vasodilation as well as interstitial ATP from contracting muscles and sympathetic nerve terminals activating P_2X receptors resulting in vasoconstriction. For example, ATP in the lumen of blood vessels can bind to P_2Y purinergic receptor subtypes in endothelial cells which act through G-protein coupled receptors to release Ca^{2+}, activating nitric oxide synthase and leading to vasodilation (35, 123). Conversely ATP binding to P_2X receptor subtypes in vascular smooth muscle cells leads to channel opening and the direct influx of Ca^{2+} resulting in vasoconstriction (13). Opposing responses to a single stimulus suggests that the vascular response to purinergic signaling occurs in distinct local domains where the subtype expressed varies depending on the tissue type examined (96). Purinergic signaling plays an important role in local control of vessel tone however, they appear to be complimentary to the hyperemic response and not wholly responsible for it (26).

Nitric Oxide and Prostanoids

Downstream effectors of vessel caliber including NO, EDHF, and prostanoids are all implicated as potential contributors to vasodilation and increased blood flow due to stimulation by Ach, ATP and mechanical factors. Nitric oxide synthase (NOS) is the enzyme responsible for catalyzing the formation of NO from L-arginine and is constitutively expressed as endothelial NOS (eNOS) and neuronal NOS (nNOS). NOS

are expressed in skeletal muscles of all mammals and aid in regulating force production, and autoregulation of blood flow, as well as redox related systems (143). For human muscle these are localized to the endothelium where they contribute to blood flow autoregulation (51) and the skeletal muscle where NO may influence contraction (60, 87, 113). NOS are responsive to several controlling mechanisms including receptor binding, shear stress of the endothelial cells (118), Ca^{2+} concentrations and phosphorylation status at a serine residue in the reductase domain (Ser^{1177}) as well as a threonine residue in the calmodulin-binding domain (Thr^{495}) (46). Free NO has a short half-life unless it can be transported via interaction with proteins to form *s*-nitrosothiols with hemoglobin, or form nitrites or nitrates (1). NO affects vessel caliber by stimulating guanylate cyclase to produce cyclic-guanylate monophosphate (cGMP) which activates specific phosphodiesterases and ion channels leading to vasodilation (38). NO has been characterized as a contributor to resting vascular tone possibly stimulating, in addition to guanylate cyclase, prostaglandins (130). NO may also play a role in conducted vasodilation along arteriolar endothelium (24). Not surprisingly, a variety of studies relying on different NOS inhibitors, in different muscle groups, under different exercise paradigms led to a variety of opinions as to the potential role of NO in exercise hyperemia. An emergent theme is that blunting of NOS alone is not sufficient to blunt the hyperemic response to exercise, suggesting compensatory action of other mechanisms in response to skeletal muscle activity.

Production of prostanoids is regulated by the availability of arachidonic acid (AA) and the activity of cyclooxygenase (COX) enzymes. COX catalyzes the conversion of AA to prostaglandin H_2, from which prostacyclin, prostaglandin E_2, and thromboxane E_2

are derived. Important to vessel dilation are prostacyclin and prostaglandin; the production of both is influenced by purinergic signaling as well as the contraction of skeletal muscles (146). Their concentrations in venous effluent following forearm exercise of man (155) are elevated. Importantly, in canine hindlimb stimulation, prostaglandin E_2 release was elevated but found not to contribute to the hyperemic response after inhibition with indomethacin (156). Further, within the interstitial space of muscle prostaglandin E_2 as well as prostacyclin have been found to increase in proportion to exercise intensity (78). However, several investigations aimed at blocking the hyperemic response through COX inhibition, as well as simultaneous COX and NOS inhibition were unsuccessful, suggesting a non-essential role for these substances in the response.

Neural Control

A neural regulatory mechanism has been viewed as an attractive mediator of vascular caliber due to the rapidity with which such an effect could be mediated relative to the supposed slower speed with which metabolic control could elicit a response. Early investigations linking the proportional response of the vascular system to increases in muscular activity found that the vasculature had a diminished response to adrenergic stimulation during exercise compared with rest (124). Attenuation of sympathetic vasoconstriction can potentially be mediated prejunctionally by reducing neurotransmitter release as well as postjunctionally by interfering with receptor binding and/or action within the smooth muscle cells (147). Limited support for a prejunctional mechanism is provided by exercise-mediated attenuation of vasoconstriction in response to sympathetic nerve stimulation but an absence of such a response to exogenous norepinephrine (25).

More recent investigations have identified that, in addition to norepinephrine release from sympathetic nerves, ATP co-release occurs and acts as a neurotransmitter in vascular smooth muscle (27, 71). In binding with P_{2X} purinergic receptors located primarily in the vascular smooth muscle, ATP could mediate a direct vasoconstriction through a different mechanism than norepinephrine.

Several studies support postjunctional modulation of vasoconstrictor response to exogenous norepinephrine and other α-adrenoreceptor agonists in exercising muscle (6, 12, 23, 41, 124, 147). At moderate levels of exercise, α_2 constriction is preferentially attenuated by local vasodilatory substances leading to small arteriolar dilation while at higher exercise levels both α_2 as well as α_1 constriction are blunted (6). These results were subsequently supported by infusion of the α_1-specific agonist phenylephrine and α_2-selective agonist clonidine into surgically instrumented dogs during mild and heavy exercise. Only α_2-adrenergic-receptor responsiveness was affected during mild exercise while both α_1/α_2-adrenergic receptor responsiveness were affected during heavy exercise (23). To explore the role NO may contribute to the blunting of sympathetic vasoconstriction in humans the same α_1/α_2-adrenergic receptor agonists were used along with tyramine to stimulate endogenous release of norepinephrine and N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME) to inhibit NOS. By antagonizing NOS during tyramine infusion it was determined that NO is not obligatory for functional sympatholysis in contracting skeletal muscle (41). Further experimental evidence suggests that NO is not obligatory in humans. With prior elevation of forearm blood flow to levels seen during exercise with sodium nitroprusside (SNP), a precursor for NO, subsequent α-adrenergic vasoconstriction remained intact further indicating NO

was not effective in blunting sympathetic constriction (127). By administration of a ganglionic blocker prior to exercise onset Buckwalter & Clifford (22) were able to observe blood flow in the external iliac arteries during treadmill walking in canines with and without neural contributions. There were immediate increases in iliac blood flow in both control and ganglionic blockade with no differences in hindlimb conductance over the first 20 s. This provides further evidence that the immediate rise in conductance at the onset of exercise is not dependent on the autonomic nervous system.

Acetylcholine (Ach) Spillover

The appeal for Ach as mechanism lies in its feed-forward potential as a vascular regulator. In this hypothesis, Ach released during activation of the motor nerves escapes the motor end plate and is capable of stimulating the vasculature of skeletal muscle (153). This dilatory mechanism would then be directly correlated to increases in work rate and/or metabolic rate, thus coupling blood flow to force development. One experimental approach to this question utilized atropine as a muscarinic receptor blocker in the skeletal muscle vasculature of the forearm in humans performing either a single hand grip or exposure to a single blood pressure cuff inflation to 120 mmHg. The infusion of atropine prior to either experimental protocol had no effect on peak forearm blood flow or total hyperemia as compared to control (20). Alternative observations utilizing neuromuscular blockade of nicotinic receptors in human forearm (43) as well as during sciatic nerve stimulation in the instrumented canine hind limb (112) found no increase in blood flow following attempted contraction or nerve stimulation. Nicotinic receptor blockade prevents the contributing factors of the muscle contraction *per se* while still allowing for normal Ach release. The lack of response in these separate investigations suggests that

Ach spillover is not the explanatory mechanism for rapid dilation in the vasculature of skeletal muscle at the onset of contraction.

Myogenic Control

A link connecting the initiation of exercise with increased blood flow via a singular putative vasoactive substance has, to this point, been impossible to identify. This difficulty in resolving spatial and temporal issues relating to either metabolite or neural control ultimately led to investigations of mechanical factors that could fill the gap in explaining increased conductance as well as maintenance of flow during skeletal muscle contraction. Hypotheses of this type suppose that the contraction of the muscle itself contributes directly and independently to perfusion of the muscle while also interacting with the vasculature to regulate flow. Similar in concept to the benefits of neural control or Ach spillover, mechanical effects of muscle contraction would constitute a feed-forward mechanism that could promote a rapid increase in blood flow (76, 88).

It has been difficult to demonstrate a hyperemic response to extravascular pressure in the absence of an actual muscular contraction (42,805). A creative instrumentation of isolated canine hindlimb by Mohrman and Sparks (109) allowed for interrogation of this question by placing an intramuscular pressure transducer deep within the muscle and a cuff around the muscle belly. In comparing the vascular conductance following tetanic contraction to that observed following the cuff occlusion it was concluded that a portion, but not all, of the vasodilation could be accounted for in this way. This response was observed following high extravascular pressures, raising the question of their *in vivo* application. In humans exercising at moderate running speeds,

intramuscular pressures of 270 mmHg have been recorded (11) and during maximal contraction pressures as high as 570 mmHg have been reported (134). To elicit similar myogenic responses in rat soleus arterioles, pressures of 600 mmHg were required (31) whereas for an observable response in canine gracilis, extravascular pressures of only 100 mmHg were required (10). Importantly, in the canine gastrocnemius *in situ* preparation performing isometric contractions with tetanic stimulation via sciatic nerve, regional pressure differences have been reported (3). The pressures were recorded superficially and deep near the origin, mid-muscle, and insertion with deep pressures of 586, 1,676, and 993 mmHg and superficial pressures of 170, 371, and 351 mmHg respectively. The observations of increased interstitial pressure in proportion to contraction intensity (121) as well as the intensity dependent magnitude of vasodilation (33, 148, 149) suggest a graded response to the mechanical actions of skeletal muscle. These results suggest that compressive changes within the vasculature contribute to the immediate adaptation of blood flow rather than myogenic dilation following contraction.

Muscle Pump

The inadequacy of the previous mechanisms to fully resolve the spatial and temporal alterations in local skeletal muscle blood flow at least partially led to a muscle pump hypothesis and subsequent investigations of the muscle pump contribution. According to the muscle pump hypothesis, skeletal muscle contraction effectively compresses the contents of its blood vessels which in turn expels blood while also increasing the arterio-venous pressure gradient increasing local blood flow (91). There are several mechanisms through which the muscle pump may contribute to muscle blood flow. However three predominate: 1) Within dependent limbs the local arterio-venous

pressure gradient may be increased by the transient reduction of hydrostatic pressure in the venous circuit; 2) The addition of peripheral kinetic energy to the blood aids in the return of blood to the heart in a healthy vasculature with functioning venous valves (fighting gravity); and 3) The elastic recoil of the relaxing muscles may rapidly open the tethered veins creating a suction of blood from the arterial inflow. Interestingly, even if the muscle pump is not directly implicated in the immediate matching of metabolism to perfusion in an exercising muscle the benefits could still be surmised indirectly. An ancillary contribution of the muscle pump, separate from potential direct contributions to muscle blood flow, is to reduce muscle blood volume and return it centrally which allows for normal increases in cardiac output throughout exercise, a phenomenon that is absent in persons lacking venous valves (128).

Observations of the contribution of muscular contraction to blood flow existed prior to the early 1900's and include those of Harvey (1628), Hunter (1794), and Gaskell (1869) as previously mentioned. However, pioneering investigations that laid the framework for a "muscle pump" hypothesis came from work in humans by Leonard Hill (1909), D.R. Hooker (1911), and August Krogh (1929) (see references in Loring Rowell, 2004). In upright humans, as they begin to walk, there is a reduction in pressure in the superficial veins below that of hydrostatic pressure. Pollack and Wood (119) noted that it took six to seven steps to reduce ankle vein pressure from 90 to ~25 mmHg. Based on the previous works it was thought that the slow reduction in venous pressure was due to the superficial blood being forced from small perforating veins to deep veins. For this to occur a high driving pressure would be necessary with a negative pressure in the deep veins compared to superficial, a phenomenon which could be observed during the

skeletal muscle contraction/relaxation cycle. These early observations set the stage for subsequent investigations into the exact mechanisms of the muscle pump as well as its potential contribution to immediate versus sustained hyperemia.

Various investigations have found that skeletal muscle contraction can raise intramuscular pressure. In some of the earliest, stimulated frog gastrocnemii were found to increase pressure from 100 to 300 mmHg (68). Interestingly, sustained isometric contractions of rat calf muscles produced increases in pressure linearly correlated with increases in force of contraction. Similar to the results of Ameredes et al. (3), higher average pressure was observed in the central area of the rat calf muscle (220$\pm$80 mmHg) as opposed to the peripheral regions (85$\pm$56 mmHg) (84). The result of these local increases in perfusion pressure, in conjunction with functioning venous valves oriented toward the heart, is that blood flows from the compressed segment toward the heart. During rhythmic contractions of skeletal muscle this unidirectional pumping action imparts kinetic energy during the contraction phase while potentially enhancing refilling during the relaxation phase.

An interesting study into the intra-thoracic and intra-abdominal pressures in man while running in place found that the pressure developed in the abdomen to stabilize the core raised inferior caval pressure sufficiently to impede venous return from the legs. However, the force imparted to the blood from the contracting muscles was sufficient to pump blood past this functional obstruction (145). This phenomenon was further examined during a series brief tetanic contractions at a rate mimicking the stride frequency of running in cat muscle (47). In this paradigm, arterial inflow occurred only during the relaxation phase while venous outflow occurred primarily during the

contraction phase in the dependent limbs. The average blood flow through the muscle was observed to be as great, or greater, than the flow in the immediate post-exercise period. The venous pressure reduction between contractions suggests a gain in local perfusion pressure attributed to the pumping action of the skeletal muscle (47). Extension of these findings to humans resulted from experiments that were performed at rest and during exercise while subjects were tilted from supine to upright. A shift from supine to upright during post-exercise hyperemia did not significantly increase flow through the muscles; however, the same shift performed during the exercise increased maximal flow approximately 60% (48). This observation did not offer mechanistic insight for the muscle pump but provided support for the role of muscle contraction in dependent limbs to increase local perfusion.

These findings have been further evaluated more recently in both healthy and diseased humans. Comparison of plantar flexion in the upright seated versus supine position found differential increases in blood flow through the femoral artery for single as well as repeated contractions that were proportional to increases in force production. Femoral artery flow was increased to a greater degree in the upright seated position relative to supine however, the changes in the venous volume due to hydrostatic pressure changes were insufficient to fully explain the total blood flow, indicating that local changes in vascular resistance are requisite (95). With continuous monitoring of femoral artery inflow utilizing Doppler ultrasound, Rådegran and Saltin (121) were able to establish temporal resolution of the incoming arterial versus the outflowing venous blood. Their conclusion, based on curve fitting the increase in blood velocity through contraction cycles, was that the elevation in blood flow during the initial contractions is

due to mechanical factors whereas the vasodilators that are triggered during the first contractions potentiate further amplification in blood flow. In another study, the reduction in venous pressure at the ankle previously seen in upright walking was tested in upright cycling. Ankle vein pressures in healthy subjects declined 45 mmHg leading to a 5.3-fold increase in femoral artery blood flow while that of the CHF patients only dropped 36mmHg and femoral artery blood flow increased only 1.7-fold during the same exercise (138). These results indicate that there are mechanical contributions to blood flow in dependent limbs, a dynamic relationship between central versus peripheral pump contributions, and a likely interplay between muscle pumping and vasodilatory agents previously discussed.

Recent investigations have shed important light on contributions of the muscle pump during the initial hyperemic response from rest to activity as well as the impact on maximal blood flow for exercising muscle. Thus far, a myriad of vasoactive substances have been discussed collectively implying a necessity for vasodilation concurrent to an observable muscle pump effect. In an inventive attempt to circumvent the identification of a single vasoactive substance, Hamann et al. (62) reasoned that several of these substances might cause vasodilation via a final common pathway; specifically by hyperpolarizing the membrane of smooth muscle cells. With the working hypothesis that rapid increases in blood flow at exercise onset require vasodilation this investigation aimed to prevent dilation by holding the membrane potential in a depolarized state, thus any immediate changes on blood flow following a single tetanic contraction could be attributed to a muscle pump effect. An additional strength of this investigation was the creative instrumentation of the canine subjects, thus allowing upright body positioning as

well as controlled sciatic nerve stimulation. Intra-arterial infusion of K^+ effectively holds the membrane potential of smooth muscle cells in a depolarized state (86), this reduces resting blood flow as well as blunts the dilator response to hyperpolarizing substances. For comparison, in separate trials, phenylephrine was infused to reduce baseline blood flow to the same level as that observed during K^+ infusion; phenylephrine is a selective α_1-adrenergic receptor agonist. Under control conditions a marked increase in blood flow over baseline was observed immediately following a single contraction. During phenylephrine infusion baseline flow was reduced approximately 50% but an immediate increase in flow remained present following a single contraction. Conversely, K^+ infusion reduced baseline flow approximately 50% while also ablating the hyperemic response to a single stimulated muscle contraction. During all trial contractions force was preserved indicating no intrinsic reduction in skeletal muscle activity due to infusion. This would suggest that the muscle pump operating by itself is not responsible for the immediate hyperemia following a single tetanic contraction and that both vasodilation resulting from membrane hyperpolarization and muscle contraction generate the increased flow *in vivo.*

The contribution of the muscle pump to skeletal muscle auto-perfusion has been investigated with regard to both its role in the immediate response and its role in the steady state of exercise/contraction hyperemia. Commonly, a transient reduction of the hydrostatic column in venous circulation is posited as the primary benefit from the muscle pump. This lowers venous pressure within the exercising muscle during relaxation thus increasing the pressure gradient promoting additional blood flow to the muscle. An experimental hurdle to quantifying this contribution is the requirement of a

sufficient hydrostatic column, normal exercise and recruitment characteristics, and the instrumentation necessary to quantify force and conductance. In an elegantly instrumented canine model, Hamann et al. measured canine hindlimb blood flow during treadmill running with intra-arterial infusion of ADO (64). In control conditions the experimental limb blood flow increased approximately 4-fold 10 s after the initiation of moderate intensity exercise. When resting blood flows were raised to observed exercising levels with ADO prior to exercise initiation there was no additional increase in flow from the muscular contraction. One limitation of this investigation is that the results are based solely on vasodilation from ADO infusion. However this still provides strong evidence that vasodilation is sufficient by itself, independent of any muscle pump effect, to cause the full effect of exercise hyperemia.

To identify the potential contributions of the muscle pump to maximal blood flow experiments have been conducted with stimulated contractions following prior vasodilation. To establish maximal vasodilation, Dobson et al. (42) first clamped venous outflow for five minutes to allow any local vasodilating agents to accumulate. Additional infusion of ADO and sodium nitroprusside with the release of the occlusion generated baseline blood flows in this paradigm that were as great as flows observed during maximal exercise. Importantly, no direct instrumentation of the muscle's arterial supply were used as this has been suggested to negatively affect the normal vascular response under investigation (88). The gastrocnemius vasculature was fully dilated prior to contractions as described above followed by four consecutive muscle contractions stimulated at a rate of one contraction per second. In this model, the average venous outflow was found to be lower across the four contraction period than the corresponding

averages during the induced hyperemia and post-contraction periods. If the muscle pump were important in perfusion of the muscle, one would have expected an increase in blood flow with contraction onset. Therefore, these findings argue against the efficacy of the muscle pump to contribute to peak skeletal muscle blood flow in the presence of maximal vasodilation.

As discussed, the muscle pump hypothesis predicts that if skeletal muscle begins rhythmic contraction after maximal vasodilation is already achieved, blood flow should increase further due to the mechanical contributions. This was examined with tetanic contractions in addition to maximal vasodilation with sodium nitroprusside (SNP) (92). Similar to the results of Dobson et al., tetanic contractions in an already maximally vasodilated muscle bed did not significantly increase blood flow. Further, in this preparation the ability of the muscle to autoregulate was abolished leading these investigators to question the efficacy of *in situ* preparations for studies of exercise hyperemia. Supporting these experimental findings, further evidence in several *in situ* studies reported no significant muscle pump effect for skeletal muscle blood flow (102, 111, 140). Contrarily, other studies with conscious animals and conscious humans exercising on treadmills, provide evidence for the muscle pump as a contributor to maintaining skeletal muscle blood flow during exercise (136, 149). These findings call into question the mechanisms by which the muscle pump may contribute as well as inherent problems within the *in situ* design and instrumentation.

In describing the muscle pump it is important to consider that conductance or resistance characteristics do not exist across a pump. For differences in conductance to be attributed to a muscle pump effect the contraction must increase energy in the

circulation and not result from relaxation smooth muscle or resistance vessels (92). The muscle pump may increase blood flow to exercising muscle by increasing total kinetic energy or decreasing the venous pressure during contraction, thus increasing the energy gradient forcing blood through the vascular bed. In support of the muscle pump contribution to maintaining elevated flow during exercise reports have indicated higher blood flows to deep, highly oxidative muscles during normal exercise than those that could be achieved through vasodilation alone (89, 90). Additionally, there is evidence that rhythmic tetanic stimulation is able to simulate the magnitude of blood flow that has been observed during normal locomotory exercise, indicating efficacy for stimulated muscle preparations in muscle pump investigations (100). Taken together these results support the role of the muscle pump in blood flow maintenance during exercise as well as the experimental potential for the *in situ* preparation.

The implication for the muscle pump as a contributor to both the immediate hyperemic responses as well as continued conductance during rhythmic contractions remains controversial. The purposes of the present study are to identify the contribution of the muscle pump on a contraction by contraction basis from rest to a variety metabolic rates as well as during the transition from a low metabolic rate to a higher rate. Further, we aimed to identify a predicted conductive response by fitting the blood flow response curve from initiation through steady state exercise

II. JOURNAL MANUSCRIPT

Abstract

The aim of this study was to characterize the contribution of skeletal muscle contraction to the immediate hyperemic blood flow response as well as the continued involvement in matching tissue perfusion to elevated metabolic rates. There exists a substantial reserve for increased blood flow within skeletal muscle in response to dynamic exercise however the interaction of neural regulation, vasoactive metabolites, and mechanical characteristics are incompletely understood. To address questions concerning blood flow in response to transitions from rest to various metabolic rates an isolated canine gastrocnemius *in situ* model was employed. Seven canines were used for this investigation with the gastrocnemius muscles isolated for isometric contractions with tetanic stimulation. Measures were made for blood flow, blood pressure, force, and near infrared spectrophotometric analyses under conditions of spontaneous blood flow. The following transitions were investigated: from rest to tetanic contractions of 1/3 s, rest to 2/3 s, rest to 1/1 s and during the transition from 1/3 s to 2/3s all with spontaneous blood flow response intact. Additionally, an estimation for the blood flow response with no mechanical contribution from the muscle pump was made with determination for the kinetics of the estimate. The time constant (tau) for the blood flow response was not significantly different between the measured flow with contraction (Q_{wc}) and the estimate with no contraction (Q_{nc}) for the 1/3 s stimulation rate (12.8 ± 5.5 s vs 11.8 ± 3.2 s

respectively), the transition from the high baseline (1/3 s) to a higher rate ($2/3_{HB}$) (21.2 ± 3.4 s vs 21.7 ± 4.8 s respectively), from rest to 2/3 s (25.6 ± 12.0 s vs 22.1 ± 1.3 s respectively), or from rest to 1/1 s (16.7 ± 3.0 s vs 22.1 ± 1.3 s respectively). Initially, for this model, there is a positive contribution to total blood flow provided by the contracting skeletal muscle, however this diminishes within the first few contractions. At higher stimulation rates the net effect of the contracting muscle is to limit local blood flow in the exercising muscle. In conclusion, the muscle pump may contribute to local perfusion at exercise onset with diminishing returns as rhythmic contractions continue. In the steady state the main contributions of the muscle pump is to aid in the maintenance of central hemodynamics.

Introduction

The mechanical effect of muscular contraction to initiate an immediate increase in local skeletal muscle perfusion as well as to facilitate increased blood flow during rhythmic types of exercise are collectively referred to as the "muscle pump". Indirectly, muscle contraction in the dependent limbs leads to expulsion and central return of peripheral venous blood which increases cardiac filling pressure, stroke volume, and thus cardiac output (135). In such a manner the action of the muscle pump increases central blood volume facilitating a normal rise in cardiac output by the Frank-Starling effect, which can then be directed to the exercising muscle, thereby indirectly promoting muscle hyperemia. In the current investigation, the focus is on direct effects by which the muscle pump may contribute to immediate and sustained muscle perfusion.

The muscle pump hypothesis grew from observations of venous pressure reductions in the ankles of men when transitioning from standing to walking (119). These early observations were furthered by showing that blood flow during heavy rhythmic contractions in human calf muscles was greater when the subjects were standing than when they were in the supine position (48). Subsequent investigations aimed to quantify a muscle pump contribution, reasoning that contraction-induced changes in venous pressures would be greater with the limb below the heart than at or above the heart. An enhanced flow from a single contraction with the limb below the heart (149) as well as during rhythmic exercise (95, 139) were attributed to the benefit of the skeletal muscle pump.

The muscle pump is believed to contribute to muscle perfusion through three primary mechanisms: 1) Compression of the contents of skeletal muscle blood vessels, transiently reducing hydrostatic pressure in the venous circulation during relaxation; 2) Addition of peripheral kinetic energy to the blood in healthy vasculature, thus aiding in the central return of blood (noted above); and 3) Elastic recoil of relaxing muscles to rapidly open veins tethered to the muscle, thereby creating a suction of blood from arterial inflow (47, 88, 91, 137). There is ample evidence for an immediate increase in blood flow at the onset of exercise (33, 95, 112, 139, 149, 150). However there is controversy as to whether these immediate increases in blood flow are accompanied by an immediate vasodilation (33, 112, 139, 149) or responsive vasodilation that occurs after a latency of up to five seconds (9, 56, 75, 104, 136).

Recent investigations have shed important light on contributions of the muscle pump during the initial hyperemic response from rest to activity as well as its impact on

maximal blood flow for exercising muscle. In an inventive attempt to circumvent the contribution of a single vasoactive substance, Hamann et al. (62) that several of these substances might cause vasodilation via a final common pathway; specifically by hyperpolarizing the membrane of smooth muscle cells. With the working hypothesis that rapid increases in blood flow at exercise onset require vasodilation, this investigation aimed to prevent dilation by holding the membrane potential in a depolarized state. By clamping smooth muscle membrane potential, immediate changes in blood flow following a single tetanic contraction could be attributed to a mechanical muscle pump effect alone. An additional strength of this investigation was the creative instrumentation of the canine subjects, thus allowing upright body positioning as well as controlled sciatic nerve stimulation. Intra-arterial infusion of K^+ effectively holds the membrane potential of smooth muscle cells in a depolarized state (86), thus reducing resting blood flow as well as blunting the dilator response to hyperpolarizing substances. For comparison, in separate trials, phenylephrine (a selective α_1-adrenergic receptor agonist) was infused to reduce baseline blood flow to the same level as that observed during K^+ infusion. Under control conditions a marked increase in blood flow over baseline was observed immediately following a single contraction. During phenylephrine infusion, baseline flow was reduced by approximately 50% but an immediate increase in flow remained present following a single contraction. However, K^+ infusion reduced baseline flow approximately 50% while also ablating the hyperemic response to a single stimulated muscle contraction. During all trial contractions force was preserved, indicating no intrinsic reduction in skeletal muscle activity due to infusion. This suggests that the muscle pump operating by itself is not responsible for the immediate hyperemia

following a single tetanic contraction and that the combination of vasodilation resulting from membrane hyperpolarization and muscle contraction act together to generate the increased flow *in vivo.*

The contribution of the muscle pump to skeletal muscle auto-perfusion has been investigated with regard to both its role in the immediate response and its role in the steady state of exercise/contraction hyperemia. Commonly, a transient reduction of the hydrostatic column in venous circulation is posited as the primary benefit from the muscle pump. This lowers venous pressure within the exercising muscle during relaxation thus increasing the pressure gradient across the muscle vasculature. This increase in driving pressure should promote additional blood flow to the muscle. An experimental hurdle to quantifying this contribution is the requirement of a sufficient hydrostatic column, normal exercise and recruitment characteristics, and the instrumentation necessary to quantify force and conductance. Again, in an elegantly instrumented canine model, Hamann et al. (64) measured canine hindlimb blood flow during treadmill locomotion with intra-arterial infusion of ADO. In control conditions the experimental limb blood flow increased approximately 4-fold 10 s after the initiation of moderate intensity exercise. When resting blood flows were raised to the observed exercising levels with ADO prior to exercise initiation there was no additional increase in flow from the muscular contraction; e.g., no muscle pump effect was observed. One limitation of this investigation is that the results are based solely on vasodilation from ADO infusion. However this still provides strong evidence that vasodilation is sufficient by itself, independent of any muscle pump effect, to elicit the full effect of exercise hyperemia.

To identify the potential contributions of the muscle pump to maximal blood flow, experiments have been conducted with stimulated contractions following prior vasodilation. To establish maximal vasodilation in the surgically isolated canine gastrocnemius complex, Dobson et al. employed the combined effects of vasodilator agents and vascular occlusion. First, the muscle was infused briefly with ADO and sodium nitroprusside, then both the arterial inflow and venous outflow were clamped for five minutes; this allowed further accumulation of local, endogenous vasodilating agents (42). Upon release of the occlusion, blood flows reached the peak values observed in this preparation during rhythmic isometric contractions that elicit $\dot{V}O_{2peak}$. Importantly, no direct instrumentation of the muscle's arterial supply was used as this has been suggested to negatively affect the normal vascular response under investigation (88). With the gastrocnemius vasculature fully dilated prior to contractions as described above, four consecutive muscle contractions were stimulated at a rate of one contraction per second. In this model, the average venous outflow was found to be lower across the four-contraction period than the corresponding averages during the induced hyperemia and post-contraction periods. If the muscle pump were important in maximal perfusion of the muscle, one would expect an increase in blood flow when the contractions were elicited during the peak hyperemic period following release of the occlusion. Therefore, these findings argue against the efficacy of the muscle pump to contribute to peak skeletal muscle blood flow in the presence of maximal vasodilation.

As discussed, the muscle pump hypothesis predicts that if skeletal muscle begins rhythmic contraction after maximal vasodilation is already achieved, blood flow should increase further due to the mechanical contributions. In a rat hindlimb preparation, by

performing tetanic contractions after achieving maximal vasodilation with sodium nitroprusside (SNP), no additive effect was observed from the contractions (92). In this preparation the ability of the muscle to autoregulate was abolished leading these investigators to question the efficacy of *in situ* preparations for studies of exercise hyperemia. The ablation of autoregulation in this experimental set up, and small animal preparations like it, is likely due to the intrusion of measurement equipment into the small vessels being studied. In the larger canine gastrocnemius *in situ* preparation, Dobson et al. (42) also showed no increase in blood flow with muscle contraction after prior maximal vasodilation had been achieved. In canine preparations instrumentation has not reduced the muscles ability to autoregulate during dynamic upright exercise (64) or following a single tetanic stimulation in the isolated preparation (62). Additional evidence in several *in situ* studies reported no significant muscle pump effect for skeletal muscle blood flow; however, in these studies resting as well as vasodilated blood flow were substantially lower than those of other investigations (102, 111, 140). Despite the reports described above indicating no muscle pump contribution, other studies performed on conscious animals and conscious humans exercising on treadmills, provide evidence for the muscle pump as a contributor to maintaining skeletal muscle blood flow during exercise (136, 149). Clearly, controversy remains with regard to efficacy of the muscle pump.

In describing the muscle pump it is important to consider that conductance or resistance characteristics do not exist across a pump. For differences in conductance to be attributed to a muscle pump effect the contraction must increase energy in the circulation and not result from relaxation of smooth muscle or resistance vessels (92).

The muscle pump may increase blood flow to exercising muscle by increasing total kinetic energy or decreasing the venous pressure during contraction. This increases the driving pressure aiding blood flow into the vascular bed independently from smooth muscle relaxation or increased vessel diameter. In support of the muscle pump contribution to maintaining elevated flow during exercise, reports have indicated higher blood flows to deep, highly oxidative muscles during normal exercise than those that could be achieved through vasodilation alone (89, 90). While blood flows are typically higher for dynamic exercise than for stimulated muscle, evidence that rhythmic tetanic stimulation is able to simulate comparable blood flow was reported when comparing blood flow to different muscle groups during tetanic stimulation in rat hindlimb (100).

Additional evidence supports the efficacy of the muscle pump influence on blood flow in tetanically stimulated skeletal muscle. In an inventive study to separate muscle contribution to auto-perfusion from increased arterial flow due to cardiac responses, extra-corporeal tubing was used to allow venous blood from contracting muscle to be selectively returned to the muscle's own arterial inflow. In this way the mechanical forces of contraction were observed to be sufficient for initiation and maintenance of blood flow within the muscle with no cardiac contribution. In this preparation the blood flow responses to a stimulation rate of 1/ s increased to approximately 80% of the values observed during the normal cardiac-supported perfusion response (137). Collectively, these results indicate a potential role for the muscle pump as a contributor to skeletal muscle blood flow and further support the use of the large animal *in situ* preparation as a means for investigation.

As the preceding discussion indicates, the role of the muscle pump as a contributor to both the immediate hyperemic responses as well as continued conductance during rhythmic contractions remains controversial. Therefore, the purposes of the present study are to identify the contribution of the muscle pump on a contraction by contraction basis from 1) rest to a variety of contraction rates that engender different metabolic rates, and 2) from a low contraction rate to a higher rate. In order to estimate the effects of the muscle pump, we collected blood flow data at a high frequency and then made assumptions about the flow profile with regard to which portion was strictly due to the mechanical effects of contraction versus a portion due to all other spontaneous vasoactive, neural, metabolic and myogenic responses.

Methods

Animals and ethical approval

This study was conducted with the approval of the Institutional Animal Care and Use Committee of Auburn University (Auburn, AL), where the experiments were performed. Nine adult dogs of either sex were initially anesthetized with pentobarbital sodium (30 $mg \cdot kg^{-1}$) via injection into a prominent cephalic vein of the forelimb. After confirming that a surgical plane of anesthesia had been attained, a jugular vein was isolated, and all subsequent doses (65–100 mg) were delivered directly through this vein to maintain a deep surgical plane of anesthesia. All dogs were treated with an initial bolus of heparin (1,500 $U \cdot kg^{-1}$) upon jugular isolation with two subsequent equal doses given at two hour intervals to a total of 3,000 $U \cdot kg^{-1}$. Dogs were intubated with an endotracheal tube and mechanically ventilated (model 613, Harvard Apparatus, Holliston, MA). Rectal temperature was maintained at 37°C with a heating pad and heating lamps.

Mechanical ventilation was initiated at 20 $ml \cdot kg^{-1}$, 15 $breaths \cdot minute^{-1}$, 50% inspiratory cycle and adjusted from this baseline to maintain normal arterial Po_2, Pco_2, and pH levels.

Some of the data from experiments on the animals used here have been previously analyzed and reported (Wüst et al., 2014); however, that publication dealt with oxygen uptake on-kinetics. In the current investigation, some data from these same animals have been used in a separate, independent analysis for determination of muscle pump contribution to the immediate and steady state blood flow at varying metabolic rates and transitions.

Surgical preparation

The surgical preparation was similar to the methods previously described in detail (67, 142). In short, the left gastrocnemius plus superficial digital flexor muscle group (GS) was surgically isolated from surrounding muscles. All muscles that overlie the GS (sartorius, gracilis, semitendinosus, and semimembranosus) were cut with a cauterizing blade (electric soldering gun) at their insertions and laid back. A portion of the calcaneus, with the two tendons from the GS attached, was cut away at the heel and clamped around a metal rod for connection to an isometric myograph via a load cell (Interface SM-250, Scottsdale, AZ, USA) and a universal joint coupler. The universal joint coupler allowed the muscle generate force directly in line with the load cell while generating minimal torque. The proximal end of the muscle remained attached to its origin. The hind limb was fixed at the knee and ankle with bone nails placed into the femur and tibial bones then attached to a fixed platform. A turnbuckle strut was inserted between the tibial bone nail and the arm of the myograph to further minimize limb

movement during contractions. The muscle was covered with saline-soaked gauze and a thin plastic sheet to reduce drying and cooling. The left sciatic nerve, which innervates the GS, was isolated near the GS, doubly ligated, cut between the ties, and the distal end secured in an epoxy-resin loop containing stimulating electrodes.

Venous outflow from the GS was isolated by ligating all veins draining into the popliteal vein except the GS veins. Similarly, arterial inflow was only from the popliteal artery with all other arterial branches ligated. The popliteal vein was cannulated, and this venous blood flow was returned to the animal via a reservoir attached to a cannula in the left jugular vein. An indwelling oximeter probe (Oximetrix 3, Abbott, North Chicago, IL) and flowmeter (T206, Transonic Systems, Ithaca, NY) were inserted in-line with the cannulated vein to continuously monitor the fraction of saturated hemoglobin (Hb) and blood flow, respectively. Perfusion pressure was measured via a cannula in the right, contralateral femoral artery.

After all experiments, the muscle was removed from the animal, cleared of surface connective tissue, and weighed before (wet) as well as after drying. The wet weight was used to normalize physiological variables to muscle mass (e.g., blood flow and $\dot{V}O_2$). All dogs were euthanized at the end of the experimental series with an overdose of pentobarbital sodium and saturated potassium chloride.

Protocol

At the start of each experiment, the muscle was set at an optimal length (*L*o) by progressive lengthening until a peak in developed tension was obtained during stimulation at 0.2 Hz. Once *L*o was determined, at least 5 min of rest was given before

experiments began. Before the start of each experimental condition the resting muscle tension was checked, and the muscle reset to *L*o as necessary. Isometric tetanic contractions (0.2 ms pulses at 50 Hz; 200 ms duration) were elicited at different frequencies via maximal nerve stimulation (6-8 V). Four different contractile transitions were studied: 1) from rest to 0.33 Hz (1/3 s), 2) from rest to 0.67 Hz (2/3 s), 3) from rest to 1.0 Hz (1/1 s), and 4) from 0.33 Hz to 0.67 Hz (2/3 from high baseline, $2/3_{HB}$). These rates of tetanic trains are expected to result in metabolic rates that are ≈40% (0.33 Hz), ≈60% (0.67 Hz), and ≈100% (1.0 Hz) of $\dot{V}O_{2peak}$ for this muscle (2, 81) although in practice the absolute rates will vary somewhat due to previous stimulation history (e.g., fatigue). Between all trials the muscle was allowed to recover for 35 minutes at the resting perfusion rate. For all trials muscle blood flow was spontaneous.

Measurements

Maximal tension (T_{max}) was determined as the highest value during the contraction (load cell 90% response time was <1 ms). The indwelling flowmeter was set to its highest pulsatile cut-off frequency of 100 Hz, and manually calibrated with a graduated cylinder before, during and after each series of contractions. Arterial samples collected before and after each series of contractions were analyzed immediately at 37°C for Po_2, Pco_2, pH, and lactate concentration ($[La^+]$) by a blood gas, pH, metabolite analyzer (GEM Premier 3000, Instrumentation Laboratories, Lexington, MA) and for hemoglobin concentration ([Hb]), and percent saturation of Hb with O_2 (SO_2) by a CO-Oximeter (IL 682, Instrumentation Laboratories, Lexington, MA) set for dog blood.

Venous O_2 saturation was measured by an indwelling oximeter and along with arterial O_2 values and blood flow measurements was used to calculate contraction-by-

contraction $\dot{V}O_2$ as described previously (67). Muscle oxygenation was determined using continuous-wave near-infrared spectroscopy (NIRS; Oxymon MkIII, Artinis Medical Systems, Zetten, the Netherlands). Data for $\dot{V}O_2$ and muscle oxygenation on-kinetics from some of the current experiments have been published previously and will only be referred to secondarily in the present communication.

Calculations and kinetics analysis

Analysis of blood flows

The onset of contractions was determined with a Microsoft Excel macro written in-house and blood flow data were fitted using OriginLab 9.1 (Northhampton, MA, USA) from contraction onset with the following mono-exponential function:

$$y_{(t)} = y_{bas} + A\,[1 - e^{-(t-TD)/tau}] \quad (\textit{equation 1})$$

The variable value (y) any time (t) after the onset of contractions was characterized by the steady state increment ([y]ss), above the baseline ([y]b), and TD and represent the delay and time constant of the exponential response respectively. Y_{bas} was held constant as the 30 s average value prior to the first contraction. The other parameters (A0, TD and tau) were allowed to float until the "best fit" was seen. The criteria for "best fit" have been described by Whipp et al. (74). Briefly, this is accomplished by moving the exponential fitting window to minimize the 95% confidence interval (C95), maximize the flatness of the residual, and minimize the reduced $\chi 2$ of the fit (in that order). Basically, Chi^2, residuals of the fit, and the 95% confidence interval for tau were observed until the best combination of minimal values was achieved. Muscle blood flow was identified on a

contraction-by-contraction basis with the in-house macro and using the muscle weights, blood flow data were expressed as:

$$\dot{Q} = ml \cdot 100g^{-1} \cdot min^{-1} \text{ (}\textit{equation 2}\text{)}$$

Blood flow estimation

Figures 1-4 illustrate the same two single contraction cycles at the 2/3 s stimulation rate, the first contraction and the 80th contraction, representing the final contraction during these data collection periods. Blood flow for each contraction cycle is determined by integrating the entire flow curve and dividing by the time of the cycle (e.g., ≈1.5 s for the 2/3 s stimulation rate); we refer to this as blood flow with contraction (Q_{wc}; see shaded area in Figures 1 and 2). In order to estimate what the blood flow might have been in the absence of the mechanical effects of the contractile force, we assumed that the blood flow would have traversed a route from its value just prior to each contraction (specifically 0.04 s prior to each contraction cycle with the cycle onset being determined by the rise in force) to the blood flow at the end of each contraction cycle (see line labeled Q_{nc} in Figures 3 and 4). Note that the ending blood flow of one contraction cycle is the blood flow immediately prior to the following contraction. This "no contraction" flow rate (Q_{nc}) was then calculated by integrating the area under the line connecting blood flow prior to the contraction cycle with blood flow at the end of that cycle and then dividing by the time of the cycle (see shaded area in Figures 3 and 4).

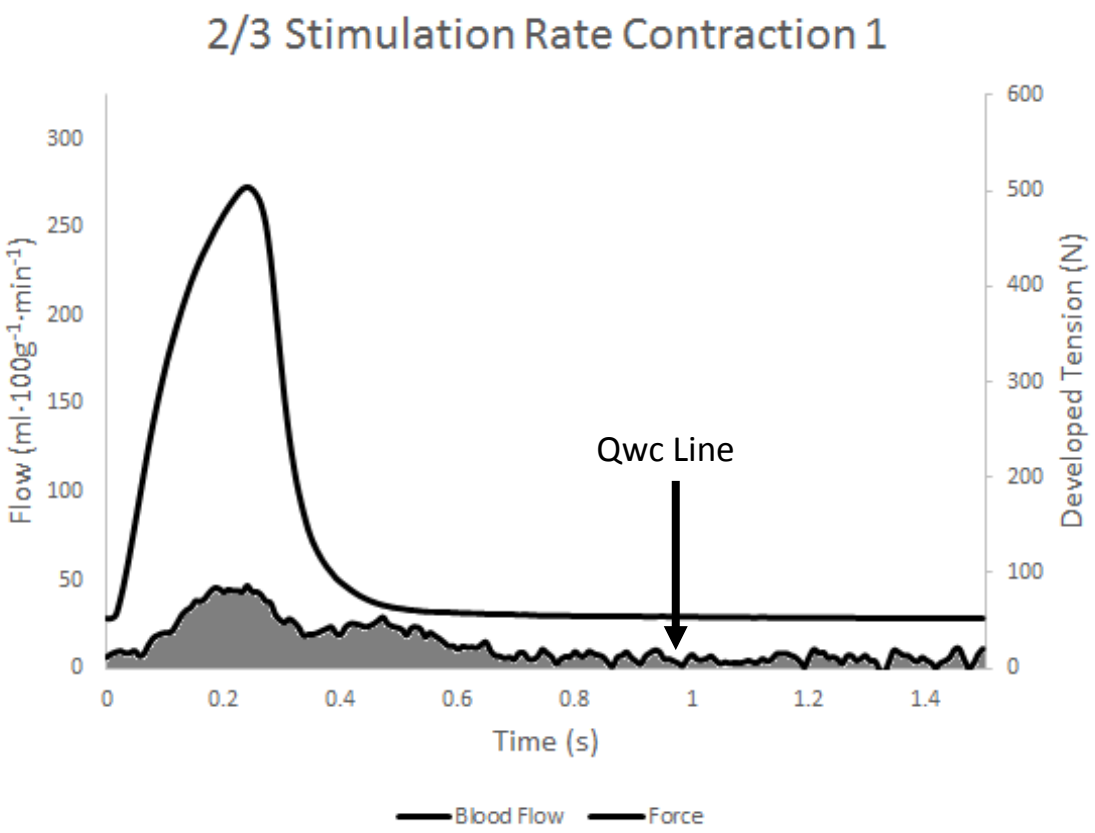

Figure 1. Representative image for measured blood flow (Q_{wc}) superimposed with tension development during the first contraction of 2/3 s stimulation rate. Q_{wc} line indicates the actual blood flow tracing from a single dog during the 2/3 s trial.

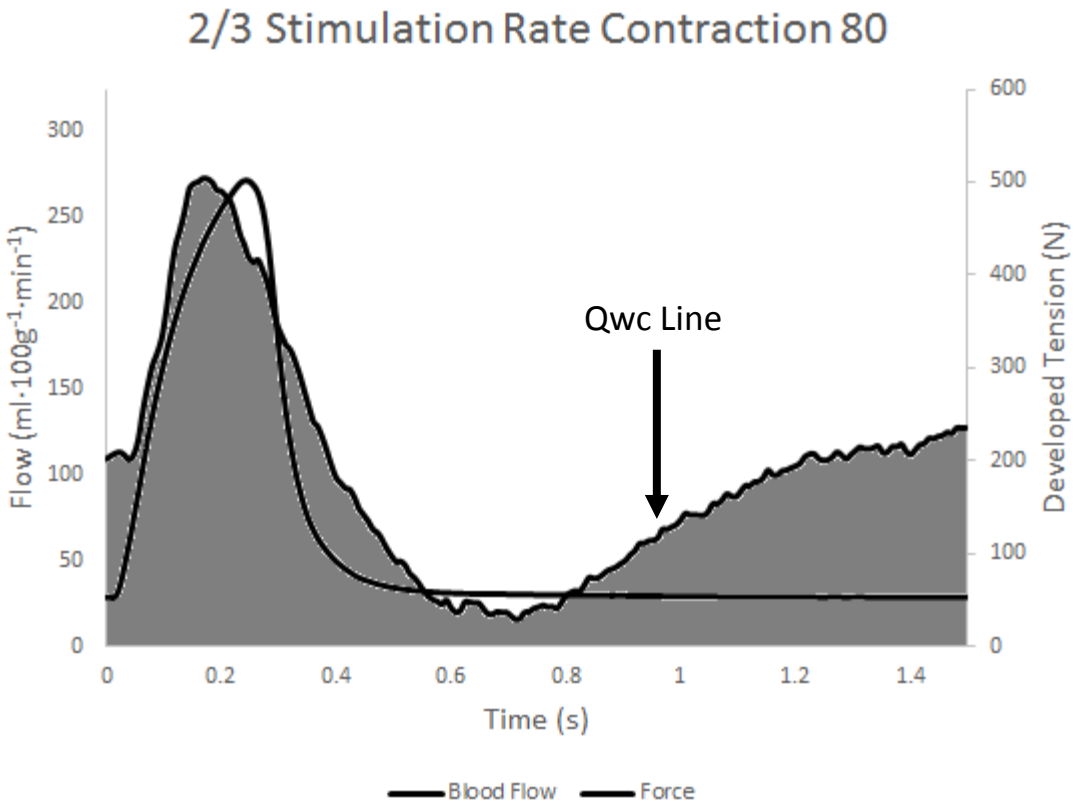

Figure 2. Representative image for measured blood flow (Q_{wc}) superimposed with tension development during the 80th contraction of 2/3 s stimulation rate. Q_{wc} line indicates the actual blood flow tracing from a single dog during the 2/3 s trial.

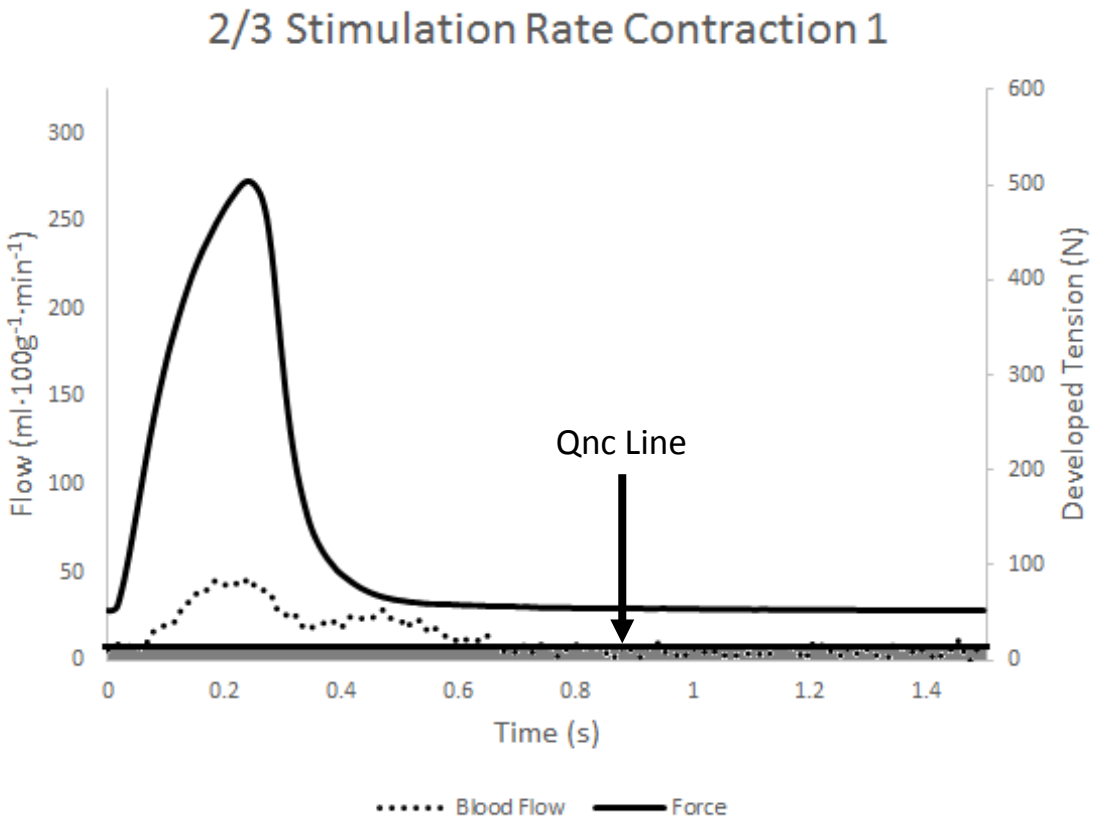

Figure 3. Representative image for measured blood flow (Q_{nc}) superimposed with tension development during the first contraction of 2/3 s stimulation rate. Area under the Q_{nc} line indicates the estimated blood flow that would have occurred during this period with only the mechanical effect of contraction pumping removed.

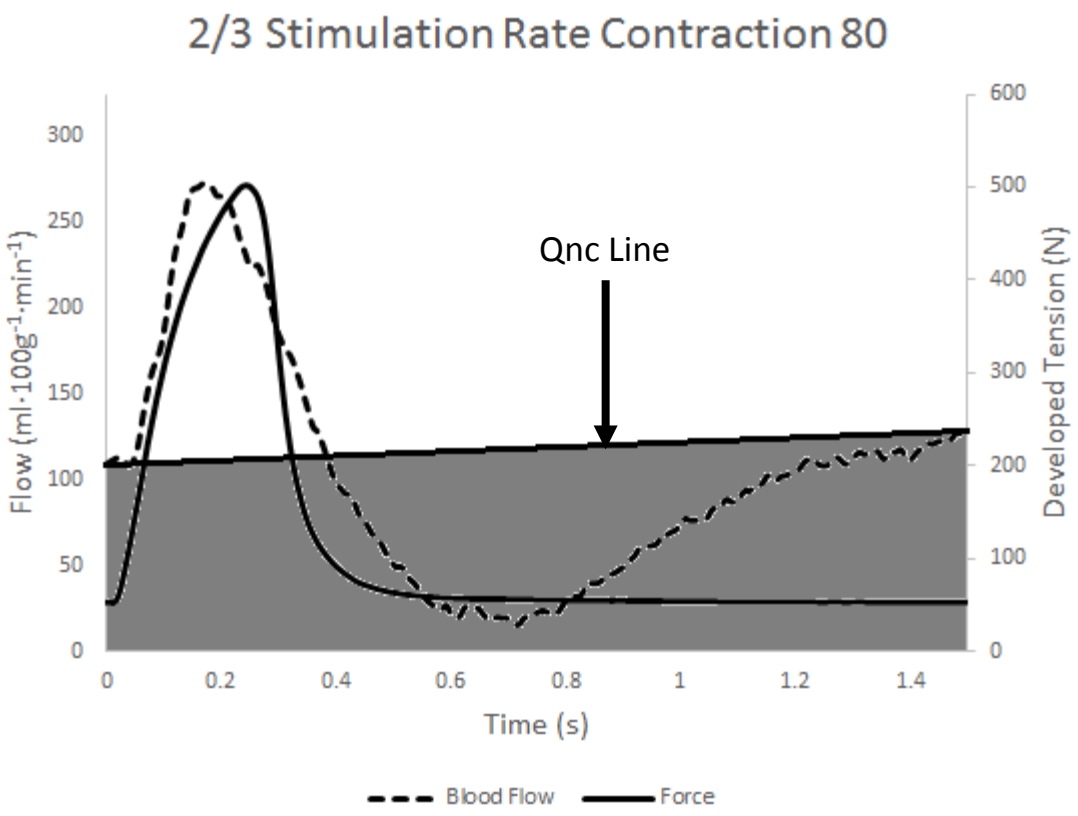

Figure 4. Representative image for measured blood flow (Q_{nc}) superimposed with tension development during the 80th contraction of 2/3 s stimulation rate. Area under the Q_{nc} line indicates the estimated blood flow that would have occurred during this period with only the mechanical effect of contraction pumping removed.

Statistical analysis

Data are presented as means ± SDs. Separate one-way repeated-measures analysis of variance (ANOVA) was used to compare measures made within each trial, as well as to compare the measurements in different trials. Data were collected on seven total dogs for 1/3 s and $2/3_{HB}$ trials with six used for analysis. Due to venous sampling during the transition from 1/3 s to $2/3_{HB}$ blood flow in one animal was not analyzed for

blood flow. Six animals were included for the 2/3 s trial and three trials were performed at the 1/1 s stimulation rate. The level of significance was set at $p < 0.05$. If a difference was found, post-hoc analysis with Bonferroni correction was performed to identify the locations of specific differences between pairs in the dataset. Where appropriate, a paired *t*-test was performed and all statistical analyses were performed in SPSS 22.0 (IBM Corp., Armonk, NY, USA).

Results

The average muscle weight (n = 7) of the gastrocnemius complex was 75 ± 15 g and the average percentage of water was 77.1 ± 1.1%. These data are very similar to previous findings from this lab utilizing this model. One of the muscles used for analysis was slightly edematous following pump perfusion trials with perfusion pressure > 160 mmHg (data in a separate report); wet weight for this muscle was corrected to the average percent water of the other six muscles and included with these as a group average.

All trials were compared from the first contraction until the contraction occurring at the two minute mark (e.g.-120 s data periods). Maximal tension (T_{max}) during the 1/3 s stimulation protocol tended to increase from the first (617 ± 231 N) to the last contraction (653 ± 243 N) representing a modest change in force (106 ± 6%; $P = 0.06$). During the 2/3 s protocol T_{max} showed no indication of fatigue from first (578 ± 233 N) to final contraction (541 ± 188 N) maintaining force output (94 ± 6%; $P = 0.23$). The limited number of samples from the 1/1 s protocol (n = 3) precluded adequate statistical analysis however force declined from an initial (590 ± 293 N) to (459 ± 159 N), maintaining (78 ± 11%; $P = 0.24$) of initial force. A significant decline in force was observed in the $2/3_{HB}$

trial where force declined from (638 ± 233 N) to (548 ± 180 N) maintaining only (86 ± 4%; *P* =0.006). The observation of fatigue in this trial is likely a result of a time effect as this trial was begun following the three minute elevated baseline period of 1/3 s contractions.

Table 1 contains blood gas parameters for the different stimulation protocols. There were no differences in the arterial measures at any time points, post-test venous blood gasses were different than pre-test baseline as anticipated. Baseline S_{O2} in the 1/1 s trial (74 ± 5%) appear lower than the 1/3 s baseline (88 ± 3%), possibly due to an elevated resting $\dot{V}O_2$ stemming from the prior contractile bouts but limited by the sample number of the 1/1 s trials (n = 3). Lactate values were greater at post-test in the 2/3 s (2.3 ± 0.3mM) relative to baseline (p = 0.01) and $2/3_{HB}$ (2.7 ± 0.2mM) relative to its baseline (p = 0.01) as anticipated with the increased work rate.

Table 1. Arterial and venous blood gas, pH, and blood metabolites for rest and at steady state for various stimulation rates

	1/3 Contraction		2/3 Contraction		1/1 Contraction		$2/3_{HB}$ Contraction	
	BL	**Post**	**BL**	**Post**	**BL**	**Post**	**BL**	**Post**
Arterial								
S_{O2} *(%)*	97 ± 1	-	97 ± 0.3	97 ± 0.2	97 ± 0.1	97 ± 0.4	-	97 ± 1
P_{O2} *(mmHg)*	106 ± 11	-	105 ± 7	112 ± 10	104 ± 13	106 ± 13	-	108 ± 10
P_{CO2} *(mmHg)*	31 ± 1	-	30 ± 2	30 ± 2	30 ± 2	31 ± 1	-	30 ± 2
pH	7.41 ± 0.02	-	7.41 ± 0.03	7.40 ± 0.03	7.38 ± 0.01	7.37 ± 0.02	-	7.40 ± 0.02
[Lactate$^-$] (mM)	1.2 ± 0.5	-	1.4 ± 0.4	1.5 ± 0.6	1.7 ± 0.6	1.8 ± 0.5	-	1.3 ± 0.4
Venous	**BL**	**SS**	**BL**	**SS**	**BL**	**SS**	**BL**	**SS**
S_{O2} *(%)*	88 ± 3	22 ± 5[a]	84 ± 4	22 ± 6[a]	74 ± 5[b]	17 ± 7[a]	-	25 ± 9[a]
P_{O2} *(mmHg)*	61 ± 5	17 ± 2[a]	51 ± 3	17 ± 3[a]	44 ± 6	14 ± 2[a]	-	19 ± 4[a]
P_{CO2} *(mmHg)*	32 ± 1	50 ± 2[a]	32 ± 2	57 ± 3[a]	33 ± 0	60 ± 7[a]	-	59 ± 3[a]
pH	7.39 ± 0.02	7.33 ± 0.01[a]	7.39 ± 0.03	7.27 ± 0.04[a]	7.38 ± 0.01	7.25 ± 0.02[a]	-	7.26 ± 0.02[a]
[Lactate$^-$] (mM)	1.4 ± 0.4	1.6 ± 0.3	1.6 ± 0.5	2.3 ± 0.3[c]	1.9 ± 0.5	2.7 ± 0.2[a]	-	2.2 ± 0.3[c]

Values are mean ± S.D for 1/3 (n = 7), 2/3 (n = 6), 1/1 (n = 3), and $2/3_{HB}$ (n = 7). Abbreviations: S_{O2}, oxygen saturation; P, partial pressure; mmHg, millimeters of mercury; mM, millimolar; BL, baseline prior to individual trial; SS, steady state value; Post, following final contraction; 1/3, one contraction per three seconds; 2/3, two contractions per three seconds; 1/1, one contraction per second; $2/3_{HB}$, two contractions per three seconds beginning from high baseline. Arterial values are baseline prior to contraction onset and following the final contraction. No significance found across all arterial measures at any time point sampled. Significant difference ($P \leq 0.05$) compared to ([a]) different from all baseline values, ([b]) different from 1/3 baseline, likely due to slightly elevated resting $\dot{V}O_2$ due to previous contractions. ([c]) different from 1/3 and 2/3 baselines.

The data for the average blood flow responses as well as the average estimated blood flow responses without a mechanical effect ($\dot{Q}_{nc}$) for all stimulation frequencies along with the respective mono-exponential fits are illustrated as group means for each condition in Figures 5-12. Tables 2 and 3 contain the blood flow kinetics parameters associated with the fitting for the Q_{wc} and Q_{nc} trials respectively, corresponding to four different transitions; rest to 1/3 s, rest to 2/3 s, rest to 1/1s and from a lower contraction (metabolic) rate to a higher one (1/3 s to $2/3_{HB}$). The responses were determined with the mono-exponential fitting procedures previously described.

A repeated measures ANOVA with Greenhouse-Geisser correction indicated differences in baseline (A0) between stimulation protocols ($F(1.51, 7.54) = 103.74$, $p < 0.001$). Post hoc analysis with Bonferroni correction showed that the A0 was higher for $2/3_{HB}$ Q_{nc} and Q_{wc} than all other groups (59.0 ± 11.8 ml·100g^{-1}·min^{-1} vs 58.7 ± 8.6 ml·100g^{-1}·min^{-1} respectively, $p < 0.005$), though not different from each other ($p = 1.0$). Additionally, A0 in Q_{nc} 2/3 s (6.7 ± 3.7) was significantly lower than A0 in Q_{wc} 2/3 s (14.3 ± 2.2 ml·100g^{-1}·min^{-1}, $p = 1.012$). The repeated measures ANOVA analysis with Greenhouse-Geisser correction indicated amplitude change ($\Delta\dot{Q}_{ss}$) based on stimulation group ($F(1.37, 6.84) = 23.62$, $p < 0.001$). Post hoc analysis with Bonferroni correction revealed $\Delta\dot{Q}_{ss}$ in Q_{nc} 1/3 s (49.9 ± 8.1 ml·100g^{-1}·min^{-1}) to be greater than $\Delta\dot{Q}_{ss}$ in Q_{wc} 1/3 s (45.5 ± 7.6 ml·100g^{-1}·min^{-1}, $p = 0.03$) and $\Delta\dot{Q}_{ss}$ in Q_{nc} 2/3 s (96.8 ± 29.5 ml·100g^{-1}·min^{-1}) to be greater than $\Delta\dot{Q}_{ss}$ in Q_{wc} 2/3 s (78.1 ± 25.4 ml·100g^{-1}·min^{-1}, $p = 0.003$). For the current experiments, these data suggest faster kinetics responses for blood flow at the slowest stimulation frequency (e.g. -1/3 s). The key component of these results is the contribution of the mechanical effect of force production; e.g., the muscle pump.

Table 2. Kinetics parameters for measured blood flow ($\dot{Q}_{wc}$) for step transitions during multiple stimulation protocols

	Units	1/3	2/3	1/1	$2/3_{HB}$
$\dot{Q}_{bl}$	$ml \cdot 100g^{-1} \cdot min^{-1}$	14.9 ± 2.3	14.3 ± 2.2[b]	10.5 ± 1.8	58.7 ± 8.6[a]
$\Delta\dot{Q}_{ss}$	$ml \cdot 100g^{-1} \cdot min^{-1}$	45.5 ± 7.6[c]	78.1±25.4[d]	87.8 ± 26.3	38.5 ± 15.3
TD $\dot{Q}$	s	1.8 ± 2.8	1.2 ± 1.3	3.1 ± 2.7	2.4 ± 1.9
$\tau\dot{Q}$	s	12.8 ± 5.5	25.6 ± 12.0	16.7 ± 3.0	21.2 ± 3.4
MRT $\dot{Q}$	s	14.6 ± 3.9	26.8 ± 11.8	19.8 ± 5.1	23.6 ± 3.4

Values are mean $\pm$ S.D for 1/3 (n = 7), 2/3 (n = 6), 1/1 (n = 3), and $2/3_{HB}$ (n = 7). Abbreviations: $\dot{Q}_{bl}$, baseline blood flow; $\Delta\dot{Q}_{ss}$, change in steady state blood flow from baseline; TD $\dot{Q}$, time delay in blood flow response; $\tau\dot{Q}$, time constant for blood flow response; MRT $\dot{Q}$, mean response time (sum of TD + τ); 1/3, one contraction per three seconds; 2/3, two contractions per three seconds; 1/1, one contraction per second; $2/3_{HB}$, two contractions per three seconds beginning from high baseline. Significant difference ($P \leq 0.05$) compared to ([a]) different from all other baselines, ([b]) 2/3 s Q_{nc} baseline different from 2/3 Q_{wc} baseline, ([c]) 1/3 s Q_{nc} amplitude significantly greater than 1/3 s Q_{wc} amplitude, ([d]) 2/3 s Q_{nc} amplitude significantly different from 2/3 s Q_{wc} amplitude.

Table 3. Kinetics parameters for estimated blood flow ($\dot{Q}_{nc}$) for step transitions during multiple stimulation protocols

	Units	1/3	2/3	1/1	$2/3_{HB}$
$\dot{Q}_{bl}$	$ml \cdot 100g^{-1} \cdot min^{-1}$	14.0 ± 4.0	6.7 ± 3.7[b]	5.8 ± 1.8	59.0 ± 11.8[a]
$\Delta\dot{Q}_{ss}$	$ml \cdot 100g^{-1} \cdot min^{-1}$	49.9 ± 8.1[c]	96.8±29.4[d]	90.2 ± 36.4	50.1 ± 13.1
$TD\ \dot{Q}$	s	1.9 ± 1.5	0.6 ± 1.0	3.3 ± 1.7	3.2 ± 1.6
$\tau\dot{Q}$	s	11.8 ± 3.2	26.9 ± 10.2	22.1 ± 1.3	21.7 ± 4.8
$MRT\ \dot{Q}$	s	13.5 ± 3.6	27.5 ± 10.4	25.3 ± 1.8	25.0 ± 3.8

Values are mean $\pm$ S.D for 1/3 (n = 7), 2/3 (n = 6), 1/1 (n = 3), and $2/3_{HB}$ (n = 7). 1/1 were excluded from statistical analysis and reported here for reference. Abbreviations: $\dot{Q}_{bl}$, baseline blood flow; $\Delta\dot{Q}_{ss}$, change in steady state blood flow from baseline; TD $\dot{Q}$, time delay in blood flow response; $\tau\dot{Q}$, time constant for blood flow response; MRT $\dot{Q}$, mean response time (sum of TD + τ); 1/3, one contraction per three seconds; 2/3, two contractions per three seconds; 1/1, one contraction per second; $2/3_{HB}$, two contractions per three seconds beginning from high baseline. Significant difference ($P \leq 0.05$) compared to ([a]) different from all other baselines, ([b]) 2/3 s Q_{nc} baseline different from 2/3 Q_{wc} baseline, ([c]) 1/3 s Q_{nc} amplitude significantly greater than 1/3 s Q_{wc} amplitude, ([d]) 2/3 s Q_{nc} amplitude significantly different from 2/3 s Q_{wc} amplitude.

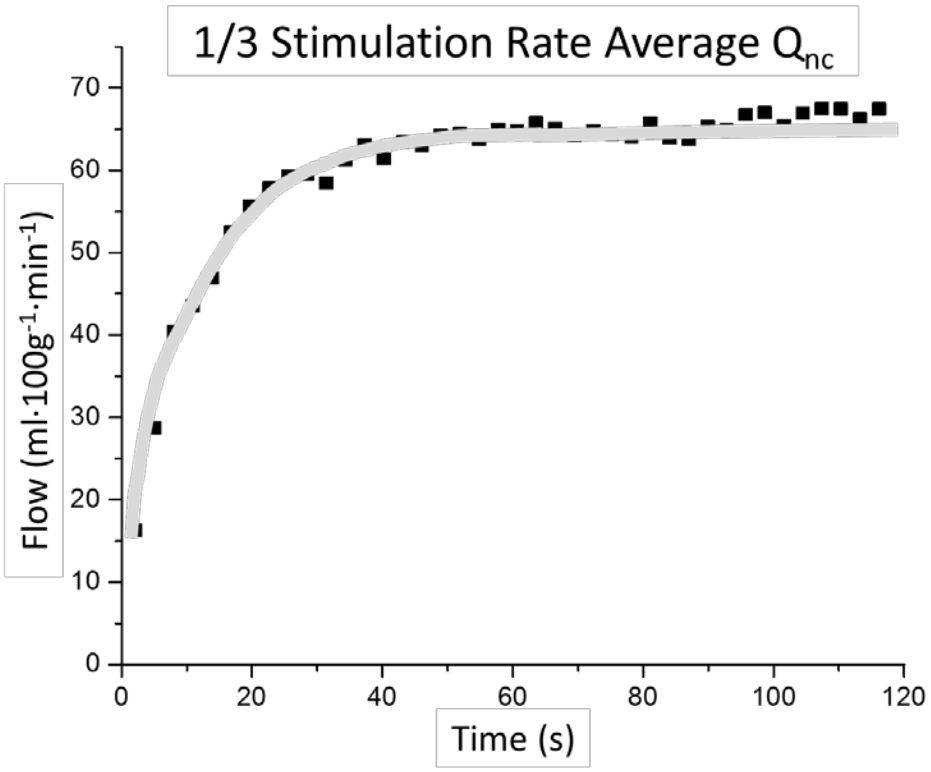

Figure 5. Average blood flow from all 1/3 s trials for estimated blood flow (Q_{nc}) with fitted kinetics.

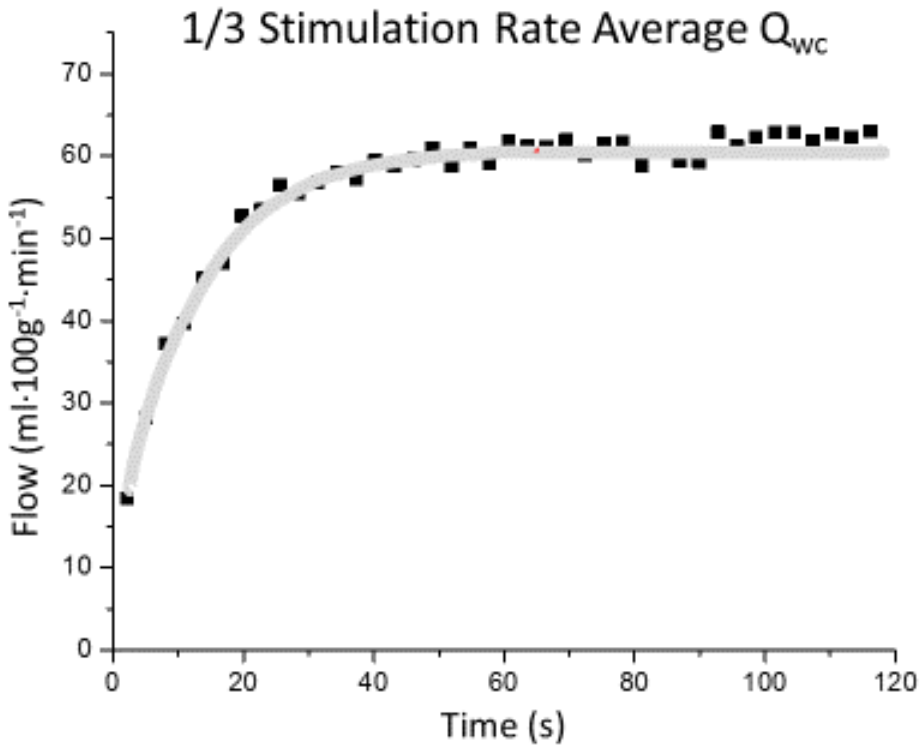

Figure 6. Average blood flow from all 1/3 s trials for estimated blood flow (Q_{wc}) with fitted kinetics.

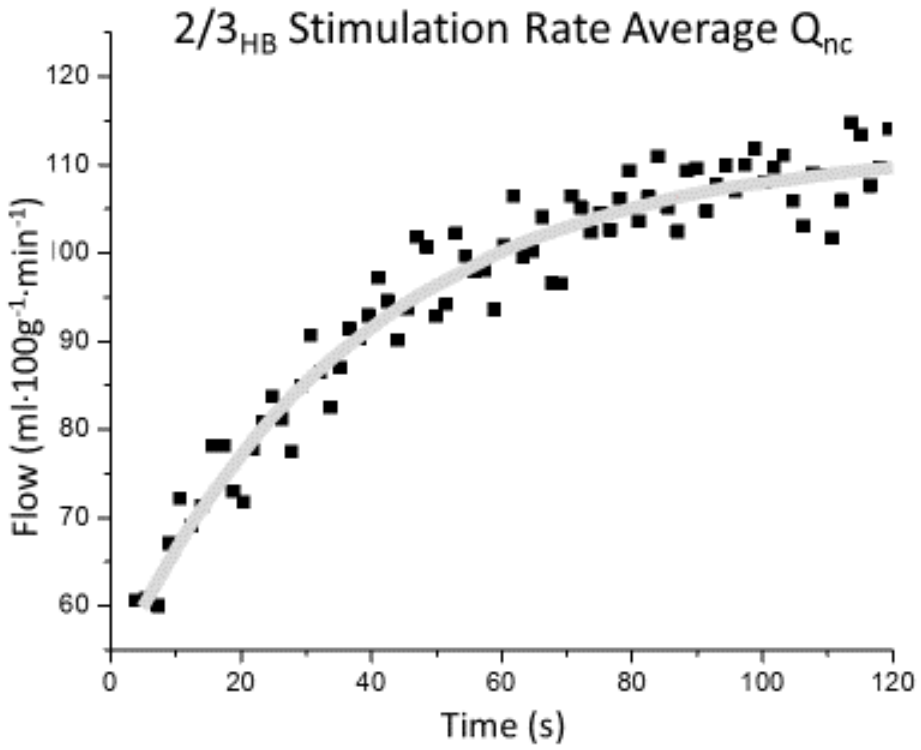

Figure 7. Average blood flow from all $2/3_{HB}$ s trials for estimated blood flow (Q_{nc}) with fitted kinetics.

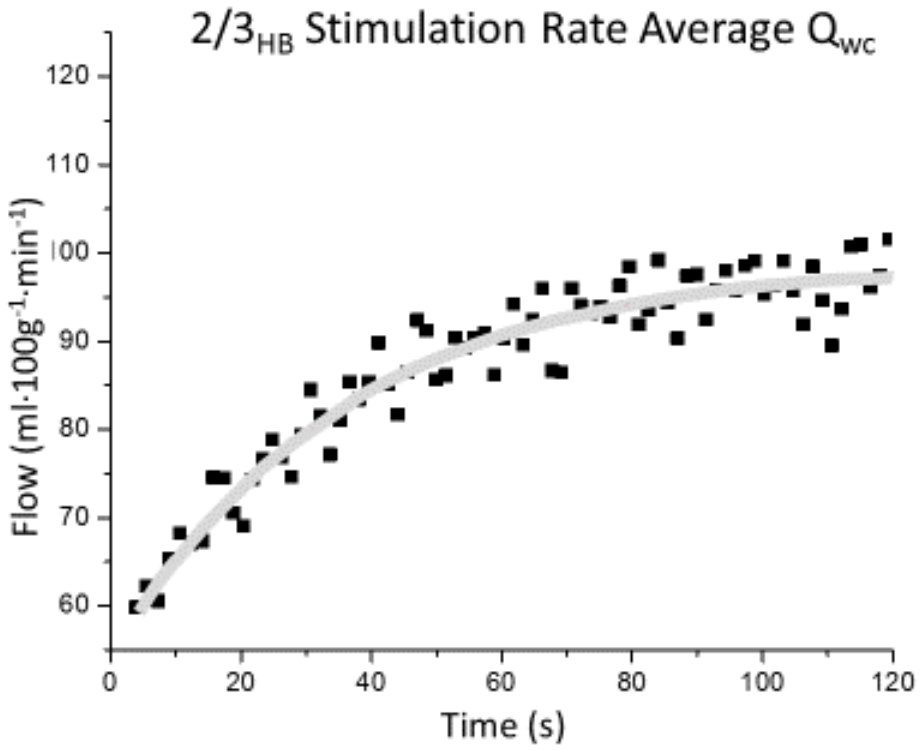

Figure 8. Average blood flow from all $2/3_{HB}$ s trials for estimated blood flow (Q_{wc}) with fitted kinetics.

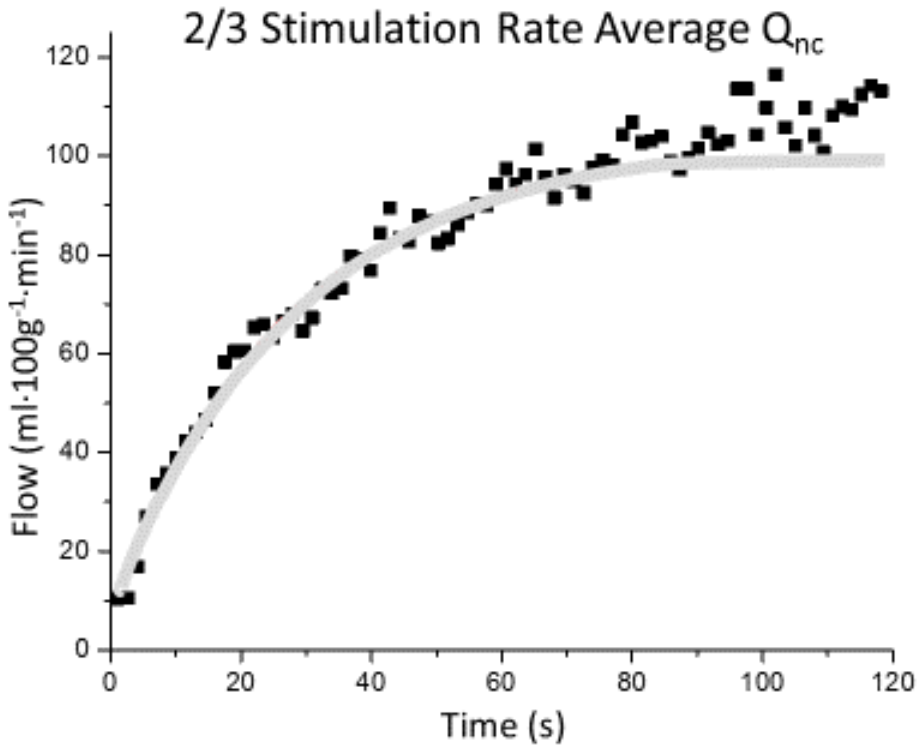

Figure 9. Average blood flow from all 2/3 s trials for estimated blood flow (Q_{nc}) with fitted kinetics.

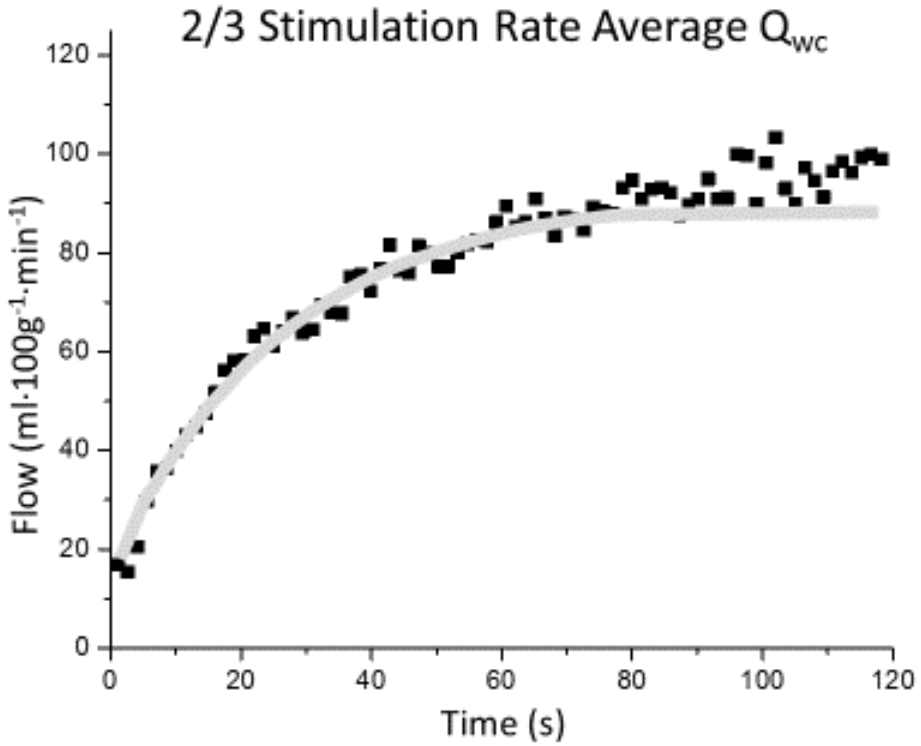

Figure 10. Average blood flow from all 2/3 s trials for estimated blood flow (Q_{wc}) with fitted kinetics.

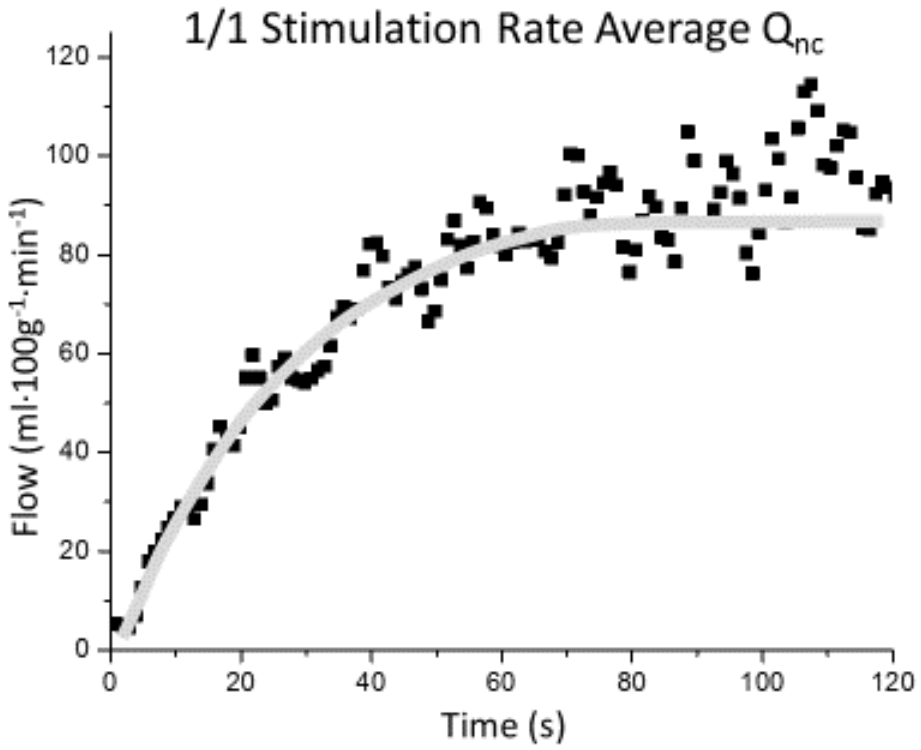

Figure 11. Average blood flow from all 1/1 s trials for estimated blood flow (Q_{nc}) with fitted kinetics.

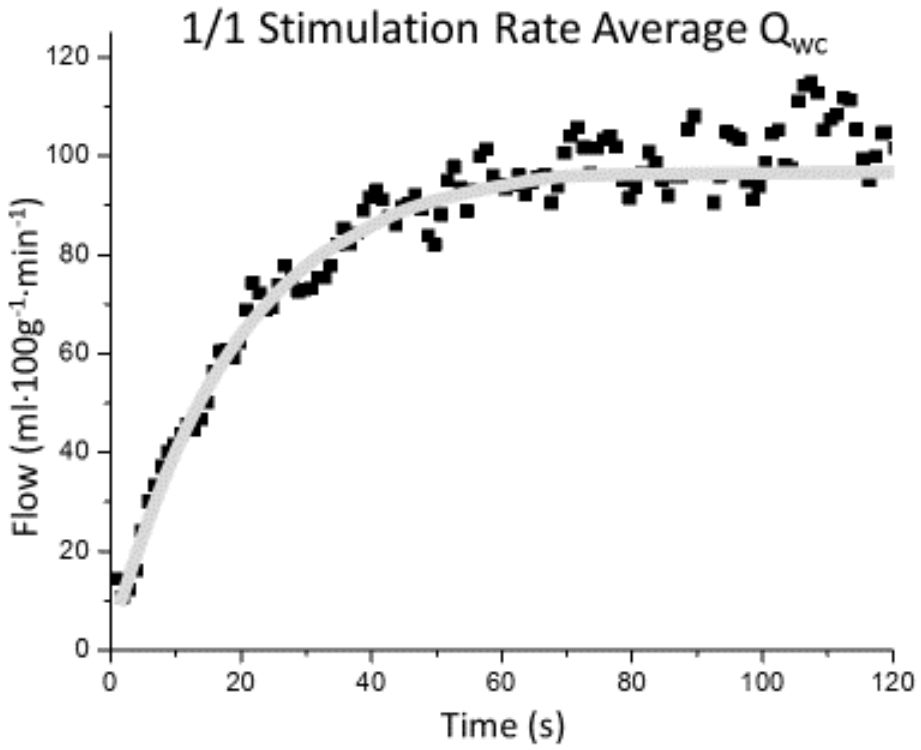

Figure 12. Average blood flow from all 1/1 s trials for estimated blood flow (Q_{wc}) with fitted kinetics.

Figures 13 and 14 highlight the frequency dependence in quantifying the muscle pump contribution. The muscle pump contribution was converted to a percentage as shown by the following equation:

$$\% \text{ Muscle Pump Contribution} = (\dot{Q}_{wc} - \dot{Q}_{nc})/\dot{Q}_{wc} \times 100 \quad (\textit{equation 3})$$

By quantifying the muscle pump in this way, positive values represent a positive contribution to total blood flow while negative values indicate a reduction in flow relative to that which would have been observed with vasodilation alone. The adjustment of blood flow in the 1/3 s trials indicated a reduced blood flow by the second contraction

($Q_{nc} > Q_{wc}$) in all but one animal (fourth contraction) suggesting the muscle pump was not contributing to the hyperemic response beyond this point. Conversely, in the 2/3 s protocol during at least the first six contractions a positive muscle pump effect was observed. In the 2/3 s protocol this effect declined rapidly (tau = 4.4 ± 2.1 s) and beyond 20 seconds total blood flow was reduced by contraction relative to the estimated blood flow from vasodilation alone.

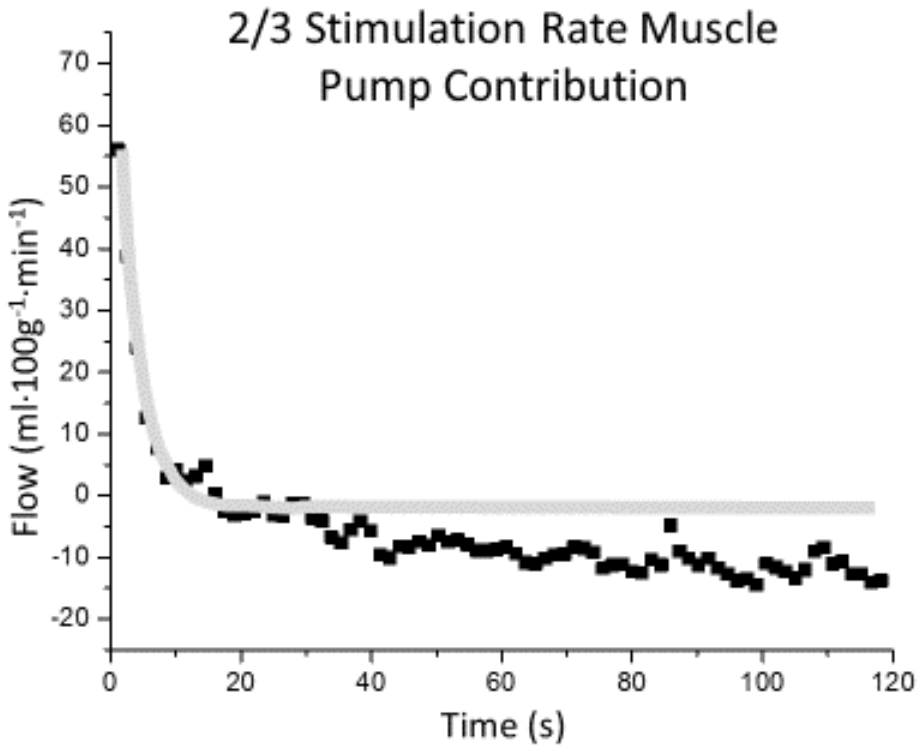

Figure 13. The kinetics response for the percent muscle pump contribution from onset to two minutes of contractions for the 2/3 s stimulation rate. Notice the slow component and negative values for muscle pump contribution in the 2/3 s trial. See text for discussion of frequency dependence in muscle pump contribution.

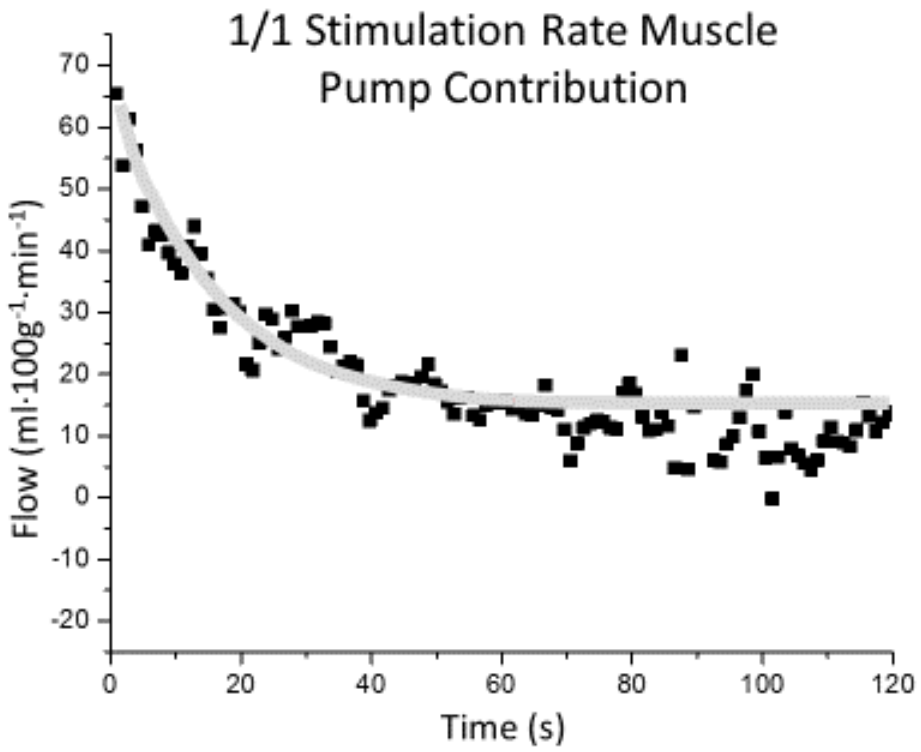

Figure 14. The kinetics responses for the percent muscle pump contribution from onset to two minutes of contractions for the 1/1 s stimulation rate. Notice the positive contribution attributed to the muscle pump at all points during the 1/1 s trials. See text for discussion of frequency dependence in muscle pump contribution.

This is likely due to the majority of blood flow occurring in the passive "vasoactive" period during low frequency contractions relative to the blood flow ejected during the rapid "contraction" phase. For the 1/1 s stimulation rate a slower adjustment in the muscle pump contribution was observed (tau = 18 ± 10 s) as well as positive values throughout the two minute stimulation period.

Discussion:

For this investigation we examined the spontaneous blood flow response of a surgically isolated skeletal muscle during transitions from rest to contraction rates eliciting various metabolic rates as well as from a low contraction rate to a higher rate during tetanic stimulation. Further, based on the estimated spontaneous blood flow response that excludes the mechanical effects of muscle contraction, we characterized the kinetics response of the directly measured blood flow to the kinetics response of the estimated "no contraction" blood flow on a contraction by contraction basis.

The contribution of the muscle pump to skeletal muscle perfusion has been a long debated topic. Efforts to establish this contribution have generally relied on prior vasodilation, preventing vasodilation, or limb position relative to the heart to determine if the contracting muscle was capable of increasing its own blood flow. To our knowledge, this is the first investigation to attempt to separate the spontaneous blood flow response to a variety of contraction frequencies from an estimate of the blood flow response due only to vasodilation. Additionally, based upon this estimate we aimed to characterize the kinetics response for blood flow and the estimated blood flow from rest through two minutes on a contraction by contraction basis. The muscle pump hypothesis posits that skeletal muscle contraction aids in its own perfusion by translocating blood volume from the muscle into the venous vasculature towards central circulation. This movement transiently reduces the venous pressure during the relaxation phase of each contraction. Veins, tethered to skeletal muscle that rapidly returns to resting size/shape, may quickly create negative pressure in the veins and potentiate the flow from the arterial circuit (47, 88, 136). Our contraction by contraction approach to identify the muscle pump

contribution, from exercise onset through steady state, in spontaneously self-perfusing skeletal muscle is a novel attempt to examine this hypothesis.

The pressure generated within the vasculature during skeletal muscle contractions causes the forceful expulsion of blood from the muscle bed into the venous circulation while concurrently creating an impediment to incoming flow from the arterial vasculature. In this investigation it was assumed that with all vasoactive and myogenic responses intact an estimate could be made to compare the blood flow from vasodilation to that which occurs with the cyclic ejection/obstruction pattern observed during rhythmic contractions. Greater measured blood flow than estimated "no contraction" blood flow at any contraction was interpreted as a net benefit to total skeletal muscle perfusion contributed by the muscle pump. During high frequency contractile stimuli, a greater portion of the total blood flow during each contraction cycle is mobilized during the fast "contraction" phase relative to the slow "vasoactive" phase. During a high frequency contraction cycle the subsequent contraction is superimposed early on during the vasoactive phase thus reducing its contribution to total flow. With slower contraction frequency a greater portion of the total flow occurs during the vasoactive phase allowing for faster adjustment to the steady state of blood flow. The impediment to flow that occurs during skeletal muscle contraction outweighs the increased flow ejected therein; this creates a limitation to total flow that is exacerbated by high frequency contraction cycles.

Because no single vasodilatory substance has been identified that fully explains the changes in blood flow following muscle contraction, alternative mechanisms have been sought. The muscle pump hypothesis is appealing for the immediate hyperemic

response because with the first contraction a decrease in venous pressure could lead to an increase in perfusion. According to this hypothesis, by emptying the volume of blood present in resting skeletal muscle with contraction, the pressure gradient across that muscle bed is increased, thus promoting additional arterial inflow (47, 88, 91, 136). Evidence in support of a muscle pump effect comes from investigations in which equivalent exercise bouts were initiated with limb position either above or below the heart. Our data would indicate that at exercise onset the muscle pump does aid in increasing blood flow beyond vasodilation alone, but that this benefit quickly diminishes and is further largely dependent on contraction frequency.

With prior maximal vasodilation achieved, tilting subjects from supine to an upright position during post-exercise hyperemia did not enhance blood flow while the same shift performed during rhythmic exercise significantly raised flow (48). This observation was attributed to the lowering of venous pressure by muscle contraction in the dependent position and an increase in local perfusion pressure. Comparison of hyperemic responses to repeated forearm cuff inflation, simulating only the mechanical contribution to venous emptying, were greater when performed with the arm below the heart compared with above (149). However, vasodilation in addition to muscle pumping appeared requisite as blood flow responses to muscle contraction were greater for both positions when compared to the cuff inflation trials. Importance of the vasodilation at exercise onset is further illustrated by increases in mean blood velocity from rest to exercise during handgrip exercise with the arm positioned below the heart in the supine position. The gain in blood flow was not wholly explained by the difference in blood

pressure between the positions indicating concurrent vasodilation is needed with the reduction in venous pressure to observe an immediate increase in blood flow (139).

Hypotheses aimed at separating the potential muscle pump contribution from spontaneous adjustments in vessel diameter have reasonably assumed that for the pump to be a positive contributor, blood flow should increase with the onset of contractions in a previously dilated resting vascular bed. This hypothesis has been tested with the addition of stimulated muscle contractions subsequent to vasodilation in canine diaphragm (111) as well as in the isolated gastrocnemius (42, 111). By maintaining experimental control over the arterial flow with constant pressure, Naamani et al. (111) concluded that the muscle pump had little direct effect on muscle blood flow and primarily contributed to the observed reductions in venous pressure. A possible limitation of that study was the relatively low blood flow values attained in their vasodilation protocols. To achieve a blood flow with vasodilation comparable to maximal exercise, Dobson et al. (42) combined infusion of SNP with ADO and further occluded arterial as well as venous flow. This method "trapped" all exogenous and endogenous vasodilating agents in the muscle for a period of five minutes. Upon release, the flows achieved were similar to those elicited during contraction rates inducing $\dot{V}O_{2peak}$ for this muscle preparation. The addition of contractions in the previously dilated skeletal muscle vasculature led to a reduction in total blood flow indicating no benefit of the muscle pump in achieving maximal blood flow. In the current study, we characterized the kinetic responses of blood flow and found that Q_{wc} was greater than Q_{nc} in the 1/1 s trials while the inverse was true for all other protocols. We interpret this as a frequency dependency for the

muscle pump effect because the imposition of rapid contractions precludes an adequate filling time and thus a reduction in our estimate of total blood flow.

The potential for the muscle pump to contribute to muscle hyperemia is dependent on both vasodilation within the tissue, allowing for increased blood volume as well as a contraction frequency allowing for adequate filling time. It is well known that blood flow to skeletal muscle increases in response to elevated contractile activity. In rats performing treadmill running, blood flow was increased selectively in oxidative fiber types at slower running speeds and in all fiber types with increasing running speed (90). Further, blood flow increased across a variety of running speeds from 15 to 75 m/min with increased flow directly related to high-oxidative fiber type content at greater speeds. Fiber type specific differences in blood flow were confirmed in a subsequent investigation indicating blood flow heterogeneity within a single muscle as well as indicating a strong correlation between total blood flow and contraction frequency (99). With a stimulation pattern of one per second, aimed at simulating running stride frequency in the calf of the cat, blood flow through the rhythmically contracting muscle was found to exceed that of spontaneous flow during the immediate post-exercise period. This was taken as evidence for the muscle pump contribution (47). In examining the influence of cycling cadence in relation to blood flow at a fixed workload, Gotshall et al. (57) attributed greater vascular conductance at higher cadences to more effective muscle pumping.

Additional evidence suggests that the relationship between contraction frequency and metabolic cost may be more complex. Metabolic byproducts, including many putative vasodilators, increase with increasing contractile work. The connection between

blood flow and $\dot{V}O_2$ has been demonstrated (2, 4, 5, 108); however, there appear to be conditions related to contraction duration which can dissociate this relationship. With matched contractile work performed, contractions of short duration elicited greater $\dot{V}O_2$ and rates of ATP use than long duration contractions (14, 29, 70). This potentially relates to the ATP cost of ion transport during muscle activation and relaxation. In one of the aforementioned studies Hogan et al. utilized a tetanic contraction-to-rest-ratio of 0.25s/0.75s for short duration versus 1s/3s for long duration with constant blood flow. The short duration stimulation pattern resulted in higher $\dot{V}O_2$ than the long duration under these conditions in spite of the matched tension-time integral. With spontaneous perfusion the same stimulus protocol was utilized to determine if blood flow was more closely matched to contractile work or metabolic rate. In this setting the spontaneous blood flow response was more closely associated with muscle metabolism than the total work performed (63). In the current study, as blood flow approached its steady state within each protocol, the frequency of contraction was paramount in determining a muscle pump contribution.

Limitations

Laughlin et al. (7) proposed that during investigations into the effect of the muscle pump, the very instrumentation required for measurement may abolish the effect. However, that supposition was based on investigations in the rat hindlimb where the delicate vasculature no longer exhibited auto-regulatory control after instrumentation. It is unlikely that large animal preparations similar to that utilized in the current study limit the auto-perfusive response of the skeletal muscle vasculature. Concerns regarding the use of isometric, tetanically stimulated muscle have been raised questioning the ability to

extrapolate these findings to those of dynamically recruited muscle *in vivo*. Hamann et al. (64) addressed this limitation by using a chronically instrumented canine model in which the animals were able to perform dynamic exercise on a treadmill with simultaneous measures of blood flow and blood pressure. Prior to the onset of mild-intensity treadmill exercise vasodilation was induced with ADO infusion. The onset of normal, spontaneously recruited muscle contraction in the previously vasodilated hindlimb failed to further increase blood flow indicating that without concurrent vasodilation the mechanical action of contracting muscle was no benefit to muscle blood flow.

Although we believe our model offers some significant advantages for the assessment of potential muscle pump contributions to skeletal muscle blood flow, we recognize its limitations. The complete activation of all fibers with tetanic stimulation represents a stimulus that is not likely observed in a normal dynamic exercise condition. Perfusion in skeletal muscle is heterogeneous and is directed preferentially to fiber types based on the intensity of the exercise (90, 99). This response of normal perfusion in dynamic exercise is eliminated during rhythmic tetanic stimulation and may influence the muscle pump contribution. An additional consideration during dynamic muscle activation is the presence of antagonistic contractions generating a “push/pull” of blood between muscle groups, a characteristic that is absent in this context. Also, as has been noted, the benefit of the muscle pump may only be fully realized when investigated in the distal segments of animals with a significant hydrostatic column.

Conclusions

The results of the current investigation suggest that during simulated exercise, in spontaneously perfused muscle, the contribution of the muscle pump to skeletal muscle perfusion is limited. In this study we utilized an analytic technique in which all naturally occurring vascular adaptations associated with exercise were included in our estimation of the blood flow response to various metabolic rates. The time course for blood flow response was not different between the measured flow and the estimated flow suggesting that with vascular responses intact, vasodilation is obligatory. The time course in the adjustment of blood flow between the actual flow and the estimated flow indicated no significant differences. In all but one animal (fourth contraction), by the second contraction of the 1/3 s stimulation protocol we observed no additional contribution to blood flow as a result of skeletal muscle contraction, similarly in the 2/3 s protocol during at least the first six contractions a positive muscle pump effect was observed. For the 2/3 s protocol this effect declined rapidly (tau = 4.4 ± 2.1 s) and beyond 20 seconds total blood flow was reduced by contractions relative to the estimated blood flow from vasodilation alone. At a stimulation frequency of 1/1 s there appears to be a positive muscle pump contribution throughout the stimulation protocol. Similar to previously mentioned investigations we have identified the frequency of contraction as a key component to the efficacy of the muscle pump hypothesis. During high frequency contraction rates the blood that is expelled during the forceful phase represents a greater proportion of the total blood flow during each contraction cycle. During low frequency contraction rates a greater fraction of the blood flow occurs in the post contraction "vasoactive" period. Rapid skeletal muscle contraction rates quickly reduce the

contribution of the muscle pump to total blood flow during the steady state, actually impeding blood flow rather than enhancing it.

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153. **Welsh DG, and Segal SS**. Coactivation of resistance vessels and muscle fibers with acetylcholine release from motor nerves. *American Journal of Physiology-Heart and Circulatory Physiology* 273: H156-H163, 1997.
154. **Whipp BJ, and Wasserman K**. Oxygen uptake kinetics for various intensities of constant-load work. *J Appl Physiol* 33: 351-356, 1972.
155. **Wilson JR, and Kapoor SC**. Contribution of prostaglandins to exercise-induced vasodilation in humans. *American Journal of Physiology-Heart and Circulatory Physiology* 265: H171-H175, 1993.
156. **Young EW, and Sparks HV**. Prostaglandins and exercise hyperemia of dog skeletal muscle. *American Journal of Physiology-Heart and Circulatory Physiology* 238: H191-H195, 1980.

Appendix A - Institutional Animal Care and Use Committee Approval

GENERAL INFORMATION AND INSTRUCTIONS

SUBMIT ORIGINAL AND 16 COPIES TO:

ANIMAL RESOURCES, 307 Samford Hall, MAIN CAMPUS

University policy requires that all research, teaching, production/maintenance, and demonstration activities involving vertebrate animals be approved by the Auburn University Institutional Animal Care and Use Committee (IACUC) prior to initiation of the project. The Auburn University IACUC policy is available from the Animal Resources office and website: www.auburn.edu/research/vpr/animals/documents/policy.pdf. This policy is in accordance with federal regulations and guidelines.

When submitting the original and 16 copies, the General Information and Instructions and the Additional Information sections should be omitted.

The IACUC meets the first and third Thursdays of each calendar month. Protocols received at least seven days prior to a scheduled meeting date (e.g. by 11:30 a.m. on Thursday of the week prior to a scheduled Thursday p.m. meeting) will be placed on the agenda. Approved protocols will be assigned a PRN (protocol review number). Approved Animal Subjects Review Forms will remain in the official files of the University for not less than three years beyond the completion of the project. If a project is not funded, please notify ANIMAL RESOURCES AS SOON AS POSSIBLE (844-5978).

Annual review of all protocols is required. An Annual Review Form will be sent to the Principal Investigator approximately 30 days prior to the anniversary date of the approved PRN. Please complete and return the form promptly to ANIMAL RESOURCES, 307 Samford Hall. This form is also available on the Animal Resources website http://www.auburn.edu/research/vpr/animals/forms.htm.

Animal users are encouraged to become familiar with all guidelines and regulations pertaining to the care and use of animals in research and teaching by visiting the Animal Welfare Information Center (AWIC) on the World Wide Web at http://www.nal.usda.gov/awic/

An Animal Subjects Review Form may be obtained by downloading it from the Animal Resources website http://www.auburn.edu/research/vpr/animals/forms.htm.

Alternatively, this form may be obtained at either of the following offices: Animal Resources, 307 Samford Hall, Main Campus or the Division of Laboratory Animal Health, 311 Greene Hall Annex, College of Veterinary Medicine.

Complete this form by providing **BOLD TYPED** answers to <u>each item</u> listed. If an item is not applicable, please indicate NA.

ANIMAL SUBJECTS REVIEW FORM

PRINCIPAL INVESTIGATOR: **L. Bruce Gladden, Ph.D.**

RANK/TITLE: **Alumni Professor**

DEPARTMENT: **Kinesiology**

COLLEGE/SCHOOL: **Education**

CAMPUS ADDRESS: **2050 Memorial Coliseum** E-MAIL ADDRESS: **gladdlb@auburn.edu**

CAMPUS PHONE #: **844-1466** FAX #: **844-1467**

_____ Check if PI will serve as faculty advisor to the Lead Graduate Student or Resident associated with this activity.

LEAD GRADUATE STUDENT/RESIDENT: **N/A**

RANK/TITLE:

DEPARTMENT: E-MAIL ADDRESS:

CAMPUS PHONE #: FAX #:

CO-INVESTIGATOR: **N/A**

RANK/TITLE:

DEPARTMENT: E-MAIL ADDRESS:

CAMPUS PHONE #: FAX #:

PROJECT TITLE: Oxygen uptake on-kinetics in canine skeletal muscle: metabolic rate steps

STARTING DATE: July 1, 2011 **EXPIRATION DATE: June 30, 2014**

(Must not be prior to IACUC approval) (Must not exceed three years)

Is any part of the funding from a U.S. Public Health Service Agency: Yes

No _XX__

REQUIRED SIGNATURES

The information contained on this form provides an accurate description of the animal care and use protocol which will be followed. I agree to abide by governmental regulations and university policies concerning the use of animals. I will allow veterinary oversight to be provided to animals showing evidence of pain or illness. If the

information provided for this project concerning animal use should be revised, or procedures changed, I will so notify the committee of those changes in writing, and no proposed changes will be implemented until full IACUC approval has been granted.

_____

Principal Investigator Date

Medical care for animals will be available and provided as indicated by a qualified veterinarian. By accepting this responsibility, the veterinarian is providing assurance that any personal interest he/she might have in the project will not conflict with his/her responsibility for the provision of adequate veterinary care for the animals. Furthermore, the veterinarian provides assurance of review and consultation on the proper use of anesthetics and pain relieving medications for any painful procedures.

_____ **Dr. Pat Rynders**

Project Veterinarian Date **Project Veterinarian** (Please type or print)

_____

Departmental Chairperson Date

_____

Lead Graduate Student/Resident Date

***IACUC Chair Date**

*IACUC Chair signs the protocol after IACUC approval has been granted.

PLEASE TYPE IN BOLD FONT AND COMPLETE THE FOLLOWING FORM IN FULL.

1. Will the animals be used in:

Teaching _____

Research **XX**

Demonstration_____

Production _____

If Teaching, give the course number:

2.

Species Common Name	**Total Number**	**Source**	**Housing Locatio**
Canis lupus familiaris	**12 – project total**		

(Dog)		**Division of Laboratory Animal Health**	**Division of Laboratory Animal Health Kennels**

3. Will animals be maintained for a period of 12 or more consecutive hours in a location other than the housing location mentioned in Item 2? (*See Item 3 of Additional Information at the end of this form.*)

Yes _____ No **XX**

If Yes, specify the location and reason.

4. PERSONNEL QUALIFICATIONS (*See Item 4 of Additional Information at the end of this form.*)

A. Indicate who will provide daily care and maintenance of the animal(s). Indicate name(s) or identify the particular unit staff.

DLAH personnel under the direction of Andrea Brown.

B. List the names of all individuals who will conduct procedures involving animals on this protocol. If all individuals are not currently known, please indicate as such.

Dr. L. Bruce Gladden, PI for this project.

James R. McDonald, a graduate student trained by Dr. Gladden.

Yi Sun, a graduate student trained by Dr. Gladden.

Matt Rogatzki, a graduate student undergoing training with Dr. Gladden.

Brian Ferguson, a graduate student undergoing training with Dr. Gladden.

Potentially, collaborators from other universities who are well-trained and experienced in the type of experiments to be conducted.

C. Principal Investigator Certifications

My signature on page 1 of this form certifies that:

1) Individuals performing animal procedures on this protocol are or will be qualified to perform their particular animal related duties through training and/or experience (individuals will be supervised until adequate training has occurred). Training and/or experience must encompass the following: *biology, handling, and care of the species; aseptic surgical methods and techniques (if applicable); the concept, availability, and use of research or testing methods that limit the use of animals or minimize distress; the proper use of anesthetics, analgesics, and tranquilizers (if applicable); and procedures for reporting animal welfare concerns. Informative links regarding training resources have been provided for assistance as needed at http://www.auburn.edu/research/vpr/animals.

2) All individuals working with animals, animal tissues, or animal products on this protocol will be informed of relevant *occupational health and safety issues prior to performing their duties. * Informative links have been provided for assistance in this and other areas as needed at http://www.auburn.edu/research/vpr/animals.

5. State how or why you selected the species to be used in this project.

Dogs are used because vascular isolation of the gastrocnemius is a well-established method in these animals. As a result, there is an abundance of baseline data with which to compare our results. This is an excellent model for the study of exercise metabolism and the physiological effects of changes in blood flow and metabolic stimulators/inhibitors. Oxygen delivery can be precisely controlled and varied, a distinct advantage for the study of metabolic on-kinetics. Dog muscles are highly oxidative (Maxwell et al., 1977); this characteristic results in a slow fatigue rate thus facilitating the establishment of a steady state of muscle contractions and metabolic rate. Metabolic measurements are more reliable and more valid when the muscle is in a steady state. Other models (rats, cats, rabbits) have their uses but in the present experiments suffer a variety of disadvantages (amount and quality of blood flow to active muscles, fiber types, need for artificial perfusate, etc.).

6. STUDY/ACTIVITY JUSTIFICATION AND OBJECTIVES:

A. Justification:

Skeletal muscle has a remarkable ability to cope with rapid and dramatic changes in metabolic rate. However, our understanding of the regulatory factors underlying and controlling this ability is far from complete. It has been known for

decades that upon a step transition from rest to exercise, or from a lower to a higher work rate, oxygen uptake (VO_2) by skeletal muscle lags behind the power output or energy requirement increase. The resultant increase in VO_2 during these transitions follows a time course that has been termed "VO_2 on-kinetics".

My laboratory, along with collaborators, has previously published extensively on this topic, employing the canine gastrocnemius model described in this protocol (two papers by Grassi et al. 1998, Grassi et al. 2000, Grassi et al. 2002, Grassi et al. 2005, Zoladz et al. 2008, two papers by Hernández et al. 2010, Grassi et al. 2011).

In human studies, it has been demonstrated that if exercise is initiated from a raised work rate/metabolic rate, the VO_2 on-kinetics response is slowed in comparison to beginning from a resting/unloaded baseline (Brittain et al. 2001, MacPhee et al. 2005). These necessarily indirect studies in humans suggest that the VO_2 on-kinetics from a raised work rate/metabolic rate may be slowed by a slower blood flow response, recruitment of fast twitch muscle fibers which are slower to respond metabolically, and/or a less favorable energetic state. With the canine gastrocnemius model, blood flow and muscle fiber recruitment can be controlled and energetic state can be assessed via muscle sampling. This will either confirm or eliminate these factors as causative mechanisms in the control of the VO_2 transition between work rates.

B. Objectives:

As a continuation of this line of research, it is the purpose of the present project to compare the VO_2 on-kinetics response to contractions begun from the resting condition versus an increase in contraction frequency from a prior contracting baseline. Electrical stimulation will be supramaximal so that all motor units are recruited in all conditions and blood flow will be controlled via pump perfusion. Therefore, these two factors will be eliminated as causative agents in the resulting VO_2 on-kinetics response.

7. SUMMARY OF PROPOSED ACTIVITY: USE LAY TERMS to give a description of the proposed activity. *From reading this section it should be possible for a non-scientist to determine exactly how animals will be used in the context of the proposed activity.*

This section should include a clear description of the experimental design (research protocols) or activities involving animals (teaching, demonstration, or production/maintenance protocols). This section should also include a brief description of each phase of activities involving animals and should make it possible to account for all animals requested in Item 2. Justification for animal numbers is required to assure that only the necessary number of animals is being used. (*See Item 7 of Additional Information at the end of this form for guidance in providing the appropriate information.)*

In previous experiments we have studied the effects of O_2 delivery (hypoxia), metabolic activation, and metabolic inhibition on the VO_2 on-kinetics (e.g., PRN 2007-1185). Reduced O_2 delivery at the onset of contractions slowed VO_2 on-kinetics but hypoxia with no change in O_2 delivery had no effect on the on-kinetics. We have also collected data that we expect will allow us to correct for transport time from mid-capillary to our venous sampling site – these data are still being analyzed and modeled. We are planning to calculate cellular response times from this modeling approach. As a side-benefit of the experiments in this previous protocol, we were able to add continuous monitoring of venous O_2 concentration and calculate VO_2 on a contraction-by-contraction basis. However, none of the experiments from this previous protocol addressed the questions of the present protocol; i.e., a direct comparison of VO_2 on-kinetics within the same muscle preparation at different contraction rates, and more importantly, from one contraction rate directly to another. Studies in humans suggest that the VO_2 on-kinetics are significantly slowed when going from a lower exercise intensity and metabolic rate (directly with no rest period in between) to a higher one; that is the question that will be investigated in the present experiments, in a model in which blood flow (and thereby O_2 delivery) can be controlled and muscle motor unit recruitment is maximal.

In the present experiments, the gastrocnemius muscles of anesthetized dogs will be surgically isolated to allow measurement of metabolite exchange (e.g., O_2 uptake, CO_2 output, glucose uptake, lactate uptake/output, etc.) across the muscle as well as

the sampling of muscle biopsies. In all cases, a primary measurement will be VO_2 on-kinetics. To determine the on-kinetics, venous outflow will be measured continuously via a flowthrough ultrasonic probe and flowmeter while venous O_2 content will be measured continuously via an inline oximeter probe (Opticath model no. U425C, size 4 F, Hospira, Lake Forest, IL)). Myoglobin deoxygenation will be measured continuously with a non-invasive near-infrared Oxymon spectrometer (Artinis, Zetten, Netherlands). Arterial and venous blood samples (< 2 ml) will be taken before and after each series of contractions and analyzed for hemoglobin concentration, percent saturation of hemoglobin with O_2, partial pressure of O_2 and CO_2, pH, and in some cases, metabolite concentrations (lactate, pyruvate, alanine, glucose, etc.). Arterial blood samples are also drawn periodically throughout the entire experimental time period to check for maintenance of normal blood gas levels and acid-base balance. Additionally, venous samples are drawn at the end of each trial period for calibration of the indwelling oximeter probe. The total volume for all blood samples will be less than 70 ml. Muscle samples will be taken at rest, and then at 15 s and 180 s for each contraction period, and subsequently analyzed for phosphocreatine, ATP, glycogen, lactate, pyruvate, and alanine concentrations.

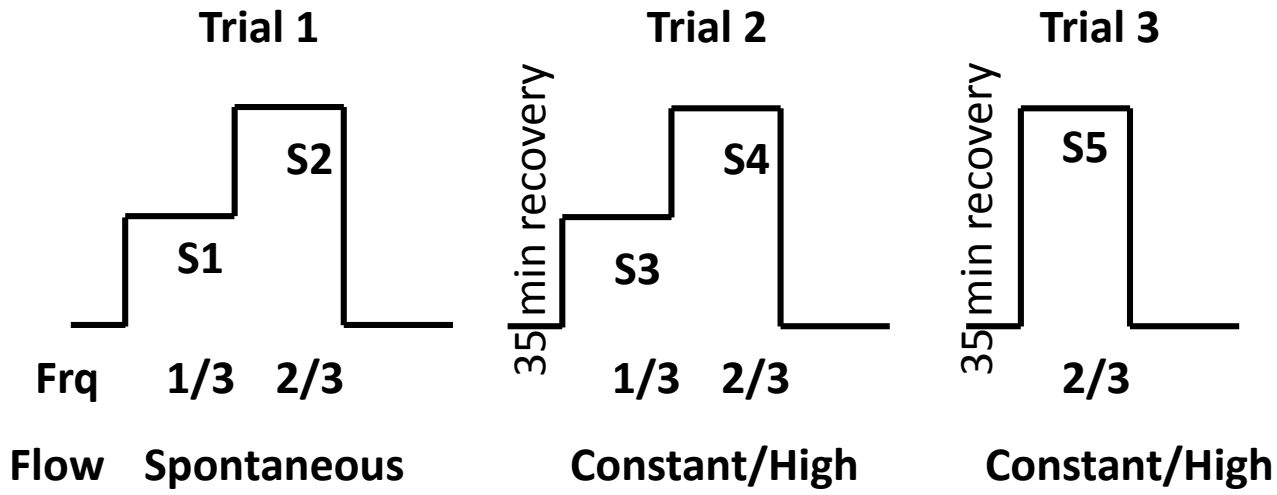
In all cases, the animals are anesthetized and intubated at the Division of Laboratory Animal Health facility at the Veterinary School campus. After animals have been monitored and determined to be in a surgical plane of anesthesia, they are placed in a plastic cart with a closed cover and transported via automobile from the Veterinary School campus to the Beard-Eaves-Memorial Coliseum. If the car is a sedan, the cart is placed in the rear seat; if it is a station wagon, the cart is placed

in the rear compartment behind the second seat. The animals remain in the closed cart until they are removed and placed on a surgical table in the Muscle Physiology Laboratory. The dogs are ventilated and warmed with a heating pad while they are kept in a deep, surgical plane of anesthesia (additional sodium pentobarbital given as required to maintain absence of pedal, palpebral, and corneal reflexes). The heating pad is used to maintain normal body temperature. The animals are euthanized while still under anesthesia at the end of the experiments.

The following protocol will be used:

Under spontaneous, self-perfused conditions, VO_2 on-kinetics will be determined from rest (no contractions) to a low submaximal metabolic rate (1 tetanic contraction per 3 s) and then, without stopping the contractions, from the low submaximal metabolic rate to a high submaximal rate (2 tetanic contractions per 3 s). Then, during a 35 minute recovery period, tubing and a pump will be inserted into the arterial inflow to the muscle. Subsequently, the same protocol will be repeated except that the blood flow (and therefore O_2 delivery) will be set prior to any contractions at the level previously determined for the higher metabolic rate. This will eliminate any effect of altered blood flow responses when elevating from a resting versus submaximal contraction baseline to a higher metabolic rate. Also, since the stimulation voltage is always supramaximal, there will be no change in motor unit recruitment from one metabolic rate to another. Finally, following another 35 minute recovery period, VO_2 on-kinetics will be determined, again with a fixed, high blood flow, from rest to the high submaximal metabolic rate (2 tetanic contractions per 3 s). These experiments will require 12 dogs.

Each contraction period (1/3 s and 2/3 s) will last 4 minutes. When blood flow is elevated prior to contractions, adenosine (105 – 210 µmol•kg wet muscle^{-1}•min^{-1}) will be infused to maintain blood pressure at the spontaneous level. The protocol is illustrated below.



8. A. Select pain/distress category relevant to the use of animals in this study. (*See Item 8A of Additional Information at the end of this form.)*

C _____ D ____**X**____ E _____ _____

B. If category D or E was chosen in 8A, please complete the following. (*See Item 8B of Additional Information at the end of this form.)*

1) Database(s) searched or other sources consulted to determine the availability of alternatives.

Database Searched		Date of Search	Years Covered
Medline	**X**	June 7-8, 2011	1950-2011
Agricola			
CABA			
Altweb	**X**	June 7-8, 2011	1950-2011
Other (describe)	**AWIC**	June 7-8, 2011	1950-2011
	PubMed	June 7-8, 2011	1950-2011

2) Keywords and search strategy used when considering alternatives to the painful or distressful procedure(s).

Keywords: oxygen, kinetics, uptake, muscle, alternative, dogs

Strategy: Began with a broad search on "oxygen uptake," then narrowed with "kinetics," and "muscle", inspecting the long lists of each result. Searched "dogs" plus "muscle" plus "alternative" in the Altweb site. Studied the descriptions of

databases, strategies, and resources in AWIC. Went to several links for further information. Attempted to determine whether applicable, alternative methods of studying oxygen uptake on-kinetics in muscle with regard to physiological manipulations such as blood flow, etc. are available.

3) A succinct written narrative based on results of the database search, that will permit the IACUC to readily assess whether the search topics were appropriate and whether the search was sufficiently thorough. This narrative must address the following:

Reduction:

Replacement:

Refinement:

Due to the invasive nature of these studies, the most direct information can be obtained on animals instead of humans. Measurement of blood flow and arterial and venous metabolite concentrations, and measurement of muscle metabolite concentrations all entail considerable risks, particularly when combined with exercise. Accurate measures of blood flow and the distribution of that blood flow are also much more easily obtained in this animal model than in the whole human system where indirect methods are less reliable. While we will be using some *in*

***vitro*-like methods, these investigations require the isolation of materials from living organisms, and cellular and sub-cellular studies would not answer some of the questions being posed. In order to study the effects of blood flow and exercise (and in turn, the effects of various other perturbations) on the rapidity of oxygen uptake adjustment, it is necessary to use a more intact preparation in which there actually is a blood flow and measurable metabolism.**

Dogs are being used because there is a great deal of background information on the muscle preparation in these animals and the difficult surgical task of blood flow isolation of the gastrocnemius muscles is relatively convenient. This canine muscle also has a fiber type profile that facilitates the type of experiments being done; i.e., they fatigue slowly and recover relatively quickly so that repeated bouts of contractions can be performed. The number of animals to be used is proposed on the basis of numerous previous studies, including my own personal experience. These studies have shown that 6-12 animals per experimental alteration are needed to account for potential failed experimental trials and to statistically determine significant differences. The use of less than six animals is not advised since this is near the minimum number required for the application of analysis of variance to the data. We plan to use 12 animals in our experimental groups for adequate statistical power while at the same time <u>reducing</u> the number of animals to the lowest reasonable level. Additionally, we always attempt to <u>reduce</u> the number of animals used by employing repeated measures designs whenever experimentally possible. If the preliminary results suggest that 6 animals is adequate, then that is all that will be used.

Finally, we have closely examined the existing body of literature to assure that there are no additional <u>refinements</u> available to reduce stress and/or discomfort to the animals, no <u>replacement</u> techniques to answer the experimental questions posed, and that the number of animals requested is <u>reduced</u> to the statistically allowable limits. The literature was examined by searching PubMed, Altweb, and AWIC as indicated above; PubMed includes Medline and other life sciences journals. In addition, I have been involved in studies on the subject of VO_2 on-kinetics for almost 15 years now; nine studies are already published and two others are in preparation. Further, I have organized and co-chaired two symposia on VO_2 on-kinetics at the national annual meeting of the American College of Sports Medicine. All of this is to say that I am intimately familiar with the literature in this area, and qualified to address <u>R</u>eduction, <u>R</u>eplacement, and <u>R</u>efinement.

4) If alternatives are available but will not be used, please provide a justification.

N/A

5) If pain/distress category E is to be employed, please provide a justification for withholding pain and/or distress relieving drugs.

N/A

9. SURGERY:

Will surgery be performed?

Yes **X** No _____

If yes, please address the following, as applicable:

A. Nonsurvival surgery - Describe all surgical procedures, including surgical preparation. Indicate where surgery will be performed (building and rooms). Identify the person(s) and describe their qualifications for performing the particular surgical procedure(s).

All surgical procedures will be performed in the Muscle Physiology Laboratory located in Room 2129A, Beard-Eaves-Memorial Coliseum. Mongrel dogs, of both sexes will be used. All surgical techniques will require isolation of the gastrocnemius (GP) muscle (Grassi, Gladden, Stary, Wagner, and Hogan, 1998.). The left GP is typically used. Briefly, the animals are anesthetized with sodium pentobarbital (30mg•kg^{-1} iv); additional doses are given as required. The animals are intubated with an endotracheal tube and ventilated with a respirator. A heating pad is placed under the animal and adjusted as needed to maintain the rectal

temperature near 37 degrees Centigrade. Although the normal canine core temperature is approximately 39 degrees Centigrade, maintenance at 37 degrees eliminates the necessity of correcting blood gas, and pH values (instruments are set at 37 degrees). The surgical site is prepared by clipping the animal's hair very closely using an electric clipper for animals. In the unusual occurrence of a dirty animal, a moist cloth is used to clean the exposed skin. These are terminal experiments, so it is not necessary to establish a sterile field. If the GP is to be perfused via a pump in the arterial inflow, all branches of the popliteal artery which are not supplying the GP are ligated. The right brachial artery or the left carotid artery is cannulated and its outflow is directed by tubing through a pump and then through a cannula into the left popliteal artery supplying the isolated GP. This allows blood flow to the muscle to be controlled at any constant level desired, and infusion of drugs, pharmacological agents, metabolites, and/or hormones can be made directly into the arterial inflow into the muscle.

All branches of the popliteal vein that do not come from the GP are ligated and all venous connections from the muscle that do not go directly to the popliteal vein are ligated. Therefore, all of the venous outflow from the muscle is by way of the popliteal vein. The vein is cannulated, and the flow is measured with a cannulating-type ultrasonic probe and flowmeter. Venous outflow is returned to the animal via the left jugular vein. Blood coagulation is prevented by intravenous heparin (2,000 U/kg) every 4 hours.

The Achilles tendon is sectioned and secured in an aluminum clamp for attachment to an isometric myograph. The other end of the muscle is left attached to the femur,

which is fixed to the base of the myograph by two bone nails. The sciatic nerve is exposed and isolated near the muscle. The distal nerve stump, about 1.5-3.0 cm in length, is pulled into a small tubular electrode for stimulation. The release and/or uptake of oxygen, lactate, pyruvate, glucose, alanine, or any other fuel or metabolite by the muscles can be calculated from the venous blood outflow rate and the arteriovenous concentration difference of the substance across the muscle. Muscle samples are taken by biopsy when needed, and immediately frozen in liquid nitrogen. The samples are stored at –80 degrees Centigrade or are freeze-dried until analyzed.

The anesthetization of animals and all surgical procedures will be performed either by me (Dr. L. Bruce Gladden) or other technicians/graduate students who are trained by me. I have 35 years of experience with these techniques.

B. Survival surgery - Describe all surgical procedures, including surgical preparation and post-surgical care. Please indicate that aseptic technique will be followed if the procedure is a survival surgical procedure. Indicate where surgery will be performed and what postoperative care will be provided (building and rooms). Identify the person(s) and describe their qualifications for performing the particular surgical procedure(s).

N/A

10. Administration of analgesics, anesthetics, tranquilizing drugs, and/or neuromuscular blocking agents (Indicate generic name, dose, route of administration and frequency; if by inhalation, method of scavenging waste anesthetic gases.)

Sodium pentobarbital (30 mg•kg^{-1}) with additional doses (typically about 3.5-4.0 mg•kg^{-1}) as required, administered via intravenous injection. Animals are maintained in a deep, surgical plane of anesthesia (additional sodium pentobarbital given as required to maintain absence of pedal, palpebral, and corneal reflexes).

11. Administration of reagents, cells, drugs (other than anesthetics or analgesics), infectious agents, carcinogens, recombinant DNA, etc. (Indicate generic name, dose, route of administration and frequency, anticipated side effects, monitoring protocol.)

Conscious animals will be given only sodium pentobarbital. All other reagents or drugs listed below will be given to the animals <u>after</u> they have been anesthetized:

- Sodium Heparin (2000 U•kg^{-1}) will be given intravenously every four hours to prevent coagulation of blood.

- Bicarbonate (1.0 N) or hydrochloric acid (0.3 N) for adjusting blood pH as necessary to maintain normal limits, administered intravenously (Cain and Adams 1983). We almost never have to use the acid.

- Adenosine (Adenine-9-ß-ribofuranoside) will be infused into the tubing supplying the popliteal artery of the gastrocnemius muscle to effect (maintenance of normal perfusion pressure); this is typically at a rate of 105 – 210 µmol•kg wet muscle^{-1}•min^{-1}.

12. ASSURANCES:

A. Provide a brief statement to confirm that proposed activities involving animals do not duplicate previous experiments unnecessarily.

As noted previously, recent literature searches in various databases confirm my own prior knowledge that these are unique experiments with a high likelihood of producing unique results that will contribute to our understanding of basic muscle metabolism. Also, as noted earlier, I have performed research in this area for almost 15 years with nine published studies, two studies in preparation, and I have chaired two symposia at national/international meetings. These experiments are only being performed because I anticipate unique, new information to result, and to contribute to the scientific literature in premier, peer-reviewed journals.

B. My signature on page 1 of this form certifies that exercise of caged dogs will be accomplished according to the Animal Welfare Act (AWA) or cage size

provides adequate space for exercise to meet AWA requirements.

Alternatively, explain why an exception should be approved by the IACUC.

No exception requested.

C. Will wild caught or endangered animals be utilized?

Yes _____ No **X**

If Yes, the investigator is responsible for obtaining and maintaining valid permits (if required) for collecting, purchasing, transporting, and holding of these animals. List applicable federal and/or state permit numbers and expiration dates.

13. HAZARDOUS AGENTS

Use of hazardous agents in animals may require approval of the appropriate institutional committee. Contact the Department of Risk Management and Safety (844-4870) for specific information.

Hazardous Agent	**Yes**	**No**	**Agent**	**Date of Committ** **Approval and BU**
Radioisotopes		**X**		
Biological Agents		**X**		
Hazardous Chemicals or Drugs		**X**		
Recombinant DNA		**X**		

Describe the practices and procedures required for the safe handling and disposal of contaminated animals and material associated with this study. Also describe methods for removal of radioactive waste and, if applicable, the monitoring of radioactivity.

N/A

14. What will be the disposition of the animals at the termination of the project? If euthanasia is to be performed, what will be the method of carcass disposal?

All animals will be utilized in the aforementioned experiments, and will then be euthanized as described in question #15 below. All carcasses and tissues are subsequently incinerated in the incinerator at the College of Veterinary Medicine campus.

15 All protocols must include the method of euthanasia that will be used during the normal course of the protocol or in the event of unforeseen circumstances resulting from illness or injury. Please specify the method, agent, dose, and route of administration. The euthanasia method must be consistent with the AVMA Panel on Euthanasia or justification for deviation should be indicated. This document is available on the Animal Resources website, http://www.auburn.edu/research/vpr/animals/ resources/res_index.htm and in the Journal of the American Veterinary Medical Association (Vol. 218, No. 5, Pages 669-696, 2001).

All invasive experimental procedures will be preceded by anesthesia. After the experiments, the animal will be euthanized. Since all animals will be under

sodium pentobarbital anesthesia throughout each experiment, maintained in a deep surgical plane of anesthesia, they will be euthanized by administering an overdose of sodium pentobarbital (typically approximately 17 mg•kg^{-1}) along with saturated KCl intravenously (typically about 1.5 ml•kg^{-1} into the jugular vein) (2000 Report of the AVMA Panel on Euthanasia). In all cases, blood pressure will be monitored until it drops to a level incompatible with life and there is no pulse; and a double pneumothorax will be performed as a final precaution.

REFERENCE LIST:

1. **2000 Report of the AVMA Panel on Euthanasia. Journal of the American Veterinary Medical Association 218(5):669-696, 2001.**

2. **Brittain, C.J., H.B. Rossiter, J.M. Kowalchuk, and B.J. Whipp. Effect of prior metabolic rate on the kinetics of oxygen uptake during moderate-intensity exercise. European Journal of Applied Physiology 86:125-134, 2001.**

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ADDITIONAL INFORMATION

THIS PAGE NEED NOT BE INCLUDED WHEN SUBMITTING FORM FOR REVIEW

3. The IACUC is required to inspect animal housing areas and laboratories (at least twice per year) where animals are kept for 12 or more hours.

4. PERSONNEL QUALIFICATIONS:

Federal regulations require institutions to ensure that people caring for or using animals are qualified to do so through documented training or experience. This training is to include investigators, technical personnel, trainees, visiting investigators, and any other individuals who may perform animal husbandry, anesthesia, surgery, or other experimental manipulations involving animals.

7. Please use this procedure list for guidance in providing the necessary information. Please note that this is not meant to be an exhaustive list, but only a guide.

$ **Body fluid sampling** (e.g. blood, cerebrospinal fluid, ascites, urine — describe method of collection, amount, frequency).

$ **Antibody production** (indicate route of administration, volume administered per site, number of sites, adjuvant use and frequency, consideration of alternatives to Freund's adjuvant, anticipated side effects, monitoring protocol).

$ **Ascites method for monoclonal antibody production**. Auburn University requires adherence to the Office for Protection from Research Risks (OPRR) policies concerning the production of monoclonal antibodies using the mouse ascites method. Please refer to the OPRR document http://oacu.od.nih.gov/ARAC/ascites.htm. Use of the ascites method requires justification as to why in vitro systems cannot be used.

$ **Special diets** (describe any anticipated nutritional deficit or other health concerns).

$ **Indwelling catheters or implants** (describe type, maintenance/monitoring protocol).

$ **Restraint of an unanesthetized animal** other than that associated with brief routine procedures such as for the collection of blood (describe method, duration, frequency).

$ **Tumor transplantation** (describe any anticipated functional deficit to the animal, monitoring protocol, endpoint).

$ **Food or fluid restriction** (e.g. greater than that associated with pre-anesthetic procedures — describe, include justification and monitoring protocol.)

$ **Special housing, equipment, animal care** (e.g. describe special caging, water, feed, waste disposal, etc.)

$ **Experimental endpoint criteria** (list the criteria to be used to determine when euthanasia is to be performed. Death as an endpoint must always be scientifically justified.)

8A. USDA promulgated PAIN/DISTRESS CATEGORIES - Please use the following categories when categorizing the pain/distress level.

C <u>Pain or Distress - None or Minor</u>

These include studies that DO NOT involve surgery; induction of painful or stressful disease conditions, or pain or distress in excess of that associated with routine injections or blood collection. Included are induction or transplantation of tumors in animals (as long as the tumors do not cause pain and the animals are terminated prior to becoming ill), administration of mildly toxic substances or pathogenic agents that cause no significant disease or distress, polyclonal antibody production (antigen inoculations and blood collection) as long as significant disease does not result, mild food restriction, and, typically, the collection of animals from the wild or from experimental units (i.e. fish in

earthen ponds) for minor procedures. NOTE: If blood is to be collected via the retro-orbital or intracardiac methods, then anesthesia is required and Pain/Distress D must be selected. Also, if *in vivo* monoclonal antibody production is to be performed, the pain category D must be selected.

D Pain or Distress Relieved by Appropriate Measures

A major concern of the reviewers of these protocols is the degree of pain and/or distress imposed on the animals in the studies, and the methods the investigators will use to prevent, relieve, or minimize such pain or distress.

Following is a partial list of procedures known to involve significant pain and/or distress:

Surgical procedures such as biopsies, gonadectomy, exposure of blood vessels, chronic catheter implementation, laparotomy, or laparoscopy

2. Administration of any chemical or organism that would be expected to produce pains or distress but which will be alleviated by analgesics

3. Intracardiac or retro-orbital blood collections

4. Monoclonal antibody production (ascites method)

5. Other procedures which would be painful or distressful to the animal if performed without the benefit of anesthesia, analgesic, and/or tranquilization (e.g., exsanguination).

E Pain or Distress without Anesthesia, Analgesia or Tranquilizers

If the nature of the study prohibits the use of pain and/or distress relieving drugs, or if unavoidable and unalleviable pain or distress will be produced, you must provide a written justification. (Include this in your response to Item 8, B, 5.) Such procedures include: direct stimulation of central nervous system pain tracts, nociceptor stimulation by physical or chemical means that cause severe pain (e.g., corneal abrasions), or any potentially painful procedure if performed without chemical relief of pain.

8B. The Animal Welfare Act (AWA) requires that the Principal Investigator (PI) consider alternatives and provide a written narrative of the sources consulted to determine whether or not alternatives exist to procedures which may cause pain or distress.

According to the Animal Welfare Information Center (AWIC) of the U.S. Department of Agriculture (USDA), an alternative to procedures that may cause more than momentary pain or distress to animals is any procedure which results in REDUCTION in number of animals used, REFINEMENT of techniques to alleviate such pain or distress, or REPLACEMENT of animals (e.g. with an insentient model such as might be accomplished through use of cell culture or computer simulation). For assistance in conducting database/network searches, as required by the AWA when procedures may cause more than momentary pain or distress to animals, investigators may contact the AU Library On-Line Services (844-1748). Alternatively, to explore a variety of resources for

evaluating alternatives investigators may consult the following website:

http://www.aaalac.org/alts.htm

PI Checklist for Animal Subjects Review Form

General:

- ❒ Did you use the newest version of the Animal Subjects Review Form?
- ❒ Did you spell out all acronyms the first time they were used?
- ❒ Did you verify the spelling of all drugs used?
- ❒ Did you include a copy of any referenced Standard Operating Procedures (SOPs) and/or existing protocols?
- ❒ Did you omit all irrelevant information when using a previous protocol file to create a new Animal Subjects Review Form?

All Protocols:

- ❒ Did you check yes or no to Public Health Service funding source?
- ❒ #2- Did you clarify animal numbers as “per year” or “project total”?
- ❒ #2 and #7- Did you make sure animal numbers in these two sections agree?
- ❒ #2- Did you name the commercial sources?
- ❒ #2- Did you provide the specific housing facility?
- ❒ #4 - Did you list all individuals involved in study by their names (if known)?
- ❒ #7- Did you address how the animal numbers were **determined** and/or **justify** these numbers?

- ❒ #7- Could the study design be presented more clearly using a table?
- ❒ #7 and #11- Did you, if applicable, include the route of administration and/or dosage for all drugs used?
- ❒ #7- Did you, if applicable, include the technique, location, and/or volume of blood drawn?
- ❒ #7- Did you, if applicable, provide the method of transportation and/ or the method of restraint?
- ❒ #8.B.3. - Did you specify reduction, replacement, and/or refinement as they pertain to this study?
- ❒ #10 and #11- Did you, if not applicable, put None or N/A?
- ❒ #12 – Did you provide, if applicable, permit numbers and expiration dates?
- ❒ #13 – Did you include, if applicable, the Biological Use Authorization (BUA) number and date of approval or indicate that it is pending?
- ❒ #14 - Did you address method of carcass disposal and/or the location in the event that euthanasia becomes necessary?
- ❒ #15 - Did you indicate the method of euthanasia should it become necessary?

Teaching Protocols:

- ❒ #7- Did you include, if applicable, the number of students per animal, the number of animals per lab, and the number of labs per year?