EFFECTS OF ISCHEMIA AND REPERFUSION ON THE LOCAL REGULATION OF OXYGEN CONSUMPTION, TISSUE OXYGENATION AND BLOOD SUPPLY IN RAT SKELETAL MUSCLE.

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EFFECTS OF ISCHEMIA AND REPERFUSION ON THE LOCAL REGULATION OF OXYGEN CONSUMPTION, TISSUE OXYGENATION AND BLOOD SUPPLY IN RAT SKELETAL MUSCLE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at the Medical College of Virginia Campus, Virginia Commonwealth University

By

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Richmond, Virginia
April, 2013
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Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xi</td>
</tr>
<tr>
<td>Abstract</td>
<td>xii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Cardiovascular System and Role of ( \text{O}_2 )</td>
<td>2</td>
</tr>
<tr>
<td>Regulation of Blood Flow</td>
<td>3</td>
</tr>
<tr>
<td>Ischemia/Reperfusion</td>
<td>6</td>
</tr>
<tr>
<td>From Model to Mechanism</td>
<td>7</td>
</tr>
<tr>
<td>Materials &amp; Methods</td>
<td>9</td>
</tr>
<tr>
<td>Animals</td>
<td>9</td>
</tr>
<tr>
<td>Anesthesia and Euthanasia</td>
<td>10</td>
</tr>
<tr>
<td>Surgery</td>
<td>11</td>
</tr>
<tr>
<td>Femoral Vein Cannulation</td>
<td>14</td>
</tr>
<tr>
<td>Tracheostomy</td>
<td>17</td>
</tr>
<tr>
<td>Spinotrapezius Muscle Preparation</td>
<td>19</td>
</tr>
<tr>
<td>Stop-flow Device</td>
<td>22</td>
</tr>
<tr>
<td>Phosphorescence Quenching Microscopy</td>
<td>26</td>
</tr>
<tr>
<td>Pd-MTCCP ( \text{O}_2 ) Probe Preparation and Administration</td>
<td>27</td>
</tr>
<tr>
<td>Oxygen Consumption</td>
<td>28</td>
</tr>
<tr>
<td>Flow measurements</td>
<td>32</td>
</tr>
<tr>
<td>Page #</td>
<td>Section</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>35</td>
<td>Compartmental Model of Q, VO₂ and PᵢₛᵣO₂</td>
</tr>
<tr>
<td>38</td>
<td>Ischemia/Reperfusion Experimental Protocol</td>
</tr>
<tr>
<td>38</td>
<td>Ischemia/Reperfusion</td>
</tr>
<tr>
<td>38</td>
<td>PᵢₛᵣO₂ study</td>
</tr>
<tr>
<td>38</td>
<td>Q study</td>
</tr>
<tr>
<td>39</td>
<td>VO₂ study</td>
</tr>
<tr>
<td>39</td>
<td>Protocol</td>
</tr>
<tr>
<td>41</td>
<td>Statistical Methods</td>
</tr>
<tr>
<td>42</td>
<td>Results</td>
</tr>
<tr>
<td>44</td>
<td>5-second Ischemia</td>
</tr>
<tr>
<td>51</td>
<td>15-second Ischemia</td>
</tr>
<tr>
<td>58</td>
<td>30-second Ischemia</td>
</tr>
<tr>
<td>65</td>
<td>60-second Ischemia</td>
</tr>
<tr>
<td>72</td>
<td>300-second Ischemia</td>
</tr>
<tr>
<td>79</td>
<td>600-second Ischemia</td>
</tr>
<tr>
<td>86</td>
<td>Baseline Analysis</td>
</tr>
<tr>
<td>86</td>
<td>Baseline correlations to PᵢₛᵣO₂</td>
</tr>
<tr>
<td>91</td>
<td>Initial recovery rates during Reperfusion</td>
</tr>
<tr>
<td>91</td>
<td>Transient PᵢₛᵣO₂</td>
</tr>
<tr>
<td>91</td>
<td>Blood Flow</td>
</tr>
<tr>
<td>92</td>
<td>Recovery of PᵢₛᵣO₂ in VO₂ study</td>
</tr>
<tr>
<td>96</td>
<td>Percent increase in VO₂ during Reperfusion</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Ventral view of shaved regions for tracheostomy and venular cannulations</td>
<td>12</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Dorsal view of shaved region for spinotrapezius exteriorization</td>
<td>13</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Femoral vein cannulation for continuous anesthesia infusion</td>
<td>16</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Ventral view of tracheostomy site for patent airway maintenance</td>
<td>18</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Right dorso-lateral view of spinotrapezius exteriorization</td>
<td>21</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Components of the air-bag unit</td>
<td>23</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Flash-synchronized Automatic Pressure Cycler</td>
<td>24</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Completed stop-flow device on microscope</td>
<td>25</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Example PO&lt;sub&gt;2&lt;/sub&gt;/VO&lt;sub&gt;2&lt;/sub&gt; graph, 30-second ischemia/200-second reperfusion</td>
<td>30</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Example PO&lt;sub&gt;2&lt;/sub&gt;/VO&lt;sub&gt;2&lt;/sub&gt; graph, 10-minute ischemia/15-minute reperfusion</td>
<td>31</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Transient P&lt;sub&gt;ISF&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; data for 5 seconds of ischemia</td>
<td>45</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Normalized transient P&lt;sub&gt;ISF&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; data for 5 seconds of ischemia</td>
<td>46</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Flow data for 5 seconds of ischemia</td>
<td>47</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Normalized flow data for 5 seconds of ischemia</td>
<td>48</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Oxygen consumption data for 5 seconds of ischemia</td>
<td>49</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Normalized oxygen consumption data for 5 seconds of ischemia</td>
<td>50</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Transient P&lt;sub&gt;ISF&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; data for 15 seconds of ischemia</td>
<td>52</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Normalized transient P&lt;sub&gt;ISF&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; data for 15 seconds of ischemia</td>
<td>53</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Flow data for 15 seconds of ischemia</td>
<td>54</td>
</tr>
<tr>
<td>Figure 20</td>
<td>Normalized flow data for 15 seconds of ischemia</td>
<td>55</td>
</tr>
<tr>
<td>Figure 21</td>
<td>Oxygen consumption data for 15 seconds of ischemia</td>
<td>56</td>
</tr>
</tbody>
</table>
Figure 22: Normalized oxygen consumption data for 15 seconds of ischemia .......................57
Figure 23: Transient $P_{ISF}O_2$ data for 30 seconds of ischemia ..............................................59
Figure 24: Normalized transient $P_{ISF}O_2$ data for 30 seconds of ischemia .........................60
Figure 25: Flow data for 30 seconds of ischemia ...................................................................61
Figure 26: Normalized flow data for 30 seconds of ischemia ..................................................62
Figure 27: Oxygen consumption data for 30 seconds of ischemia ........................................63
Figure 28: Normalized oxygen consumption data for 30 seconds of ischemia .....................64
Figure 29: Transient $P_{ISF}O_2$ data for 60 seconds of ischemia .............................................66
Figure 30: Normalized transient $P_{ISF}O_2$ data for 60 seconds of ischemia .........................67
Figure 31: Flow data for 60 seconds of ischemia ...................................................................68
Figure 32: Normalized flow data for 60 seconds of ischemia ..................................................69
Figure 33: Oxygen consumption data for 60 seconds of ischemia ........................................70
Figure 34: Normalized oxygen consumption data for 60 seconds of ischemia .....................71
Figure 35: Transient $P_{ISF}O_2$ data for 300 seconds of ischemia .............................................73
Figure 36: Normalized transient $P_{ISF}O_2$ data for 300 seconds of ischemia ......................74
Figure 37: Flow data for 300 seconds of ischemia ...................................................................75
Figure 38: Normalized flow data for 300 seconds of ischemia ..................................................76
Figure 39: Oxygen consumption data for 300 seconds of ischemia ........................................77
Figure 40: Normalized oxygen consumption data for 300 seconds of ischemia .....................78
Figure 41: Transient $P_{ISF}O_2$ data for 600 seconds of ischemia .............................................80
Figure 42: Normalized transient $P_{ISF}O_2$ data for 600 seconds of ischemia ......................81
Figure 43: Flow data for 600 seconds of ischemia ...................................................................82
Figure 44: Normalized flow data for 600 seconds of ischemia ............................................... 83

Figure 45: Oxygen consumption data for 600 seconds of ischemia ........................................ 84

Figure 46: Normalized oxygen consumption data for 600 seconds of ischemia ...................... 85

Figure 47: Baseline oxygen consumption versus baseline interstitial fluid PO\textsubscript{2} during the VO\textsubscript{2} study .................................................................................................................... 88

Figure 48: Baseline blood flow versus baseline interstitial fluid PO\textsubscript{2} during the blood flow study .................................................................................................................... 89

Figure 49: Initial rates of recovery of P_{ISF}O\textsubscript{2} during reperfusion of the Transient P_{ISF}O\textsubscript{2} (PO\textsubscript{2} <20 mmHg) study ................................................................. 93

Figure 50: Initial rate of recovery of flow during reperfusion of the Blood Flow study .......... 94

Figure 51: Initial rate of recovery of P_{ISF}O\textsubscript{2} (<20 mmHg) during reperfusion of the Oxygen Consumption study ........................................................................................................ 95

Figure 52: Percent of VO\textsubscript{2} baseline at the earliest measurement of reperfusion .......... 97

Figure 53: Factor M calculated for 5-, 15-, 30- and 60-second ischemia groups using the normalized data .................................................................................................................. 99

Figure 54: Factor M Factor M calculated for the 300- and 600-second ischemia group using the normalized data ........................................................................................................ 100
List of Tables

Page #

Figure 1: Average baseline data for $P_{\text{ISF}}O_2$, Q, VO$_2$ studies and factor M ...........................................90
Acknowledgements

First and foremost, I would like to thank the many rats who gave their lives in the pursuit of the science presented in this work. You will forever be remembered by your unique identification numbers—especially you Rat #323631.

I would like to acknowledge Dr Roland Pittman as one of the kindest, generous and above all, supportive individuals I have had the fortune to meet. When everything started going wrong and the windowless doors shut tight around me, he gave me a chance to do something I love and for that, I owe him a debt of gratitude that I could never repay. As a guide and mentor, his keen editorial skills and ability to detangle and demystify some of the most perplexing rationale and thought processes taught me so much in the way of academia and my own journey into it. When I think about it, there has never been an instance where we concluded a meeting or finished a casual conversation where I left without having learned something—a testament to his effective and admirable teaching skill.

Dr Aleksander Golub was instrumental in the execution of this study; his sardonic and captivating storytelling skills always kept me on the edge of my seat waiting for the punch line. Whenever a microscope needed a part constructed, something fell apart and/or exploded, Alex’s particular set of skills and genius acquired over a very long career gave him the ability to look into and execute the best solution possible and I hope to follow a similar path one day.

Through tough love, a ridiculous personality and a derisive sense of humor, my predecessor Bjorn taught me the muscle preparation necessary for this work to be possible. Today, I am proud to say that I have surpassed him in the beauty of the preparation, so much so that hearts stop when the owners of those hearts observe the vascular perfusion on the monitor. I can’t count the number of times I have had to administer CPR because of it.

I would also like to thank my committee members, Drs Rakesh Kukreja and Paul Ratz for being so amenable to my bizarre and kooky requests.

Lastly, I want to dedicate this to my parents. No matter what happened to them, they never stopped believing in me. No matter what happens now, I will never give up—for them, for everyone that lent their support and put their faith in me. Thank you everyone, it has meant a lot to me.
Abstract

EFFECTS OF ISCHEMIA AND REPERFUSION ON THE LOCAL REGULATION OF OXYGEN CONSUMPTION, TISSUE OXYGENATION AND BLOOD SUPPLY IN RAT SKELETAL MUSCLE

By Sami C. Dodhy, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2013

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In resting muscle, blood flow is regulated to meet the demand for O\textsubscript{2} by the tissue. A modified ischemia (I)/reperfusion(R) investigation was systematically run and P\textsubscript{ISF}O\textsubscript{2}, P\textsubscript{a}O\textsubscript{2}, Q and VO\textsubscript{2} were observed. Twenty-nine spinotrapezius muscles from male Sprague-Dawley rats (284±20 grams) were surgically exteriorized for intravital microscopy to test a model relating blood flow, O\textsubscript{2} supply and O\textsubscript{2} demand. The model can aid in the understanding of the regulation of tissue P\textsubscript{O\textsubscript{2}}. The interstitial P\textsubscript{O\textsubscript{2}} (P\textsubscript{ISF}O\textsubscript{2}) and perivascular P\textsubscript{O\textsubscript{2}} (P\textsubscript{a}O\textsubscript{2}) measurements were made using phosphorescence quenching microscopy (PQM). O\textsubscript{2} consumption (VO\textsubscript{2}) values were obtained with a quasi-continuous, flash-synchronized, pressurized airbag to initiate ischemia and sample the rate of O\textsubscript{2} change (dP\textsubscript{O\textsubscript{2}}/dt). Centerline red blood cell velocity was measured with an Optical Doppler Velocimeter and converted to flow using vessel diameter. 5-, 15-, 30-, 60-, 300- and 600-second ischemic durations were used to observe changes in P\textsubscript{ISF}O\textsubscript{2}, Q, and VO\textsubscript{2}. A critical point was observed following 30 seconds of (I) where dP\textsubscript{ISF}O\textsubscript{2}/dt during recovery was the fastest (4.25±0.72 mmHg/s) and was 1.00±0.16 mmHg/s following 600 seconds. Flow recovery, dQ/dt, peaked to 3.88±0.64 (µl·min\textsuperscript{-1})/s after 60 seconds of (I) but significantly dropped to 2.83±0.55 (µl·min\textsuperscript{-1})/s following 300 seconds of (I) but increased to 2.92±0.45 (µl·min\textsuperscript{-1})/s following 600 seconds. This gives evidence to a no-reflow phenomenon occurring in the extended periods of ischemia. A peak in VO\textsubscript{2} to 309.2±45.0 nl O\textsubscript{2}/cm\textsuperscript{3}·s with a time course of 160 seconds occurred following 600 seconds of ischemia. As the ischemic duration decreased, the time course and peak VO\textsubscript{2} also decreased. VO\textsubscript{2} following 300 seconds of (I) was significantly higher than 5-60 seconds of (I) (p <0.05) but was not significantly different from 600 seconds of (I). The information collected during the Q and VO\textsubscript{2} studies can be incorporated into a factor, M, that relates VO\textsubscript{2}, Q and ΔP\textsubscript{O\textsubscript{2}}. M calculated for the recovery of 5- through 60-second (I) groups reasonably relates the three variables due to consistency and little variability. However, recovery in 600- and especially 300-second (I) groups showed higher variability in M which requires more consideration.
INTRODUCTION

In resting conditions, the flow of oxygen-rich blood is constantly regulated to meet the demand for oxygen by respiring tissue and to provide the energy to sustain life through a coordinated effort of the cardiovascular and respiratory systems. In instances such as exercise when the demand for oxygen initially exceeds the supply, a normal imbalance begins to occur and the organism responds with compensatory changes in ventilation and blood flow. By cutting off blood flow (vascular occlusions or collapse) or decreasing the oxygen-carrying capacity of the blood (e.g., traumatic hemorrhage or hemolysis), the prolonged supply and demand imbalance and resulting ischemia and hypoxia can cause irrevocable harm to the tissue and organism. The mechanism for the regulation of tissue oxygenation should be able to explain the relationship among flow of oxygenated blood, the amount of oxygen supplied to the tissue, and the rate at which the tissue consumes it and how changes in any of the three intrinsic characteristics are compensated. Using Fick’s principle the relationship among these three variables in the microcirculation can be mathematically modeled. If a model can be established, it would simply define what the limits of the change in one parameter are with respect to the other two. With this model, any proposed mechanism can be tested and if the results deviate from the defined limits, it can be reasoned that it is not the true mechanism or there are additional undefined variables at work. An extension of this is that it can also show how a pathology of one of the characteristics can affect the other—so long as the affected parameter is within the limits originally defined. Ultimately, an understanding of the relationship between the supply of and demand for oxygen in the microcirculation can lead us to increase the efficiency of maintaining the balance between them.
Cardiovascular System and Role of Oxygen

One of the most important functions of the cardiovascular system is to deliver oxygen to respiring cells that make up an organ and use that oxygen to generate ATP. Since oxygen is a non-polar molecule with limited solubility in aqueous solutions such as blood, it is carried in the red blood cells bound to hemoglobin. Hemoglobin A (HbA), the most abundant form of hemoglobin in adult humans, is a tetrameric protein with two alpha and beta subunits non-covalently linked with a heme group attached to each subunit. Heme groups contain iron held in a heterocyclic porphyrin ring and it is at the iron where oxygen binds. The iron will exist in either the 2+ (ferrous) state in which oxygen is able to bind or the 3+ (ferric) state or metHb which cannot bind oxygen. The two conformations of HbA, T(taut) or R(relaxed), determine its affinity for oxygen. In the lungs, where the pH is high and carbon dioxide is low, HbA will be in the relaxed form which has a high affinity for oxygen and, in areas where pH is lower and carbon dioxide is higher, such as metabolically active tissue during exercise, the T form is preferred and the affinity for oxygen decreases, so that it can be released from the hemoglobin and taken up by the adjoining tissue.

The oxygen which diffuses into cells serves as an electron acceptor within the mitochondria to facilitate the formation of adenosine triphosphate (ATP) via oxidative phosphorylation. Due to its high-energy phosphate bonds, releasing anywhere from 30-50 kilojoules of energy per mole of every phosphate split (Gajewski et al., 1986), ATP serves as the preferred energy currency of the cell.
Regulation of Blood Flow

To maintain a consistent supply of oxygen, the cardiovascular system ensures that the process by which they are supplied—the flow of blood—is tightly regulated. In periods of high respiration where the demand for oxygen increases, the perfusion of oxygenated blood to that organ increases to ensure adequate supply. Mechanisms to increase the supply of oxygen include: increased ventilation, increased heart rate and stroke volume, increased sympathetic activity and elevated hormones, such as epinephrine, norepinephrine and angiotensin II levels. Although these mechanisms will act to increase the overall supply of blood to a tissue at any given time, they do not guarantee that the demanding tissues are sufficiently supplied. Local regulation of blood flow, specifically at the level of the microcirculation, is ultimately where the amount of blood being supplied to the tissue matters most, as this is the site for nutrient and waste exchange. These mechanisms act independently of global mechanisms to ensure that tissues are perfused with a sufficient amount of blood to maintain adequate tissue oxygenation. Three examples of local blood flow regulation are: autoregulation, active (functional) hyperemia and reactive hyperemia. Hyperemia refers to the increased flow of blood in tissues and organs.

Autoregulation is the intrinsic ability of an organ or tissue to maintain a constant blood flow despite changes in perfusion pressure. Because flow is the ratio of perfusion pressure to vascular resistance, as the perfusion pressure begins to drop—as it does in ischemia—the resistance of the vascular network that is perfusing the tissue will drop to maintain the supply and prevent an oxygen imbalance. Autoregulation is prominently exhibited in the cerebral and coronary circulations (Carlson et al., 2008) where a continuous high degree of activity requires these organs to receive constant nourishment and oxygen supply.
When the cellular metabolism of a tissue increases, it begins to produce vasoactive compounds (Segal, 2005) that affect the local circulation (classic metabolic hypothesis of blood flow regulation). Occurring most notably in muscle contraction, functional hyperemia occurs when these metabolites interact with the nearby endothelium and vascular smooth muscle (Welsh & Segal, 1998) of the resistance vessels to induce vasodilation and increase perfusion. This ensures that, as the tissue increases its activity, a deficit in tissue oxygenation will not be created.

Following a brief to tolerably prolonged period of vascular occlusion to a tissue or organ, a transient increase in blood flow occurs after flow is restored. While the vessels are occluded, vasoactive metabolites such as adenosine—eventual breakdown product of ATP hydrolysis—accumulate because the tissue continues to remain active to a decreasing extent without its oxygen supply being replenished. These vasoactive compounds (Wong et al., 2003) will go on to interact with smooth muscle and cause it to relax, thereby dilating the resistance vessels, so that when perfusion is restored, flow will be elevated above baseline for a period of time. This transient increase in blood flow post-occlusion is what is referred to as reactive hyperemia. As the accumulated metabolites are washed out by the blood, the resistance vessels regain their resting vascular tone and the transient elevation of flow subsides with the return to baseline.

Two of the most widely accepted hypotheses for mechanisms of the local regulation of blood flow have been the metabolic and myogenic hypotheses. The metabolic hypothesis (DeFily & Chilian, 1995) states that the regulation of blood flow is coupled to the metabolic activity of the perfused organ or tissue. When there is an increase in tissue metabolism or a hypoxic episode, in which the demand for oxygen exceeds the supply, there is an increase in the production of certain vasoactive compounds such as: lactic acid, adenosine and potassium ions (Tang et al., 2004). Carbon dioxide (Duling, 1973), another metabolic product of respiration,
also acts as a vasodilator in the same capacity as lactic acid; when both are dissolved in aqueous blood, the hydrogen ions that dissociate act to decrease the pH of the blood eliciting a pH-sensitive central and peripheral chemoreceptor response. As these compounds accumulate, they act to decrease the vascular tone of resistance vessels causing vasodilation.

The myogenic hypothesis (Moore et al., 1994), another mechanism to explain local control of blood flow, states that the smooth muscle within resistance vessels reacts to stretch, causing the muscle to depolarize and contract, leading to vasoconstriction. When the increased stretch subsides, the smooth muscle relaxes, returning blood flow to baseline.

Although widely regarded as the standard for explaining metabolically-linked changes in blood flow, the metabolic hypothesis hinges on the fact that there are metabolites that are produced which cause changes in blood flow. With more metabolites still being discovered, the possibilities become endless which calls into question the idea that one of the metabolites identified under this hypothesis could actually be the mediator of a more complete mechanism. Functionally, the myogenic hypothesis relates the intraluminal pressure to vascular tone. Under resting conditions, this may be able to explain how blood flow is kept at a baseline level but, when it is necessary for the heart rate, contractility and blood pressure to increase, as in exercise, the explanation starts to fall apart (Schubert & Mulvany, 1999). During exercise, the cellular metabolism of tissues increases and waste production and the demand for oxygen increases.
Ischemia/Reperfusion

Ischemia is defined as the restriction of blood flow to a tissue or organ, decreasing the amount of oxygen and nutrients available to it to continue cellular metabolism (Frank et al., 2012). If the blood and oxygen supply is cut off for a prolonged period of time, an ischemic cascade is initiated that can extend well past the point where flow returns. This cascade, as expected, is initiated by cells producing lactic acid through anaerobic metabolism and eventually ATP production ceases. A lack of ATP prevents ion pumps from functioning and upon depolarization, the calcium that becomes trapped in the cell induces more calcium to enter the cell and overload it. This overload of calcium enhances the generation of reactive oxygen species and triggers the opening of the mitochondrial permeability transition pore (Brandao et al., 2003). When cytochrome c is exposed to the cytosolic environment, it dissociates from the electron transport chain, binds and activates quiescent pro-apoptotic factors which then induce apoptosis, most notably through a caspase response (Brookes et al., 2004). As the cell breaks down, it releases harmful chemicals into the interstitium which can damage and induce apoptosis in nearby cells, initiating a chain reaction of damage that continues well beyond the return of oxygenated blood flow.

A variety of pathologies have been attributed to ischemia, including: myocardial infarction, atherosclerosis, embolism and hemorrhage associated with aneurysms. Restoration of oxygenated blood flow is known as reperfusion and often times, after prolonged ischemic conditions and the buildup of harmful substances due to tissue necrosis, the washout of the chemicals spreads systemically and, because degradation of these active compounds is not immediate, they go on to cause inflammation and damage in tissue away from the site of original ischemic damage (Szokoly et al., 2006).
From Model to Mechanism

Despite the various pathologies associated with ischemia/reperfusion injury, modified protocols have repeatedly been used to understand the mechanism of tissue oxygenation and blood flow regulation. By inducing an ischemic condition in a vascular bed, changes between the supply and demand of oxygen and blood and its regulation can be observed in situ using intravital and phosphorescence quenching microscopy. Although these techniques cannot explain the underlying biochemical changes that could be mediating the regulation, they can define what the changes should look like if used correctly.

For a mechanism of the regulation of tissue oxygenation and blood flow to be complete, it should be able to explain how changes in any of the three intrinsic characteristics -- flow of oxygenated blood, the amount of oxygen supplied to the tissue and the rate at which the tissue consumes it -- are compensated. In addition to being able to describe the changes that take place, the proposed mechanism should be able to explain the relationship among these three variables.

Fick's principle, at the level of the whole organism, has been used to describe the relationship of oxygen consumption to cardiac output and the arteriovenous oxygen content difference. The principle relies on mass balance with respect to the regulation of tissue oxygenation, in other words, the total amount of oxygen that is consumed by the tissue or organ ($\dot{V}_{O_2}$) is equal to the product of the flow of blood through it ($Q$) and the difference in the incoming arterial ($[O_2]_{in}$) and outgoing venous ($[O_2]_{out}$) oxygen concentrations.

$$\dot{V}_{O_2} = Q \cdot ([O_2]_{in} - [O_2]_{out}) \quad \text{Eq. 1}$$

Using Fick's principle this study will attempt to describe the relationship among these three variables in the microcirculation with a mathematical model. If a model can be established, it would be used to define the limits of the change in one parameter with respect to the other two.
With this model, any proposed mechanism can be tested and, if the results deviate from the
defined limits, it can be reasoned that there are additional undefined variables involved and the
mechanism would need to be revised.

Because of the technical design and limitations, data were collected individually for the
three parameters and then combined. Ischemia was induced by using a pressurized airbag over
to the tissue which was rapidly inflated (<1sec) to 140 mmHg to arrest flow and extrude blood;
the airbag was then rapidly deflated (<1sec) to 5mmHg to initiate reperfusion. The oxygen
tension of the interstitial fluid was measured using phosphorescence quenching microscopy
(PQM). Using information from the oxygen dissociation curve, the concentration of
haemoglobin and its capacity to carry oxygen, oxygen tension values were converted to oxygen
contents. Blood flow in a nearby arteriole was measured by converting the centerline RBC
velocity collected from an Optical Doppler Velocimeter to flow using vessel diameter
determined simultaneously from video sequence image analysis. Using perivascular oxygen
tension measurements collected to represent arteriolar PO$_2$, the flow of oxygen was determined
by combining the information collected about oxygen contents and blood flow. Oxygen
consumption by the tissue was measured by using a quasi-continuous approach (5-sec flow
stoppage/15-sec flow recovery) to observe dPO$_2$/dt using the PQM and ischemia/reperfusion
system. Since it is known that the PQM method itself consumes oxygen, the overestimated
consumption values collected were corrected for by using a novel 1 Hz/10 Hz correction system.
Materials & Methods

Animals

Twenty-nine male Sprague-Dawley (SD) rats (Harlan, Indianapolis, IN) with an average weight of 284±20 grams were utilized in this study. The study was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee whose policies are consistent with the National Institutes of Health (NIH) Guidelines for the Humane Treatment of Laboratory Animals, as well as the American Physiological Society’s Guiding Principles in the Care and Use of Animals. The rodents were maintained on regular laboratory rodent chow and housed in the university’s animal facility in accordance with institutional guidelines. The rodents were kept two per plastic cage under a 12hr/12hr light/dark cycle with properly maintained ventilation and climate control (20-23 degrees Celsius with humidity at 40-60%) under the supervision of the Division of Animal Resources.
Anesthesia and Euthanasia

The rodents were initially anesthetized with an intraperitoneal injection of a mixture of Ketamine HCl (75 mg/kg, Bioniche Teoranta, Inverin, Co Galway, Ireland) and Acepromazine (2.5 mg/kg, Butler Animal Health Supply, Dublin, OH). The Ketamine provides the necessary depth of anesthesia for surgical procedures until the Alfaxalone infusion can begin. After the initial sedation, the rodent was kept on a continuous intravenous administration of Alfaxan (Alfaxalone 10 mg/ml, Vetoquinol UK LTD, Buckingham, MK18 1PA, UK) via an infusion pump (SP120p, World Precision Instruments Inc, Sarasota, FL) at a rate of 0.35 ml/hr. Upon completion of the experiment and in accordance with non-aseptic non-survival protocols, the anesthetized rodent was euthanized with 0.1 ml of Euthasol (Pentobarbital 390 mg/ml and phenytoin 50 mg/ml, Delmarva Laboratories Inc, Midlothian, VA).
Surgery

The rodents were weighed and given a lower-left quadrant intraperitoneal injection of Ketamine and Acepromazine. Upon verification of sufficient sedation using the heel-pinch technique, the rodent was transferred to a thermostabilized mat set at 37 °C for surgical preparation. Three locations were made available for use by using an electric shaver and a depilatory cream such as Nair (Church and Dwight Inc, Princeton, NJ): the dorsal area extending from the shoulder blade to the base of the ribcage so as to exteriorize the spinotrapezius muscle for optical microscopy, the skin between the mouth and clavicle for the tracheostomy, and the right ventral thigh for continuous anesthetic infusion and euthanizing agent via the femoral vein. The sites were wiped clean with isopropyl alcohol (BD, Franklin Lakes, NJ).
Figure 1. Ventral view of shaved regions for tracheostomy and venular cannulations.
Figure 2. Dorsal view of shaved region for spinotrapezius exteriorization.
Femoral Vein Cannulation

A 5 mL syringe of Alfaxan was attached to a three-way stopcock with a 10 mL syringe of 20 unit/mL heparinized saline (Baxter Pharmaceutical, Deerfield, IL) with a 10 in?? PE-50 (Polyethylene tubing, Clay Adams, Parsippany, NJ) luer-locked tube that would serve as the cannula for continuous anesthesia infusion. The heparinized syringe would serve to disintegrate clots that might form within the cannula, although because of continuous infusion, clot formation would be highly unlikely. The rodent was rotated while in the supine position so that the head was facing furthest away from the surgeon. Using scotch tape, the left hind limb was stretched by securing one end of the tape to the foot and the other to the bench top. At the location where the hind limb extends from the abdomen, an approximately 1 cm incision was made in the integument with blunt dissection scissors that extended the opening and separated the skin from the underlying superficial fascia. Once the cleft between the abdominal and leg muscles were visible, the branches of the femoral vessels could be seen running down the leg. The fascia was separated with blunt dissection scissors placed between the muscles moving toward the vertebral column. Taking note of the vessel bifurcation locations, the cannulations occurred proximal to the major bifurcation. The femoral artery, vein, and nerve are bundled together in the fascia so the first step was to separate the vessels and nerve from each other using curved, blunt forceps. Ensuring there was enough light and that the area was sufficiently moisturized with phosphate buffered saline (PBS, Sigma), the separation was performed under a stereomicroscope (Nikon SMZ645, Melville, NY) and the vein was tweezed apart. With two 12.5 cm hemostats, a 4 inch distal suture was securely looped around the vessel to prevent blood flow from the leg to the heart. The proximal suture was loosely looped and, using the hemostats, the vessel was pulled so as to provide tension on the vessel and prevent any backflow of blood. Using microdissection
scissors (Vannas, 3mm straight), a ¼ circumferential incision of the vein was made and the cannula was inserted. Successful cannulation was confirmed when a small quantity of blood was able to be withdrawn; then the Alfaxan syringe was immediately connected to the infusion pump. The incision site was sealed with a small piece of scotch tape so as to prevent desiccation.
Figure 3. Femoral vein cannulation for continuous anesthesia infusion.
Tracheostomy

Tracheal intubation allows for the maintenance of a patent airway, ensuring normal blood gas and acid-base balances (PO\textsubscript{2} ~ 85-95 mmHg, SO\textsubscript{2} ~ 90-95\%, PCO\textsubscript{2} ~ 40 mmHg, pH ~ 7.4) while under anesthesia. Immediately after the femoral vein cannulation, while ensuring the animal was sufficiently anesthetized and placed ventral side up on the thermostabilized heating pad, with the head toward the surgeon, a lateral cut (~1 cm) of the skin was made with blunt dissection scissors perpendicular to the body axis, approximately 1.5 cm below the mouth, even with the jawline of the rodent. The opening was widened until the skin was separated from the underlying connective and fat tissue. A thick, tough connective/fatty tissue layer (superficial fascia) was visible and cleared away between the mandibular glands in order to expose the underlying sternohyoid muscle. Two forceps were used to divide the muscle down along the body axis and, using the same forceps, the trachea was isolated by hooking under the elevated trachea. With the trachea elevated using one of the curved forceps, a piece of 3-0 non-sterile, braided silk (Fine Science Tools - FST, Foster City, CA) was threaded under the forceps and pulled with a hemostat to provide slight tension on the trachea for incision and cannulation. A 1/3 circumferential incision was made with the microdissection scissors between the bands of cartilage and 1 cm of a 3-cm PE-240 tracheal cannula was immediately placed into the airway and tied off with the threaded suture.
Figure 4. Ventral view of tracheostomy site for patent airway maintenance.
Spinotrapezius Muscle Preparation

For the purposes of this work, the use of intravital microscopy techniques required the surgical exteriorization of the rat spinotrapezius muscle (Gray, 1973 and Bailey et al, 2000). After placing the animal in the prone position, a dorsal midline incision was made from the lumbar to the cervical region, exposing the underlying superficial fascia. A perpendicular incision of the integument was made on both ends of the initial dorsal incision to create a skin flap. Using a low-temperature cautery device (Bovie, Bovie Medical, Clearwater, FL), bleeding originating from the surrounding connective tissue and skin was cauterized. The fascia that covers the tissue was removed so that the muscle and the thin strap muscle on the left lateral border running parallel to the spinotrapezius were exposed but not damaged. A pair of curved scissors was used to separate the spinotrapezius muscle from the underlying muscle layers starting at the left lateral border toward the muscle insertion point on the scapular spine and continuing towards the distal end of the thoracolumbar origin. 6-0 silk thread was sutured every 1 cm along the strap muscle as the muscle was separated to ensure minimal trauma to the spinotrapezius. The right lateral border was excised from the vertebral axis using the cautery to liberate the muscle from its scapular attachment and was similarly sutured. The tissue was kept moist with frequent applications of PBS. Once the spinotrapezius was successfully isolated and the attachment points set, the animal was placed onto a thermostatic animal platform (Golub and Pittman, 2003) with the muscle held in place with sutures at the attachment points on the platform (see Figure 5). The muscle was blotted with 41 grade filter paper (Whatman) to remove any excess fluid and R0 probe was immediately applied to the area to be studied. Another piece of filter paper was placed over the muscle and the entire preparation was covered with a 12 micron thick gas barrier polyvinylidene chloride film (Krehalon, Japan) to prevent desiccation. The tissue was loaded
with R0 probe by incubating it for approximately 45 minutes after which the filter paper was removed and the Krehalon film was affixed back to the preparation, ensuring that all air bubbles had been removed with the push of a cotton swab, being careful not to damage the preparation.
Figure 5. Right dorso-lateral view of spinotrapezius exteriorization and attachment to thermostatic animal platform for intravital microscopy.
Stop-flow Device to Induce Ischemia/Reperfusion

The stop-flow device (Golub et al., 2011a) was used to rapidly stop blood flow and extrude blood from most of the vessels in the spinotrapezius muscle preparation when inflated and restore blood flow when deflated. In its construction, a rubber grommet was placed around the front lens enclosure of a 20x/0.45 objective (Zeiss, Thornwood, NY). The grommet was pierced by an 18-gauge blunted needle which was connected to a pressure regulator. Krehalon film was wrapped around the grommet loosely enough to leave an air pocket, so that the film was spaced from the lens and could be quickly inflated to stop flow. A rubber O-ring was fastened around the Krehalon film and grommet and the preparation was secured with electrical tape to prevent leakage of air. The sealed device was connected to a flash-synchronized, Automatic Dual Pressure cycler to quickly switch between high and low pressures. Depending on what was to be observed, the cycler was switched to either synchronize the excitation flash of the PQM system so that data were recorded only when the bag was inflated (specifically during the oxygen consumption study) or to inflate the air-bag independently of the flash. To minimize oxygen inflow while the study was being performed, the bag was inflated to a basal 5 mmHg and, to decrease the amount of friction between the two film pieces in contact, a small drop of a water-based lubricant (KY brand) was added between the two film pieces to allow easy movement when changing measurement sites. The bag was inflated to 140 mmHg within <1sec to rapidly stop blood flow and initiate ischemia and was deflated back to 5 mmHg to restore blood flow, also within <1sec.
Figure 6. Components of the air-bag unit (from left to right): Krehalon film, 20x/0.45 objective, grommet, blunt needle, O-ring, electrical tape.
Figure 7. Flash-synchronized Automatic Pressure Cycler.
Figure 8. Completed stop-flow device on microscope.
Phosphorescence Quenching Microscopy

The method of phosphorescence quenching microscopy consisted of an Axioplan 2 intravital microscope (Zeiss, Germany) constructed with other optical equipment on an optical breadboard (Edmund Optics, Barrington, NJ). To observe changes in arteriolar diameter, the microscope was equipped with a video camera (MTI CCD72, Dage-MTI, Michigan City, IN) and a customized photomultiplier unit to register the phosphorescence signal. The phosphorescence signal was gathered by an Achromplan 20x/0.45 objective (Zeiss, Germany) and sent to an analog-to-digital converter (SCB-68, National Instruments, Austin, TX) to be analyzed with custom software written in LabVIEW (National Instruments, Austin, TX). To limit the contribution of oxygen inflow to the interstitial PO$_2$ within the illuminated tissue disc, a flash area of 600 µm was used. Perivascular PO$_2$ measurements were also taken to represent the arteriolar PO$_2$, a technique that previous data (Golub et al., 2011b) had shown to support. The flash area was decreased to 65 µm and oriented to the center of the vessel to limit the contribution of oxygen inflow from the adjacent interstitium and ensure that the phosphorescence signal came only from the region encompassed by the arteriole. In both cases of oxygen tension measurements, a xenon flash lamp (FX-249, PerkinElmer, Salem MA) was used to excite the area with an output of 167 mJ/µs at a rate of 1 Hz and 3 µs flash duration. Phosphorescence decay curves (500 data points per curve) were sampled at a rate of 500 kHz and were analyzed by fitting curves nonlinearly with the following rectangular PO$_2$ distribution model (Golub et al., 1997):

$$I(t) = I(0) \exp \left[ - (K_0 + K_q M) t \right] \cdot \sinh\left( K_q \delta t \right) / (K_q \delta t) + A \exp (-t/T) + B$$  \hspace{1cm} \text{Eq. 2}

where $t$ (µs) is the time of phosphorescence decay, $I(t)$ (V) is the magnitude of the phosphorescence signal, $I(0)$ (V) is the magnitude of the phosphorescence signal at $t = 0$, $M$ (mmHg) is the mean PO$_2$, $\delta$ (mmHg) is the half-width of the PO$_2$ distribution, $T$ (µs) is the
lifetime of the fast post-excitation transient, and \( B (V) \) is the baseline offset. \( k_0 \), the phosphorescence decay rate in the absence of oxygen, and \( k_q \), the quenching coefficient for the oxygen probe used in this study were \( 18.3 \times 10^{-4} \mu \text{s}^{-1} \) and \( 3.06 \times 10^{-4} \mu \text{s}^{-1} \text{ mmHg}^{-1} \), respectively (Zheng et al., 1996). All experiments were performed in a dark room.

Pd-MTCP O₂ Probe Preparation and Administration:

The oxygen-sensitive phosphorescent probe, palladium meso-tetra-(4-carboxyphenyl)-porphyrin (Pd-MTCP, Oxygen Enterprises, Philadelphia, PA), was bound to bovine serum albumin (Vanderkooi et al., 1987). A topical application of the probe solution was given to the surface of the spinotrapezius muscle for 45 minutes using 41 grade filter paper (Whatman). The method of application chosen provided sufficient penetration of the probe through the interstitial fluid throughout the tissue and prevented intravascular and intracellular penetration (Golub et al., 2007).
**Oxygen Consumption Measurements**

PO$_2$ in the interstitium is a function of both oxygen inflow and oxygen consumption. In normal, resting tissue, there is a balance between these parameters brought about by various regulatory systems such that the interstitial PO$_2$ (P$_{ISF}$O$_2$) represents the balance between the oxygen supply from the incoming arterioles and the oxygen consumption of the respiring tissue. When an imbalance occurs in this sort of equilibrium, a shift in the interstitial PO$_2$ will occur and by temporarily eliminating the supply of oxygen (e.g., occlusion of blood supply), the rate at which the interstitial PO$_2$ falls becomes a function of the consumption of oxygen by the tissue and the methodology. By correcting for the consumption by the PQM method using alternating excitation frequencies coupled with flow arrest (Golub & Pittman, 2008), the rate at which the tissue consumes oxygen over a given period of time can be determined (denoted by $\dot{V}_{O_2}$).

The interstitial PO$_2$ measured within the illuminated excitation disk formed by the phosphorescence quenching system is determined by three variables: the tissue oxygen consumption, $\dot{V}_{O_2}$; the oxygen consumption by the method itself; and the diffusive supply of oxygen from the tissue surrounding the disk. Utilizing the stop-flow device, the rate at which the PO$_2$ falls within five seconds can be ascribed to the rate at which the tissue is consuming oxygen, as the inflow of oxygenated blood has been halted. The following equation describes this (Golub et al., 2010):

\[
\frac{dP_n}{dn} = -V_n - KP_n + Z(p_n - P_n)
\]

Eq. 3

where $P_n$ is the PO$_2$ after the $n^{th}$ flash and $\frac{dP_n}{dn}$ is the rate at which oxygen disappears after the $n^{th}$ flash. $K$ is the consumption of oxygen by the methodology and $Z$ is the coefficient that describes the driving force of the inflow of oxygen to the flashed region by passive diffusion from the surrounding volume. As the radius of excitation increases, the contribution of passive diffusion
of oxygen decreases and, because an octagonal area of excitation of 254558.44 \( \mu m^2 \) was utilized, \( Z \) was made to be significantly smaller than \( K \) so that its impact is negligible in the calculation of \( \frac{dP_n}{dn} \) and can be set to be zero. \( V_n \) is the amount of oxygen consumed by the tissue for the \( n^{th} \) flash. Due to the insignificant contribution of \( Z \), the equation can be simplified to describe the initial rate at which oxygen is consumed:

\[
V_0 = -\frac{dP_n}{dn} - KP_n
\]

Eq. 4

The rate at which oxygen is consumed can now be attributed to the rate at which the tissue consumes oxygen and the consumption by the methodology. Once \( KP_n \) is compensated for, the equation can be further simplified to measure the rate at which the tissue consumes oxygen:

\[
\dot{V}_{O_2} = V_0 F \alpha
\]

Eq. 5

where \( F \) is the rate which the phosphor is being excited and \( \alpha \) is the solubility of oxygen within skeletal muscle (Mahler et al., 1985). By reinserting the rate at which oxygen is consumed by both the tissue and the method, a complete equation that describes the rate at which the tissue consumes oxygen can be made:

\[
\dot{V}_{O_2} = \left( -\frac{dP_n}{dn} - KP_n \right) F \alpha
\]

Eq. 6

By utilizing a quasi-continuous method for determining the rate of tissue oxygen consumption that consists of 5 seconds of bag inflation and 15 seconds of deflation, the time course at which the \( \dot{V}_{O_2} \) changes following an ischemia period can be determined, in addition to the rate at which the tissue is consuming oxygen at the beginning of an ischemic period. An example of this methodology is shown in Figures 9 and 10.
Figure 9. Example PO$_2$/VO$_2$ graph, 30 seconds of ischemia and 200 seconds of reperfusion.
Figure 10. Example PO$_2$/VO$_2$ graph, 10 minutes of ischemia and 15 minutes of reperfusion.
Flow measurements

The flow (Q) of red blood cells is important for the oxygenation of tissue. In a vessel, it is typically calculated as the product of average red cell velocity and the cross-sectional area of the vessel, as described by the following:

\[ Q = V_{avg} \cdot CSA_{vessel} \]  \hspace{1cm} \text{Eq. 7}

The flow through a vessel of radius, R, can be calculated as the integral of the product of the velocity at a certain radius, r, and the cross-sectional area over the entire radius of the lumen:

\[ Q = \int Q_{ring} = \int_0^R v(r)2\pi rdr \]  \hspace{1cm} \text{Eq. 8}

Assuming that red cell velocities have a parabolic Poiseuille dependence, the equation for v(r) that describes this can be obtained with the bluntness factor, B, that characterizes the velocity profile in the circular lumen (Pittman & Ellsworth, 1986):

\[ v(r) = v_0 \left[ 1 - B \left( \frac{r^2}{R^2} \right) \right] \]  \hspace{1cm} \text{Eq. 9}

where \( v_0 \) is the velocity along the central line. Combining v(r) and the volumetric flow rate integral to determine the average velocity over the lumen, we get:

\[ V_{avg} = \frac{\int_0^R v_0 \left[ 1 - B \left( \frac{r^2}{R^2} \right) \right] 2\pi rdr}{\pi R^2} = v_0 \left( 1 - \frac{B}{2} \right) \]  \hspace{1cm} \text{Eq. 10}

Through the use of an Optical Doppler Velocimeter (ODV, Borders & Granger, 1984), the central velocity along the long axis of the vessel as recorded by the system (\( V_{ODV} \)) is typically converted to \( V_{avg} \) by using the Baker-Wayland velocity conversion ratio, k, of 1.6 (Baker & Wayland, 1974) and was confirmed by Davis (1987):

\[ V_{avg} = \frac{V_{ODV}}{k} = \frac{V_{ODV}}{1.6} \]  \hspace{1cm} \text{Eq. 11}
The conversion ratio however does not take into consideration the shape of the velocity profile and its dependence on the velocity within the vessel, the diameter of the measured vessel, and the hematocrit within the vessel (Pittman & Ellsworth, 1986). By utilizing a streak length method, Al-Khazraji et al (2012) were able to characterize the velocity conversion ratio as a function of the vessel diameter:

\[
\frac{v_{ODV}}{v_{avg}} = 0.0071 \cdot \text{diameter} + 1.15 \quad \text{Eq. 12}
\]

By combining this equation with the average velocity over the lumen, we can determine how the bluntness profile will look as a function of the vessel radius:

\[
B = \frac{0.0142r + 0.15}{0.0071r + 0.575} \quad \text{Eq. 13}
\]

Taking \(V_{ODV}(0)\) as the velocity given by the ODV and substituting this into the Baker-Wayland model, a more generalized case of velocity measured with a finite sensor width \(w\) and bluntness profile along the centerline, the predicted value for \(V_{ODV}\) is:

\[
V_{ODV}(0) = \frac{N(0, w, B)}{D(0, w, B)} \quad \text{Eq. 14}
\]

where

\[
N(0, w, B) = v_0 \cdot 2 \left[ \frac{\left(\frac{1}{2} \lambda (1 - \lambda^2)^{3/2} + \frac{1}{2} \sin^{-1} \lambda \right)}{B} + B \left(\frac{1}{2} \lambda (1 - \lambda^2)^{3/2} - \frac{1}{2} \lambda (1 - \lambda^2)^{1/2} - \frac{1}{2} \sin^{-1} \lambda \right) \right] \quad \text{Eq. 15}
\]

\[
D(0, w, B) = v_0 \cdot 2 \left[ \frac{\left(\frac{1}{2} \lambda (1 - \lambda^2)^{1/2} + \frac{1}{2} \sin^{-1} \lambda \right)}{B} + B \left(\frac{1}{2} \lambda (1 - \lambda^2)^{1/2} - \frac{1}{4} \lambda (1 - \lambda^2)^{1/2} - \frac{1}{4} \sin^{-1} \lambda \right) \right] \quad \text{Eq. 16}
\]

and \(\lambda\) is the ratio of the fixed sensor width to the luminal diameter. By calculating the ratio of \(V_{ODV}\) and \(v_{avg}\) as a function of radius, the following equation is obtained for the conversion factor, \(k\):

\[
k = 0.00604r + 0.82705 \quad \text{Eq. 17}
\]
A more accurate determination of the average velocity and flow in a given vessel can now be made that corresponds to the velocity profile for vessels of diameter between 37 and 120 microns.
Compartmental Model of Q, VO$_2$ and P$_{ISF}$O$_2$

The regulation of tissue oxygenation is extremely important for cells in a tissue. The demand for oxygen must be met with a sufficient supply, otherwise an imbalance occurs in one direction or the other. Using Fick’s principle, the matching of oxygen supply and demand to the tissue oxygen consumption can be made (Pittman, 2011):

\[
\dot{V}_O = Q \cdot ([O_2]_in - [O_2]_{out}) \tag{Eq. 17}
\]

where \(\dot{V}_O\) is the oxygen consumption/demand by the tissue, Q is the incoming flow, \([O_2]_in\) is the concentration of oxygen that is coming into the vessel while \([O_2]_{out}\) is the concentration of oxygen that is leaving the vessel. This equation can be rearranged to show oxygen consumption as a function of the fraction of oxygen extracted from the incoming flow of oxygen:

\[
\dot{V}_O = Q \cdot [O_2]_in \cdot \left(\frac{[O_2]_in - [O_2]_{out}}{[O_2]_in}\right) \tag{Eq. 18}
\]

Consider a cylindrical blood vessel of length, L, that has flow, Q, and supplies oxygen to a nearby cylindrical sleeve of oxygen-consuming tissue (similar to the Krogh-cylinder model). The blood vessel and tissue are separated by interstitial fluid (ISF) that has a uniform partial pressure of oxygen. By equating the difference in convective flow of oxygen to the rate at which oxygen diffuses between the blood and the ISF, we get:

\[
Q\{[O_2(x + dx)] - [O_2(x)]\} = -\alpha D \left(\frac{2\pi R}{\Delta x}\right) (P_{O_2}(x) - P_{ISF}O_2) \, dx \tag{Eq. 19}
\]

where \(\alpha\) is the solubility of oxygen in the tissue, \(\frac{2\pi R}{\Delta x}\) is the surface area per unit length of vessel divided by the vessel wall thickness which can otherwise be denoted as \(\gamma\), and D is the coefficient of diffusion for oxygen at any position x along the vessel and the interstitial fluid.

The concentration of oxygen within the “linear” portion of the oxygen dissociation curve can be expressed as:
\[ [O_2] = \beta'[Hb]C_{Hb}P_{O_2} \quad \text{Eq. 20} \]

where \( \beta' \) is the slope of the “linear” region of the oxygen dissociation curve, \([Hb]\) is the concentration of hemoglobin within the blood, and \(C_{Hb}\) is the oxygen-binding capacity of hemoglobin. By substituting this back into the previous relationship with \(\beta'=\beta'C_{Hb}\), we get:

\[ Q\beta[Hb]\{P_{O_2}(x + dx) - P_{O_2}(x)\} = -aD\gamma(P_{O_2}(x) - P_{ISF}O_2)dx \quad \text{Eq. 21} \]

Rearranging this equation using the definition of the first derivative, we get the derivative of \(P_{O_2}\) which is defined as:

\[ \frac{dP_{O_2}}{dx} = -\left(\frac{aD\gamma}{Q\beta[Hb]}\right)(P_{O_2}(x) - P_{ISF}O_2) \quad \text{Eq. 22} \]

Rearranging this so it can be solved as a differential equation gives:

\[ \frac{dP_{O_2}}{(P_{O_2}(x) - P_{ISF}O_2)} = -\left(\frac{aD\gamma}{Q\beta[Hb]}\right)dx \quad \text{Eq. 23} \]

By integrating both sides with respect to the point at which blood enters vessel \((x=0)\) to the point at which the blood exits the vessel \((x=L)\), we get:

\[ \ln\left(\frac{P_{O_2}(x) - P_{ISF}O_2}{P_{O_2}(0) - P_{ISF}O_2}\right) = -\left(\frac{aD\gamma}{Q\beta[Hb]}\right)x \quad \text{Eq. 24} \]

To make \(x\) dimensionless, the right side can be multiplied by \(L/L\), turning \(\gamma = \frac{2\pi R}{\Delta x}\) to \(\Gamma = \frac{2\pi RL}{\Delta x}\) and we end up with:

\[ \ln\left(\frac{P_{O_2}(x) - P_{ISF}O_2}{P_{O_2}(0) - P_{ISF}O_2}\right) = -\left(\frac{aD\Gamma}{Q\beta[Hb]}\right)\frac{x}{L} \quad \text{Eq. 25} \]

The \(P_{O_2}\) as it is leaving the vessel \((x=L)\) can now be calculated as:

\[ P_{O_2}(L) = P_{ISF}O_2 + \{P_{O_2}(0) - P_{ISF}O_2\}e^{-\left(\frac{aD\Gamma}{Q\beta[Hb]}\right)} \quad \text{Eq. 26} \]

For all subsequent calculations, \(\frac{aD\Gamma}{Q\beta[Hb]}\) can be called \(z\). Substituting this as well as \([O_2] = \beta'[Hb]C_{Hb}P_{O_2} = \beta[Hb]P_{O_2}\) back into Fick’s equation for oxygen consumption, \(\dot{V}_{O_2}\) becomes:
\[ \dot{V}_{O_2} = Q \cdot \beta[Hb] \cdot \{P_{O_2}(0) - P_{O_2}(L)\} = Q \cdot \beta[Hb] \cdot (1 - e^{-z})[P_{O_2}(0) - P_{ISF O_2}] \]  

Eq. 27

By rearranging this equation to solve for \( P_{ISF O_2} \), we get:

\[ P_{ISF O_2} = P_{O_2}(0) - \frac{\dot{V}_{O_2}}{Q \cdot \beta[Hb] \cdot (1 - e^{-z})} \]  

Eq. 28

Eq. 28 can be rearranged in order to group \( P_{O_2}(0) \) and \( P_{ISF O_2} \) as \( \Delta P_{O_2} \) to get:

\[ \beta[Hb] \cdot (1 - e^{-z}) = \frac{\dot{V}_{O_2}}{Q \cdot \Delta P_{O_2}} \]  

Eq. 29

Under steady-state conditions, \( \beta[Hb] \cdot (1 - e^{-z}) \) should remain relatively constant and as such, can be defined as \( M \).

\[ M = \frac{\dot{V}_{O_2}}{Q \cdot \Delta P_{O_2}} \]  

Eq. 30

Since the VO\(_2\) and Q data were acquired separately, the average values, including \( \Delta P_{O_2} \), computed for the baseline of that corresponding ischemic duration were used. \( M \) can be computed to observe the consistency of its behavior—allowing a determination to be made of how a model can be ultimately fit.
Ischemia/Reperfusion Experimental Protocol

Ischemia/Reperfusion

To understand the regulatory mechanisms of blood flow and tissue oxygenation, ischemia was induced to by using a flash-synchronized, Automatic Dual Cycler that was attached to a pressurized airbag. The airbag rapidly inflated (<1 sec) to 140 mmHg over the tissue to initiate flow arrest and then rapidly deflated (<1 sec) to 5 mmHg to initiate reperfusion. Because of technical limitations, the three phases of the experiments were performed separately and, using information that was common to all three, the data were combined to be incorporated into the compartmental model. Well perfused sites were chosen that had an active capillary network and one arteriole running parallel with a preferred diameter between 40 and 90 µm.

\( P_{ISF}O_2 \) study:

Using the method of PQM, oxygen measurements were made in the interstitial fluid in a disk region of diameter 600 µm. The \( O_2 \)-sensitive probe was topically loaded for a period of no more than 45 minutes at which point the experiment began once the six sites were chosen. Data were recorded and analyzed with a sampling frequency of 1Hz using the methodology described under Phosphorescence Quenching Microscopy.

Q study:

The centerline red blood cell velocity was measured using an Optical Doppler Velocimeter \((V_{ODV})\) and, using the conversion factor that was calculated as a function of the vessel diameter, the velocity was converted to average flow. The data were recorded using a Biopac system at a sampling frequency of 1Hz to correspond with the sampling rate of the PQM system.
Perivascular baseline PO$_2$ measurements were also sampled with the PQM system by switching to an excitation disk region of diameter 65 µm.

VO$_2$ study:
By incorporating the Automatic Dual Cycler system programmed to switch between 5 seconds of inflation and flow stoppage of 15 seconds of deflation and recovery, a quasi-continuous method was used to observe dPO$_2$/dt. By incorporating the same PQM methodology, VO$_2$ was sampled every twenty seconds in the same disc region but, because of the 5 sec/15 sec limitation, baselines during the start of the experimental protocol were extended to get a minimum of three measurements. Due to the same limitation, reperfusion time was also extended to obtain at least three measurements. Based on the data collected during the P$_{ISF}$O$_2$ study, VO$_2$ sampling during the recovery began once the P$_{ISF}$O$_2$ > 20 mmHg so that the non-linear region during the very early part of reperfusion was avoided. Perivascular PO$_2$ values during baseline were also collected during the oxygen consumption study.

Protocol
Baseline values were collected for thirty seconds and in the case of VO$_2$, for sixty seconds; ischemia was initiated immediately. Ischemic durations of 5, 15, 30, 60, 300, and 600 seconds were chosen to sufficiently capture transient changes of the time course as a function of the ischemic duration during reperfusion. Reperfusion started immediately after the end of ischemia with data collected for each group at each site for about three times as long as the ischemic duration or 15-900 seconds. This ensured that any changes in the rate at which the tissue recovered could be observed. So that an experiment could be completed within a reasonable
amount of time and the risk of any anesthesia-related complications could be discounted, the 5 to
60 second and 300 to 600 second ischemic durations were done in separate animals. Within each
experiment, because of the inherent heterogeneity of the tissue, site-to-site variation was
accounted for by taking measurements at 3 locations for the shorter durations and 2 locations for
the longer durations per animal per study. Baseline $P_{ISF}O_2$ data was collected during each study
as a reference for comparison between the time courses of $P_{ISF}O_2$, $Q$ and $VO_2$. 

**Statistical Methods**

Data are presented as mean ± standard error (SEM) unless otherwise specified. ANOVA and Tukey-Kramer’s Multiple Comparison tests were conducted to assess statistical significance among the baseline values collected from different ischemic durations. Two-sample Student’s t-test was used to determine the significance among the baseline, the initial reperfusion phase, and the new steady-state established with respect to the baseline. It was also used to determine the significance of the relating constant, M, between the VO₂ and Q studies. All statistical calculations were made using Origin 7.0 and statistical significance was assigned if p <0.05.
Results

Although each study in this project is independent of the others, the results will be presented in the order in which they were conducted and analyzed in each ischemic group. PQM was used to measure the oxygen tension in the interstitial fluid of vascular beds during the transient P_{SF}O_2 study, the oxygen consumption study and the baseline of the flow study. This was done in conjunction with the pressurized stop-flow device for the designated ischemic and reperfusion durations as outlined in the protocol section. Perivascular PO_2 measurements were taken during the flow and oxygen consumption studies as this was used for the analysis of the factor designated, M. To show how one variable changed in relation to the other two, the data were normalized to the baseline and averaged over sites. This also compensated for the inherent site-to-site and animal-to-animal heterogeneity of the microcirculatory variables in the spinotrapezius muscle tissue from. Plots of the averages of the data collected are also presented.

To determine how the tissue responded to the I/R protocol, measurements were taken at one vascular bed (site) per animal for the first nine animals and three for the remaining two for 5- and 60-seconds of ischemia. For the 15- and 30-second groups, one site for the first ten animals and three for the remaining two were chosen. For 300-second ischemia group, one site was chosen for six animals and two sites for each of the remaining two animals. For the 600-second ischemia group, one site was chosen for five animals and two for each of the remaining two. After observing how the tissue responded by taking one measurement per site for multiple durations, three sites were chosen per animal for the 5 to 60 second groups and 2 sites per animal for 300 to 600-second ischemia groups. Sample size information given throughout the presentation describes the number of sites observed in that particular phase of the study.
The number of sites chosen per animal during the transient study was used thereafter for the flow and oxygen consumption studies. For the flow studies, sites were chosen that had well perfused vascular beds with a single arteriole between 50 to 100 µm inside diameter. The baseline $P_{ISF}O_2$’s were taken for the vascular bed. The average arteriole in the study was $80.0\pm12.4$ (SEM) µm diameter. Video sequence analysis was used to determine the diameter changes as the velocity measurements were taken. The $V_{ODV}$ was converted to flow using vessel diameter information as outlined in the Methodology section.

Since oxygen consumption measurements could only be taken once every twenty seconds, to get an accurate baseline established and observe changes post-ischemia, the baseline was extended to 60 seconds to get three measurements and the reperfusion times were extended to get a minimum of three measurements. The correction factor, $k$, was determined to be $3.5\cdot10^{-3}$ s$^{-1}$ and used to compensate for consumption by the method (see Methods section). The presentation of the VO$_2$ data was truncated to 870 sec since the analysis process determined that VO$_2$ did not change once it reached baseline post-ischemia for the duration of reperfusion.
5-second Ischemia

Transient P_{ISF}O_2 study

The average data described in Fig. 11 demonstrates that following five seconds of ischemia, the oxygen tension went from an average baseline of 65.8±4.2 mmHg to 75% (Fig. 12) or 49.1±3.0 mmHg before returning to baseline within 15 seconds.

Flow study

The average data described in Figs. 13 and 14 demonstrate that, following five seconds of flow stoppage, the blood flow returned to a baseline of 6.00±0.74 µl/min within three seconds.

VO_2 study

Since the method by which the oxygen consumption measurements were taken was the same as the duration of this protocol, it was expected that the oxygen consumption would not vary greatly from the baseline. Figure 15 shows that the oxygen consumption values never varied by more than 4% (Fig. 16) of an average baseline of 145.8±20.6 nl O_2/cm^3·s.
Figure 11. Transient $P_{ISF}O_2$ data for 5 seconds of ischemia. Data presented as Means ± SEM with n=15.
Figure 12. Normalized transient $P_{\text{ISF}O_2}$ data for 5 seconds of ischemia. Baseline $P_{\text{ISF}O_2}=65.8\pm4.2$ mmHg. Data presented as Means ± SEM with n=15.
Figure 13. Flow data for 5 seconds of ischemia. Data presented as Means ± SEM with n=12.
Figure 14. Normalized flow data for 5 seconds of ischemia. Baseline flow=6.00±0.74 µl/min.

Data presented as Means ± SEM with n=12.
Figure 15. Oxygen consumption data for 5 seconds of ischemia. Data presented as Means ± SEM with n=12. I represents start of ischemia; R represents start of reperfusion.
Figure 16. Normalized oxygen consumption data for 5 seconds of ischemia. Baseline VO$_2$=145.8±20.6 nl O$_2$/cm$^3$·s. Data presented as Means ± SEM with n=12. I represents start of ischemia; R represents start of reperfusion.
**15-second Ischemia**

Transient $P_{\text{ISF}}O_2$ study

The average data described in Fig. 17 demonstrate that following fifteen seconds of ischemia, the interstitial oxygen tension went from an average baseline of $61.9\pm3.7$ mmHg to $34\%$ (Fig. 18) or $21.0\pm2.8$ mmHg before returning to baseline within fifteen seconds.

Flow study

The average data described in Fig. 19 demonstrate that following fifteen seconds of flow stoppage, the blood flow increased up to $10\%$ (Fig. 20) or $5.80\pm0.65 \mu l/min$ for the first 25 seconds before returning to a baseline of $5.38\pm0.62 \mu l/min$.

$VO_2$ study

The average data described in Fig. 21 demonstrates that following fifteen seconds of ischemia, tissue oxygen consumption increased up to $122\%$ or $136.5\pm20.1$ nl $O_2/cm^3\cdot s$ for 20 seconds before returning to an average baseline of $124.6\pm18.5$ nl $O_2/cm^3\cdot s$. 
Figure 17. Transient $P_{ISF}O_2$ data for 15 seconds of ischemia. Data presented as Means ± SEM with n=16.
Figure 18. Normalized transient $P_{\text{ISF}O_2}$ data for 15 seconds of ischemia. Baseline

$P_{\text{ISF}O_2}=61.9\pm3.7$ mmHg. Data presented as Means ± SEM with n=16.
Figure 19. Flow data for 15 seconds of ischemia. Data presented as Means ± SEM with n=12.
Figure 20. Normalized flow data for 15 seconds of Ischemia. Baseline blood flow=5.38±0.62 µl/min. Data presented as Means ± SEM with n=12.
Figure 21. Oxygen consumption data for 15 seconds of ischemia. Data presented as Means ± SEM with n=12. I represents start of ischemia; R represents start of reperfusion.
Figure 22. Normalized oxygen consumption data for 15 seconds of ischemia. Baseline VO$_2$=124.6±18.5 nl O$_2$/cm$^3$.s. Data presented as Means ± SEM with n=12. I represents start of ischemia; R represents start of reperfusion.
**30-second Ischemia**

Transient $P_{ISF}O_2$ study

The average data described in Fig. 23 demonstrates that following thirty seconds of ischemia, the oxygen tension went from an average baseline of 63.5±3.8 mmHg to 10% (Fig. 24) or 5.8±1.2 mmHg before returning to baseline within thirty seconds.

Flow study

The average data described in Figs. 25 and 26 demonstrate that following thirty seconds of flow stoppage, the blood flow came back to a baseline of 6.10±0.92 µl/min within eleven seconds.

$VO_2$ study

The average data described in Fig. 27 demonstrate that following thirty seconds of ischemia, tissue oxygen consumption increased up to 141% (Fig. 28) or 134.2±19.0 nl O$_2$/cm$^3$·s and within twenty seconds returned to an average baseline of 97.6±13.5 nl O$_2$/cm$^3$·s.
Figure 23. Transient $P_{ISF}O_2$ data for 30 seconds of ischemia. Data presented as Means ± SEM with n=16.
**Figure 24.** Normalized transient $P_{ISF}$ data for 30 seconds of ischemia. Baseline $P_{ISF}=63.5\pm3.8$ mmHg. Data presented as Means ± SEM with $n=16$. 


Figure 25. Flow data for 30 seconds of ischemia. Data presented as Means ± SEM with n=12.
Figure 26. Normalized flow data for 30 seconds of ischemia. Baseline blood flow=6.10±0.92 μl/min. Data presented as Means ± SEM with n=12.
Figure 27. Oxygen consumption data for 30 seconds of ischemia. Data presented as Means ± SEM with n=12. I represents start of ischemia; R represents start of reperfusion.
**Figure 28.** Normalized oxygen consumption data for 30 seconds of ischemia. Baseline \( \text{VO}_2 = 97.6 \pm 13.5 \, \text{nl O}_2/\text{cm}^3 \cdot \text{s} \). Data presented as Means ± SEM with \( n=12 \). I represents start of ischemia; R represents start of reperfusion.
60-second Ischemia

Transient $P_{ISF}O_2$ study
The average data described in Fig. 29 demonstrate that following sixty seconds of ischemia, the oxygen tension went from an average baseline of 58.5±3.8 mmHg to 2% (Fig. 30) or 1.5±0.2 mmHg before returning to baseline within sixty seconds.

Flow study
The average data described in Figs. 31 and 32 demonstrate that following sixty seconds of flow stoppage, the blood flow came back to a baseline of 5.78±0.84 µl/min within seven seconds.

VO$_2$ study
The average data described in Fig. 33 demonstrate that following sixty seconds of ischemia, tissue oxygen consumption increased up to 125% (Fig. 34) or 145.7±23.0 nl O$_2$/cm$^3$·s and within sixty seconds return to an average baseline of 110.4±16.4 nl O$_2$/cm$^3$·s.
Figure 29. Transient $P_{ISF}O_2$ data for 60 seconds of ischemia. Data presented as Means ± SEM with $n=15$. 
Figure 30. Normalized transient $P_{\text{ISF}O_2}$ data for 60 seconds of ischemia. Baseline $P_{\text{ISF}O_2}=58.5\pm3.8$ mmHg. Data presented as Means ± SEM with n=15.
Figure 31. Flow data for 60 seconds of ischemia. Data presented as Means ± SEM with n=12.
**Figure 32.** Normalized flow data for 60 seconds of ischemia. Baseline flow=5.78±0.84 µl/min.

Data presented as Means ± SEM with n=12.
Figure 33. Oxygen consumption data for 60 seconds of ischemia. Data presented as Means ± SEM with n=12. I represents start of ischemia; R represents start of reperfusion.
Figure 34. Normalized oxygen consumption data for 60 seconds of ischemia. Baseline

\[ \text{VO}_2 = 110.4 \pm 16.4 \text{ nl O}_2/\text{cm}^3 \cdot \text{s} \]. Data presented as Means ± SEM with n=12. I represents start of ischemia; R represents start of reperfusion.
300-second Ischemia

Transient $P_{ISF}O_2$ study

The average data described in Fig. 35 demonstrate that after reaching 2% of its baseline following 300 seconds of ischemia, the oxygen tension returned to an average baseline of 62.9±3.9 mmHg (Fig. 36) within 250 seconds.

Flow study

The average data described in Figs. 37 and 38 demonstrates that following 300 seconds of flow stoppage, the blood flow came back to an average baseline of 4.8±1.6 µl/min within thirty seconds.

$VO_2$ study

The average data described in Fig. 39 demonstrates that following 300 seconds of ischemia, tissue oxygen consumption increased up to 190% (Fig. 40) or $294.8±48.1$ nl O$_2$/cm$^3$.s and within 80 seconds returned to an average baseline of $176.1±35.5$ nl O$_2$/cm$^3$.s.
Figure 35. Transient $P_{ISF O_2}$ data for 300 seconds of ischemia. Data presented as Means ± SEM with $n=10$. 
Figure 36. Normalized transient $P_{ISF O_2}$ data for 300 seconds of ischemia. Baseline

$P_{ISF O_2}=62.9\pm 3.9$ mmHg. Data presented as Means ± SEM with $n=10$. 
Figure 37. Flow data for 300 seconds of ischemia. Data presented as Means ± SEM with n=8.
Figure 38. Normalized flow data for 300 seconds of ischemia. Baseline flow=4.8±1.6 µl/min.

Data presented as Means ± SEM with n=8.
Figure 39. Oxygen consumption data for 300 seconds of ischemia. Data presented as Means ± SEM with n=8. I represents start of ischemia; R represents start of reperfusion.
Figure 40. Normalized oxygen consumption data for 300 seconds of ischemia. Baseline VO$_2$=176.1±35.5 nl O$_2$/cm$^3$.s. Data presented as Means ± SEM with n=8. I represents start of ischemia; R represents start of reperfusion.
600-second Ischemia

Transient $P_{ISF}O_2$ study

The average data described in Fig. 41 demonstrates that following 600 seconds of ischemia, the oxygen tension went from an average baseline of $58.6\pm7.61$ mmHg to $3\%$ or $1.3\pm0.4$ mmHg (Fig. 42) and returned within 160 seconds.

Flow study

The average data described in Figs. 43 and 44 demonstrates that following 600 seconds of flow stoppage, the blood flow came back to a baseline of $4.35\pm0.42\ \mu l/min$ within fifteen seconds.

$VO_2$ study

The average data described in Fig. 45 demonstrates that following 600 seconds of ischemia, tissue oxygen consumption increased up to $254\%$ (Fig. 46) or $309.2\pm45.0$ nl $O_2/cm^3\cdot s$ and within 120 seconds return to an average baseline of $147.4\pm67.2$ nl $O_2/cm^3\cdot s$. 
Figure 41. Transient $P_{\text{ISF}O_2}$ data for 600 seconds of ischemia. Data presented as Means ± SEM with n=9.
Figure 42. Normalized transient $P_{\text{ISF}O_2}$ data for 600 seconds of ischemia. Baseline $P_{\text{ISF}O_2}=58.6 \pm 7.6$ mmHg. Data presented as Means ± SEM with n=9.
Figure 43. Flow data for 600 seconds of ischemia. Data presented as Means ± SEM with n=8.
Figure 44. Normalized flow data for 600 seconds of ischemia. Baseline flow=4.35±0.42 µl/min. Data presented as Means ± SEM with n=8.
Figure 45. Oxygen consumption data for 600 seconds of ischemia. Data presented as Means ± SEM with n=8. I represents start of ischemia; R represents start of reperfusion.
Figure 46. Normalized oxygen consumption data for 600 seconds of ischemia. Baseline \( \text{VO}_2 = 147.4 \pm 67.2 \, \text{nl O}_2/\text{cm}^3 \cdot \text{s} \). Data presented as Means ± SEM with \( n=8 \). I represents start of ischemia; R represents start of reperfusion.
Baseline Analysis

A multivariate ANOVA demonstrated that the average baseline $P_{ISF}O_2$ among the three studies and the six durations were not different ($F=2.82, p=0.06$). There were no significant differences among the average baseline $P_{ISF}O_2$ values of the Transient $P_{ISF}O_2$, VO$_2$, and flow studies ($F=2.82, p=0.06$); there were no significant differences among the average baseline $P_{ISF}O_2$ values of the ischemic durations within each study ($F=1.31, p=0.26$); the same ischemic durations among the studies also demonstrated that there were no significant differences ($F=0.33, p=0.97$) in $P_{ISF}O_2$. Tukey-Kramer’s tests also demonstrated that there were no differences in the effect of ischemic duration or study on the $P_{ISF}O_2$ ($p >0.05$). A one way ANOVA performed on the baseline blood flow data during the Q study demonstrated that there was no significant difference among the baseline flow values of each ischemic duration ($F=0.68, p=0.64$). Tukey’s HSD determined that there were no significant differences in average baseline flow values between any two ischemic groups. A one way ANOVA performed on the baseline oxygen consumption data during the VO$_2$ study demonstrated that there was no significant difference among the baseline consumption values of each ischemic duration ($F=1.77, p=0.13$). Tukey’s HSD determined that there were no significant differences in baseline consumption values between any two ischemic groups.

Baseline correlations to $P_{ISF}O_2$:

During the VO$_2$ study, in addition to baseline $P_{ISF}O_2$ data being collected, baseline VO$_2$ data were collected as well. To test whether there was a correlation between the two, oxygen consumption was plotted against the $P_{ISF}O_2$ as shown in Fig. 47. A regression slope test using Student’s $t$ determined that the slope of the oxygen consumption as a function of the baseline
interstitial fluid PO$_2$ was significant (p=0.006). During the blood flow study, baseline flow measurements were plotted against the baseline P$_{ISF}$O$_2$ measurements (Fig. 48) collected during the study to determine if there was a relationship between the two. A regression slope test determined that the slope of the baseline blood flow as a function of the baseline P$_{ISF}$O$_2$ was not significant (p=0.75).
Figure 47. Baseline oxygen consumption versus baseline interstitial fluid PO$_2$ during the VO$_2$ study (p=0.0596 <0.05).
Figure 48. Baseline blood flow versus baseline interstitial fluid PO$_2$ during the blood flow study (p=0.74742 >0.05).
Table 1. Average baseline data for $P_{ISF}O_2$, $Q$, $VO_2$ studies and factor $M$; all data expressed as Means ± SEM. Units: $PO_2$ in mmHg, $Q$--µl/min, $VO_2$--nl $O_2$/cm$^3$·s, $M$--nl $O_2$/nl$^2$·mmHg

<table>
<thead>
<tr>
<th>Parameter</th>
<th>5 sec</th>
<th>15 sec</th>
<th>30 sec</th>
<th>60 sec</th>
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<tr>
<td>Baseline $P_{ISF}O_2$</td>
<td>15 65.78 ± 4.23</td>
<td>16 61.89 ± 3.73</td>
<td>16 63.49 ± 3.75</td>
<td>15 58.50 ± 3.84</td>
<td>10 62.92 ± 3.87</td>
<td>9 58.60 ± 7.62</td>
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<tr>
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<td>12 5.38 ± 0.62</td>
<td>12 6.10 ± 0.92</td>
<td>12 5.78 ± 0.84</td>
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<td>12 63.53 ± 4.09</td>
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<td>12 11.80 ± 2.17</td>
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<tr>
<td>Baseline $VO_2$</td>
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<td>12 97.55 ± 13.54</td>
<td>12 110.36 ± 16.43</td>
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<tr>
<td>Baseline $P_{Art}O_2$</td>
<td>12 68.81 ± 2.50</td>
<td>12 74.19 ± 3.29</td>
<td>12 68.92 ± 3.46</td>
<td>12 68.92 ± 3.04</td>
<td>8 67.64 ± 3.96</td>
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<td>Baseline $P_{ISF}O_2$</td>
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<td>12 61.58 ± 4.30</td>
<td>12 53.92 ± 4.23</td>
<td>12 57.61 ± 4.09</td>
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<tr>
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<td>12 12.61 ± 1.89</td>
<td>12 14.99 ± 2.55</td>
<td>12 11.30 ± 1.86</td>
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<td>8 19.96 ± 4.50</td>
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<tr>
<td><strong>M</strong></td>
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<tr>
<td>From Q study data</td>
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<tr>
<td>From $VO_2$ study data</td>
<td>12 5.44 ± 1.51</td>
<td>12 2.21 ± 0.37</td>
<td>12 1.52 ± 0.35</td>
<td>12 2.36 ± 0.54</td>
<td>8 2.94 ± 0.79</td>
<td>8 3.08 ± 1.34</td>
</tr>
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</table>
Initial Recovery Rates during Reperfusion

The initial rate of recovery for the three variables in each of the ischemic durations within each study was calculated from the $P_{ISF}O_2$ data for the Transient $P_{ISF}O_2$ and $VO_2$ studies—$Q$ could not be measured simultaneously. Only the initial rate of flow recovery was calculated for the $Q$ studies since $PO_2$ could not be recorded simultaneously.

Transient $P_{ISF}O_2$

The average initial rate of recovery of $P_{ISF}O_2$ (Fig. 49) for 5 seconds of ischemia was $0.84\pm0.12\text{ mmHg/s}$, $3.09\pm0.36\text{ mmHg/s}$ for 15 seconds, $4.25\pm0.72\text{ mmHg/s}$ for 30 seconds, $3.25\pm0.42\text{ mmHg/s}$ for 60 seconds, $2.95\pm0.49\text{ mmHg/s}$ for 300 seconds and $1.00\pm0.16\text{ mmHg/s}$ for 600 seconds of ischemia. A one-way ANOVA demonstrated that the initial rates of recovery among the ischemic durations were significantly different ($F=15.19$, $p=3.92\cdot10^{-10}$). The initial rates of reperfusion during 5 seconds of ischemia were significantly different than 15, 30, 60 and 300 seconds of ischemia ($p <0.05$). The initial rates of recovery following 600 seconds of ischemia were significantly different from the rates during 30 and 300 seconds of ischemia.

Blood Flow

The average initial rate of recovery of flow during the blood flow study (Fig. 50) for 5 seconds of ischemia was $3.00\pm0.32\text{ (µl·min}^{-1}\text{/s)}$, $3.91\pm0.52\text{ (µl·min}^{-1}\text{/s)}$ for 15 seconds, $4.91\pm0.45\text{ (µl·min}^{-1}\text{/s)}$ for 30 seconds, $7.04\pm0.55\text{ (µl·min}^{-1}\text{/s)}$ for 60 seconds, $2.83\pm0.55\text{ (µl·min}^{-1}\text{/s)}$ for 300 seconds and $2.92\pm0.45\text{ (µl·min}^{-1}\text{/s)}$ for 600 seconds of ischemia. A one-way ANOVA demonstrated that the initial rates of blood flow recovery during reperfusion among the ischemic durations were significantly different ($F=12.02$, $p=5.30\cdot10^{-8}$). The initial rate of blood flow
recovery following 5 seconds of ischemia was significantly different from that of the 30 and 60 second ischemia groups (p <0.05). The initial rate of blood flow following 60 seconds of ischemia was significantly different from that of the 15-, 300- and 600-second groups (p <0.05).

Recovery of P_{ISF}O_{2} in VO_{2} study

The average initial rate of reperfusion of the P_{ISF}O_{2} in the interstitial space during the oxygen consumption study (Fig. 51) for 5 seconds of ischemia was 0.82±0.13 mmHg/s, 4.18±0.69 mmHg/s for 15 seconds, 6.23±0.68 mmHg/s for 30 seconds, 7.45±0.83 mmHg/s for 60 seconds, 3.09±0.41 mmHg/s for 300 seconds and 1.05±0.33 mmHg/s for 600 seconds of ischemia. A one-way ANOVA demonstrated that the initial rates of recovery among the ischemic durations were significantly different (F=21.40, p=2.73·10^{-11}). The initial rates of recovery following 5 seconds of ischemia were significantly different from those for 15, 30, 60 and 300 seconds of ischemia (p <0.05). The initial rates of reperfusion during 600 seconds of ischemia were significantly different than the rates during 15, 30 and 60 seconds of ischemia (p <0.05).
Figure 49. Initial rates of recovery of $P_{\text{ISF}O_2}$ during reperfusion of the Transient $P_{\text{ISF}O_2}$ (PO$_2$ <20 mmHg) study. Data presented as Means ± SEM. *Significance: Letter and/or color pairs indicate that two groups they are above are significantly (p <0.05) different; i.e., AA—5- and 300-second ischemic durations have significantly (p <0.05) different initial recovery rates.
Figure 50. Initial rate of recovery of flow during reperfusion of the Blood Flow study. Data presented as Means ± SEM. *Significance: Letter and/or color pairs indicate that two groups they are above are significantly (p <0.05) different; i.e., AA—5- and 30-second ischemic durations have significantly (p <0.05) different initial recovery rates.
Figure 51. Initial rate of recovery of $P_{\text{ISF}}O_2 (<20 \text{ mmHg})$ during reperfusion of the Oxygen Consumption study. Data presented as Means ± SEM. *Significance: Letter and/or color pairs indicate that two groups they are above are significantly ($p < 0.05$) different; i.e., AA—5- and 60-second ischemic durations have significantly ($p < 0.05$) different initial recovery rates.
Percent increase in VO\textsubscript{2} during reperfusion

A one-way ANOVA and Tukey’s HSD on the post-ischemia phase of the oxygen consumption study demonstrated that there significant differences in the consumptions between the groups (F=9.61, p=1.02·10\textsuperscript{-6}). The earliest measurement of oxygen consumption during reperfusion following 300 seconds of ischemia (Fig. 52) was significantly higher (up to 294.8±48.1 nl O\textsubscript{2}/cm\textsuperscript{3}·s) than that for 5, 15, 30 and 60 seconds of ischemia (p <0.05). Oxygen consumption during 600 seconds of ischemia (Fig. 52) was also significantly higher (up to 309.2±45.1 nl O\textsubscript{2}/cm\textsuperscript{3}·s) than the same group of durations but there was no statistically significant difference between the oxygen consumptions of the 300 and 600 second ischemia groups.
Figure 52. Percent of VO$_2$ baseline at the earliest measurement of reperfusion. 30 sec ischemia, 60 sec ischemia, 300 sec ischemia, 600 sec ischemia delayed by 3, 5, 20 and 20 seconds respectively from the start of reperfusion. Data presented as Means ± SEM. *Significantly different from 5-60 seconds of ischemia groups (p <0.05).
Relating VO$_2$, Q and P$_{ISF}$O$_2$ with the factor, M

The factor M was calculated for the baseline values of the oxygen consumption and blood flow data using Equation 30. The average value for M during the baseline of the blood flow study was 2.72±1.31 nl O$_2$/nl$^2$·mmHg. For the oxygen consumption study, the average value of M was 2.15±1.16 nl O$_2$/nl$^2$·mmHg. This demonstrated a relatively constant relationship between oxygen consumption and blood flow during the baseline period.

Since the VO$_2$ data limited how much of the duration could be characterized by M, corresponding normalized values from the VO$_2$ and Q studies at the same point in each experiment and ischemic duration were combined and plotted as a function of time. The Transient P$_{ISF}$O$_2$ study did not have P$_a$O$_2$ available so the information for ΔPO$_2$ was taken and normalized using the P$_{ISF}$O$_2$ and P$_a$O$_2$ data from the VO$_2$ study. This allowed two variables to come from the exact same site, animal, and study. Flow data was added from the Blood Flow study. The errors of each of the variables obtained was propagated and M was computed.

Figs. 53 & 54 report the factor M collected for 5-, 15-, 30-, 60-, 300- and 600-second ischemia groups. Since the data was normalized to the baseline, all points graphed began at 1.0. The 5-60 second groups noticeably recovered to baseline within 180 seconds while the 300- and 600-second ischemia groups demonstrated large changes during that time. 5 seconds of ischemia demonstrated a slower decline back to the baseline but enough data points were not collected to observe it over a longer stretch of time. 300 seconds of ischemia demonstrated the most change from the baseline and did not recover in the same manner and rate as the 600-second ischemia group.
Figure 53. Factor M calculated for 5-, 15-, 30- and 60-second ischemia groups using the normalized data. Data presented as Means ± SEM.
Figure 54. Factor M Factor M calculated for the 300- and 600-second ischemia group using the normalized data. Data presented as Means ± SEM.
Discussion

The Transient P_{ISF}O_{2} study demonstrated that although there wasn’t a significant increase in interstitial oxygen tension at the onset of reperfusion, the time course over which the P_{ISF}O_{2} returned to baseline increased as the ischemic duration increased. This occurred over fifteen seconds for 5 seconds of ischemia to 250 seconds for 300 seconds of ischemia. The blood flow study data collected has demonstrated a similar pattern to the Transient P_{ISF}O_{2} in that as the ischemic duration increased, the time course over which the flow returned to baseline following ischemia increased. However, just as it was with the Transient P_{ISF}O_{2} study, the 300sec-ischemic group had a much longer time course (30 seconds) than the 600-second ischemia group (15 second time course) over which the blood flow recovered to baseline. VO_{2} data indicated that peak oxygen consumption increased as a direct result of the ischemic duration. VO_{2} peaked at 309.2±45.0 nl O_{2}/cm^{3}·s or 254% of its baseline and recovered within 120 seconds. As the ischemic duration increased, the time course over which the VO_{2} recovered following ischemia also increased and ranged from 20 seconds following the 15-second ischemia group to 120 seconds following the 600-second ischemia group. The VO_{2} and Q data was then incorporated into finding an approximation for the factor M as a function of time in each site of every ischemic duration.

Blood flow in the cardiovascular system and microcirculation is tightly regulated to ensure that an adequate oxygen supply is available to respiring tissue as needed. When this balance in supply and demand is interrupted at a global level—through increased oxygen consumption by the tissue or decreased blood supply—regulatory mechanisms act by increasing ventilation, heart rate, stroke volume, sympathetic activity and circulating concentrations of vasoactive hormones. Although these systemic effects will act to ensure that the supply of
oxygen to the tissue at any given time is increased to the appropriate level, the microcirculation and mechanisms which control local blood flow will ultimately determine whether the tissue receives what it needs.

This study attempted to establish a relationship between three intrinsic characteristics of the local regulation of tissue oxygenation: flow of oxygenated blood (QO$_2$), the oxygenation of the tissue as reported by P$_{\text{ISF}}$O$_2$, as well as the rate of oxygen consumption by the tissue (VO$_2$). It should be noted that, although this work helps to understand how these variables are related to one another, the results themselves cannot explain the mechanism(s) by which local blood flow and tissue oxygenation are regulated. Rather, by defining the limits of change in one parameter with respect to the other two, any proposed mechanism can be tested and, if the results deviate significantly from the defined limits, it can be reasoned that an alternative mechanism should be considered or that there are additional variables at work.

Due to technical design limitations, the three variables were observed in independent studies: the Transient P$_{\text{ISF}}$O$_2$, Oxygen Consumption (VO$_2$) and Blood Flow (Q) studies. Since that was the case, P$_{\text{ISF}}$O$_2$ measurements were made during the baseline at each site in each study to be used later as the basis for comparison among the studies. If the values were statistically similar, then it could be assumed that the values found during the studies originated from similar starting conditions; despite having site-to-site and animal-to-animal heterogeneity.
The Baselines

Baseline measurements at each site were made for a period of 30 to 60 seconds (for VO₂ study) prior to the onset of ischemia and reperfusion and, as such, should have been similar throughout all of the experiments. A comparison of the baseline P₄SfO₂ measurements for each ischemic duration averaged to 62.1±1.7 mmHg during the transient study and showed that there were no significant differences in these measurements (F=0.43, p=0.83), supporting the idea that, although the protocols were performed in succession, one did not affect the other—ostensibly making each measurement independent. In other words, each experiment can be considered independently of the one before it, despite having been done in succession, and any differences in the reperfusion phase cannot be attributed to differences in the baseline P₄SfO₂. The same significance testing was done on the data collected from the VO₂ and Q studies which yielded the same results—no significant differences were demonstrated among the baseline VO₂ measurements, which averaged 130.1±8.7 nl O₂/cm³·s; and no significant differences were found among the baseline Q measurements, which averaged 5.52±0.31 µl/min.
**Transient Interstitial Fluid O₂ Tension, P_{ISF}O₂**

It was observed in the Transient P_{ISF}O₂ study that, as the ischemic duration increased, the initial rate of P_{ISF}O₂ recovery increased. The rate of recovery during 5 seconds of ischemia was significantly lower (p<0.05) than the rates during the 15 to 300 second ischemic durations. As the ischemic duration increased in steps to 30 seconds, the rate of recovery significantly increased from 0.84±0.12 mmHg/s for the 5-second group to 4.25±0.72 mmHg/s for the 30-second group. However, as shown in Fig. 47, when the ischemic duration went beyond 30 seconds of ischemia, the initial rate of P_{ISF}O₂ recovery began to diminish until it reached a point close to that found for the 5-second ischemic duration group. This recovery rate went from a maximum 4.25±0.72 mmHg/s at 30 seconds of ischemia to 1.00±0.16 mmHg/s for 600 seconds of ischemia.

Previous studies in myocardial tissue (Cai et al., 2011) related blood flow and tissue oxygenation in ischemic preconditioning and demonstrated that, as ischemic durations increased, the initial rate of tissue PO₂ recovery decreased. The present study shows that for brief (<30 sec) durations of ischemia, the initial rate of recovery increased until a limit was reached after which the initial rate began to decline as the ischemic duration increased. A possible reason for this is that, during the prolonged ischemic phase, mitochondrial activity was impaired and when blood flow returned, reactive metabolites (e.g., O₂⁻--superoxide radical, H₂O₂--hydrogen peroxide, ONOO⁻--peroxynitrite, cyclooxygenases, xanthine oxidase, NADH/NADPH) diffused to nearby parts of the tissue to further damage them. It could also be that prolonged ischemia prevented a high enough PO₂ gradient from being established between the tissue and the blood supply and, as a result, the rate at which oxygen diffused to the interstitium decreased. The P_{ISF}O₂ being able to return to baseline quickly during the shorter ischemic durations (5-60 seconds of ischemia) could
be attributed to myogenic effects in which slight hyperemic effects that caused an increase in the rate of recovery were immediately attenuated.
Blood Flow, Q

Numerous studies have been conducted to try and understand the mechanism by which changes in blood flow occur following ischemia/reperfusion. An earlier study conducted to determine the effect of occlusion duration on reactive hyperemia (Johnson et al., 1976) observed that a graded increase in the duration of vessel occlusion caused a graded increase in blood flow. Peak blood flow reached 280% above baseline following 120 seconds of occlusion in venous outflow. It was also observed that brief ischemic periods caused large (200%) increases in capillary flow. Data from the present blood flow study demonstrated that, although blood flow did not noticeably increase above the baseline following ischemia, the rate at which the flow recovered (Fig. 50) significantly increased following 5 seconds of ischemia where it was $2.99\pm0.32$ ($\mu$l·min$^{-1}$)/s to 60 seconds of ischemia where it was $7.04\pm0.55$ ($\mu$l·min$^{-1}$)/s ($p<0.05$).

However, for ischemic durations greater than 60 seconds, the rate at which blood flow recovered significantly dropped. Following 300 seconds of ischemia, the rate significantly dropped to $2.83\pm0.55$ ($\mu$l·min$^{-1}$)/s from $7.04\pm0.55$ ($\mu$l·min$^{-1}$)/s following 60 seconds, but increased to $2.92\pm0.45$ ($\mu$l·min$^{-1}$)/s following 600 seconds of ischemia.

In addition to the initiation of pro-inflammatory cascades at the onset of reperfusion, a secondary point of ischemic damage has been observed after the return of flow to baseline (Reffelmann et al., 2003). The no-reflow phenomenon (Menger et al., 1992) describes the failure of capillary perfusion that occurs once flow has returned in the larger vessels following ischemia. A number of reasons for this failure have been suggested: obstructions by clot formations, leukocyte plugging, endothelial cell swelling, increase in blood viscosity and increase in hydraulic resistance (Benz et al., 2002). The no-reflow phenomenon could
potentially be a cause of the significant decrease observed in the rate of recovery of flow during
the reperfusion phase following 300 and 600 seconds of ischemia.

The same idea can also be applied to the delay in $P_{\text{ISF}O_2}$ recovery. Although oxygen
tension eventually returned to baseline, at least during the time course we observed, the rate at
which it recovered was significantly different among the ischemic durations tested. Despite a
lack of significant differences in $P_{\text{ISF}O_2}$ between 300 and 600 second ischemic durations, there
were significant differences in the rate at which the $P_{\text{ISF}O_2}$ recovered ($p < 0.05$) between the two.
This goes to suggest that, by prolonging the ischemic duration past the observed critical point of
30 seconds, a progressive increase in capillary perfusion failure causes the rate at which the
oxygen-deprived tissue is replenished to decrease. In addition to that, certain changes occur by
300 seconds of ischemia that cause the rate at which the flow recovers to also significantly
decrease—due in part to possibly any of the reasons previously mentioned that could prevent
flow following the cessation of ischemia.
Oxygen Consumption, VO$_2$

By utilizing a novel quasi-continuous approach (5-sec flow stoppage/15-sec flow recovery) to observe dPO$_2$/dt using the PQM and ischemia/reperfusion system, VO$_2$ measurements were made for each of the ischemic durations established in the protocol. With some minor adjustments to the time when baseline started and when reperfusion observations ended, more data points could be collected as the system is designed to only acquire one measurement every 20 seconds. Oxygen consumption measurements were delayed until P$_{ISF}$O$_2$ reached >20 mmHg, because it is at this critical point where oxygen consumption becomes dependent on PO$_2$. Based on the oxygen dissociation curve, the quasi-linear range of PO$_2$ (20-40 mmHg) was used to approximate the oxygen saturation, SO$_2$ (Eq. 20), as a function of the slope of the region, the hemoglobin concentration, the O$_2$-carrying capacity of Hb, and the oxygen tension. For the model to be able to express the variables in terms of linear changes, the linear portion of the oxygen dissociation curve was used. If measurements were to be taken below 20 mmHg, oxygen consumption and the values obtained for it would become dependent on PO$_2$.

The average baseline VO$_2$ of 130.1±8.7 nl O$_2$/cm$^3$.s in resting skeletal muscle was consistent with what has been observed in previous work from this laboratory (Golub et al., 2011b), confirming the use of the technique in the present experiments. The observed pattern of oxygen consumption by the tissue following ischemia; as shown in Figs. 27, 28, 33, 34, 39, 45 and 46; behaved similarly to exercise and fatigue studies performed in human muscle tissue using near-infrared (NIR) diffuse optical methods (Gurley et al., 2012). It should be noted that Gurley et al. reported values of 20 nl O$_2$/cm$^3$.s, but the protocol and time course over which their data were collected is similar to that of the present study.
Peak oxygen consumption of 309.2±45.0 nl O₂/cm³·s occurred at the first measurement taken following 600 seconds of ischemia. Since the tissue was deprived of oxygen for such an extended period of time, it would stand to reason that the time course over which the VO₂ values recovered to baseline during reperfusion would be longer than for shorter ischemic durations. For 600 seconds of ischemia VO₂ returned to baseline within 160 seconds. The time course for recovery progressively decreased as the ischemic duration became smaller, such that it returned to baseline within 15 seconds during 5 seconds of ischemia—this also helped establish the use of the quasi-continuous VO₂ measurement system, because very little change occurred in the VO₂ measurements during that part of the study. When observing the normalized VO₂ data from the start of reperfusion and on, the 300- and 600-second groups presented themselves as being significantly higher (p <0.05) than results from the 5-, 15-, 30- and 60-second ischemia groups, however they were not different from one another (p=0.3665).
Factor M

With perivascular (P_aO_2) and interstitial (P_{ISF}O_2) oxygen tensions collected during the baseline of the VO_2 and Q studies, these two sets of data could be compared to one another using the factor M (Eq. 30). M simultaneously relates the three key variables of tissue oxygenation and blood flow regulation: VO_2, Q and ΔPO_2, where ΔPO_2 is the difference between the arteriolar (P_aO_2) and interstitial (P_{ISF}O_2) oxygen tensions:

\[ M = \frac{\dot{V}_{O_2}}{Q \cdot ∆P_{O_2}} \]

By knowing how M behaves, the behavior of \( β[Hb] \cdot (1 - e^{-z}) \) from Eq. 29 can be observed. Under steady-state conditions, each of the variables that compose M should remain relatively constant, making M constant. The value of M might also remain relatively constant during the transient recovery phase of post-ischemia reperfusion, if those variables are regulated by the same mechanism(s), but the values that compose it would not be expected to remain constant. At the start of ischemia, all variables are at their respective baseline levels, and the ratio M should remain relatively constant for all baseline periods.

Since P_aO_2 data were not collected for the Transient P_{ISF}O_2 study, data collected from the VO_2 study was used in its place. With P_aO_2 and P_{ISF}O_2 data available for every site in the VO_2 study, baseline ΔPO_2 values were determined and used in the calculation of M. With P_aO_2 data unavailable through the duration of the experiment from the start of ischemia, it was assumed that the P_aO_2 values remained relatively constant (Assumption #1) throughout the experiment. Only the P_{ISF}O_2 values should change as the supply and consumption balance changed over time during recovery and, with that, the data through the start of reperfusion (or when the first consumption measurement is taken) were normalized to the baseline values. Since the VO_2 was the limiting variable on account of only having been collected every 20 seconds, normalized
flow values were taken from the corresponding time points at which the consumption
measurements were made. With normalized ΔPO2, Q and VO2 data from every site within a
particular ischemic duration, a normalized factor M can be calculated on a site-to-site basis for
all six ischemic duration groups with respect to time.

Figures 53 and 54 show the results of the calculations for M. The recovery period of M
for the smaller ischemic durations (5 through 60 seconds) was much shorter than it was for the
longer ischemic durations. With much variability present within the calculations and a
significant increase of M 60 seconds into the recovery period of the 300- and 600-second
ischemia groups, the most important conclusion that can be drawn from this is that site-to-site
variability may play a bigger role than what was originally anticipated. It is also apparent that,
despite the ischemic duration, the value of M always returned to baseline with only the 300-
second group deviating from this pattern by not fully recovering during the observation time. If
the consequences of the no-reflow phenomenon were going to appear, it would most certainly
appear first in the 600-second group and then, depending on the degree of change to the vascular
network, to the shorter durations. The 5- through 60-second ischemic duration groups did not
display drastic changes and the changes of the 600-second group in relation to the 300-second
ischemia group were attenuated. This behavior led to the conclusion that the experiments for the
300-second ischemic group need to be repeated, to observe if this kind of behavior is consistent
or specific for that set of data. If that is the case, they will need to be approached with a different
modeling strategy.

β and [Hb] are physiological parameters that should remain relatively constant through
the duration of the experiment. In this case, any changes in the factor M represent changes in the
factor “z” since z is a function of the flow. If it can be determined that M remains reasonably
constant through the baseline and transient recovery period before approaching a new steady state, then it can be assumed that $z$ is also a constant. If it is the case that $z$ only changes with respect to the ischemic duration, then a model that relates the three variables with respect to the ischemic duration (or function of time) can be obtained.
Extensions of the Work

Although not a focus of this study, it is interesting to note that recent work (Casey et al., 2011) has indicated that following hypoxia, compensatory vasodilation occurs in which the imbalances in oxygen supply and demand are dealt with by temporarily increasing blood flow to increase the oxygen delivery. This has been a topic of much debate among researchers in microcirculation and hemodynamics for many years, but recently efforts have been made to understand the role that nitric oxide (NO) plays in the regulatory system. That has become the topic of much of the work in which this laboratory is currently engaged (Golub & Pittman, 2013) and a focus for future projects. The work from this study, in conjunction with experimental evidence for a NO-mediated compensatory mechanism, can help establish the causal relationship between NO and the regulation of local blood flow.
Future Studies

The next step in these studies will be to determine how \( z \) is influenced by blood flow and the geometry of the vessel. Although it is not expected that the geometry should permanently change, it will still be necessary to empirically demonstrate this. Since the 5-second ischemic duration has not made a substantive contribution in supporting the compartmental model, it will be removed from future experiments. The data collected so far have confirmed that the quasi-continuous \( \text{VO}_2 \)-sampling technique can effectively determine oxygen consumption. In terms of the protocol itself, no changes will be made, however, instead of looking at multiple durations at a single site, only one ischemic duration will be used at multiple sites. This will allow efficient sampling of \( \text{VO}_2 \), \( \text{P}_a\text{O}_2 \), \( \text{P}_{\text{issf}}\text{O}_2 \) and \( Q \) to be done within a reasonable amount of time. By doing this, any site-to-site heterogeneity will be eliminated because all the data necessary information will be contemporaneously recorded. It will also no longer be necessary to relate variables obtained under dissimilar conditions. Since changes in recovery were seen in the blood flow study for extended periods of ischemia, it may prove beneficial to designate experiments in which observations of leukocyte adhesion in microvenules are made, as well as any changes in capillary perfusion—enough so to affect an entire site.
Conclusions

The data presented here have been of novel work that has never incorporated so many different, non-invasive, non-destructive techniques in an attempt to understand and define the limits of how tissue oxygenation, consumption, and blood supply are regulated. These data were obtained systematically in an attempt to determine how skeletal muscle tissue responds to varying ischemic durations. Never before have the rates of reperfusion been determined which distinctly outline how the tissue is expected to recover over a certain period of time. By using velocity information that has been corrected to account for geometric changes of the vessel being observed, a novel, quasi-continuous system to measure oxygen consumption, and a highly reliable and well-respected method of determining oxygen partial pressures, strong empirical evidence has been collected for characterizing the relationships among oxygen consumption, blood supply, and tissue oxygenation.
References


Vita

Sami Dodhy was born on July 15th, 1989 in Alexandria, VA to Chand and Riffat Dodhy. He graduated from Riverbend High School in Fredericksburg, VA in May of 2007. With a knack for breaking things, he went on to graduate with a Bachelor of Science degree in Biomedical Engineering at Virginia Commonwealth University in May of 2011 and learned how to put them back together. As an undergraduate, he fell in love with the biomedical sciences and decided to pursue graduate studies at the School of Medicine of Virginia Commonwealth University starting in August of 2011. He completed a Master of Science degree in Physiology & Biophysics in May of 2013.