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PO2 DEPENDENCE OF OXYGEN CONSUMPTION IN SKELETAL MUSCLE OF DIABETIC AND NON-DIABETIC RATS

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PO$_2$ DEPENDENCE OF OXYGEN CONSUMPTION IN SKELETAL MUSCLE OF DIABETIC AND NON-DIABETIC RATS

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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Abstract

PO₂ DEPENDENCE OF OXYGEN CONSUMPTION IN SKELETAL MUSCLE OF DIABETIC AND NON-DIABETIC RATS

By: Alexander C. Liles

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

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Advisor: Roland N. Pittman, Ph.D.
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Type 2 diabetes mellitus (T2DM) is a major medical problem around the world, affecting nearly 6% of the world’s population. This study was an attempt to better understand physiological changes the disease may cause to the microcirculation and more specifically, to assess the PO₂ dependence of oxygen consumption in skeletal muscle of a diabetic animal model. The spinotrapezius muscles of Goto-Kakizaki (G-K) and Wistar control rats were used to measure interstitial PO₂ using phosphorescence quenching microscopy. The G-K rats spontaneously develop T2DM and serve as an appropriate model for the disease in humans. By rapidly arresting blood flow in the tissue and observing the resulting PO₂ changes, an oxygen disappearance curve (ODC) was created. The ODC was used to calculate oxygen consumption rate (VO₂) over the physiological range of PO₂ values. The resulting VO₂ vs PO₂ curves were analyzed using Hill’s equation to fit the data and obtain values of several key parameters to
quantitatively describe the PO₂ dependence of oxygen consumption. When compared to healthy control rats, the G-K rats exhibited a significantly higher $V_{\text{max}}$, or maximum rate of oxygen consumption, compared to the Wistar rats. The two rat sub-strains had similar values for $P_{50}$, which indicates the PO₂ at half maximal consumption. The overall higher maximal rate of consumption by the diseased animals could be explained by some disconnect in the consumption of oxygen by the mitochondria and the normal corresponding production of ATP. In conclusion, it was demonstrated that in situ muscle tissue from both diabetic and non-diabetic rats had a PO₂ dependence of oxygen consumption over a wide range of PO₂ values and the muscles of diabetic animals consumed oxygen at a higher maximal rate.
Introduction

Cellular Need for Energy

The vast array of functions that are carried out by the cell in an organism require a constant input of energy in the form of adenosine triphosphate (ATP). In the presence of oxygen, oxidative phosphorylation within the mitochondria is the most efficient mechanism for production of this high-energy molecule. Since oxygen is required for this process to take place, the delivery of O$_2$ from the atmosphere to the cell is critical for maintenance of normal cellular function.

The Blood and Circulation

The requirement for a continuous and adequate supply of oxygen to the cell for energy production by the mitochondria is accomplished through an orchestrated effort between the cardiovascular and respiratory systems. Blood is oxygenated in the pulmonary capillaries of the lungs by the diffusion of oxygen from the nearby alveoli; the oxygenated blood is pumped by the heart through the circulation (Costanzo, 1998). Almost all of the oxygen in the blood is carried by the protein hemoglobin, contained within the red blood cells (RBCs), which has four binding sites for oxygen. When the oxygen bound to hemoglobin is delivered to tissue through the capillaries, the oxygen is released from the hemoglobin and can diffuse through the RBC cytoplasm, across the RBC membrane, through the plasma and across the capillary
wall into the interstitial fluid. Here the oxygen can move into cells and be used to generate ATP (Pishchany G, 2012).

The relationship between oxygen bound to hemoglobin (oxygen saturation, SO₂) and the partial pressure of oxygen (PO₂) can be described by the oxygen saturation (or dissociation) curve. The curve is sigmoidal in shape and illustrates the binding properties of hemoglobin. As PO₂ increases, the oxygen saturation also increases. In oxygen-rich arteriolar blood, on the one hand, hemoglobin is highly saturated with oxygen. On the other hand, oxygen is unloaded from the RBCs in areas with lower PO₂, such as capillary beds of skeletal muscle (Pittman, 2011). When certain physiological changes take place in the body (e.g., altered temperature, pH, PCO₂), the dissociation curve will shift to adjust the availability of oxygen to tissues. For example, an increase in temperature due to exercise shifts the curve to the right and therefore decreases the affinity of hemoglobin for oxygen. This allows oxygen to be released more easily from hemoglobin and increases its availability to muscle cells.

After oxygen is delivered to the microcirculation by oxygenated RBCs, it must make its way into the parenchymal cells by diffusion. The diffusion is driven by partial pressure gradients in oxygen that drive oxygen out of the capillaries, into the interstitial fluid, and eventually into the cell, as needed. As the mitochondria within the cell consume oxygen to make ATP, the PO₂ within the cell decreases and draws more O₂ into the cell from the interstitial fluid (Costanzo, 1998).
Glucose

Energy is required for normal functioning of body tissues, and glucose is the main substrate used by the body. Glucose is transported throughout the body in the blood and can be measured as “blood sugar.” Serum glucose levels are normally tightly regulated through uptake and release mechanisms. Glucose can be stored in the liver as glycogen and then mobilized back to glucose as needed by the body. The β-cells of the pancreas release insulin when high blood sugar is detected in order to stimulate the uptake of glucose from the bloodstream. It has been shown that chronic exposure to high blood glucose can reduce the response of the β-cells, leading to complications like diabetes. Additionally, repeated stimulation by insulin and prolonged high levels of insulin can lead to insulin resistance, which is a major characteristic of Type 2 diabetes (White M.F., 1998).

As carbohydrates are consumed, they are broken down by the body and used to create energy. During glucose metabolism, glucose is oxidized to yield carbon dioxide and water while simultaneously producing energy in the form of ATP. The first step in the breakdown of glucose is glycolysis. As a result of glycolysis, pyruvate is produced. Under aerobic conditions, most pyruvate is oxidized and enters the Tri-carbolic acid (TCA) cycle, which has a high yield of ATP. For cellular respiration to be completed, NADH and FADH₂ produced during the earlier stages serve as electron donors in the electron transport chain (ETC). The ETC is an ordered series of proteins
embedded in the inner membrane of the mitochondria. Electrons are passed from one protein to the next in redox reactions that create a proton gradient across the membrane. This gradient is used to subsequently produce ATP. The ETC, along with this production of ATP via proton gradients, is known as oxidative phosphorylation. Oxygen is the terminal electron acceptor in the ETC and is therefore required for oxidative phosphorylation to occur. In anaerobic conditions, fermentation occurs, resulting in the production of lactate and a significantly lower ATP yield.

**PO₂ Dependence of Oxygen Consumption**

The mitochondria consume the vast majority (>95%) of oxygen that enters the cell for normal cellular respiration. The classic view of mitochondrial respiration is that consumption occurs at a constant rate regardless of shifts in the amount of oxygen in the area, unless the PO₂ falls below approximately 1 mmHg (Wilson, 1985). This view suggests that oxygen consumption is essentially independent of PO₂ under typical physiological conditions. Normal cellular respiration would be maintained even at relatively low or relatively high concentrations of oxygen as long as the PO₂ was above that critical level. This idea is supported by numerous *in vitro* studies that measured oxygen consumption rates in mitochondrial suspensions (Wilson, 1988).

The oxygen dependence of respiration was later addressed using *in vivo* methods, which demonstrated that the mitochondria do not function in such an all-or-nothing or on/off manner (Golub, 2012). Based on these experiments, the rate of oxygen consumption appears to vary over a wide range of physiological PO₂ levels.
The PO$_2$ dependence of oxygen consumption can be illustrated by plotting oxygen consumption rate (VO$_2$) versus corresponding PO$_2$ values. In the context of using Michaelis-Menten kinetics to this PO$_2$ dependence, this allows the maximal rate of consumption ($V_{\text{max}}$) and also the half-maximal rate (at PO$_2 = K_m$) to be calculated. The classic view of PO$_2$ dependence suggests a $K_m$ for oxygen consumption that is well below 1 mmHg (Wilson, 1988). However, recent in vivo studies concluded that the $K_m$ in rat spinotrapezius muscle was approximately 10 mmHg (Golub & Pittman, 2012). This is a staggering difference that demonstrates oxygen consumption depends much more on varying PO$_2$ levels than previously thought.

*Phosphorescence Quenching Microscopy*

The in vivo studies described above involve measuring PO$_2$ of the interstitial fluid of skeletal muscle in rats using phosphorescence quenching microscopy (PQM) (Pittman R.N., 2012). PQM is an innovative approach to measuring oxygen tension that involves exciting a phosphor probe molecule from the ground state to an excited state using a brief flash from a light source, such as a laser. The excited phosphor molecule can return to the ground state by either emitting a photon (phosphorescence), or being quenched by oxygen (collisional quenching). The PO$_2$ can be calculated based on this relationship using the Stern-Volmer equation. The intensity of the phosphorescent light emission can be described by the following:
\[ I_{\text{phos}}(t) = I_{\text{phos}}(0) e^{-kt} \]

In this equation, \( I_{\text{phos}}(t) \) is the intensity of the phosphorescent light emission, \( I_{\text{phos}}(0) \) is the phosphorescence intensity at \( t = 0 \), and \( k \) is the phosphorescence (exponential) decay rate. Once the decay rate \( (k) \) is obtained, the PO\(_2\) can be related to it with this formula:

\[ k = k_0 + k_q P O_2 \]

The quenching coefficient \( (k_q) \) and the decay rate at zero PO\(_2\) \( (k_0) \) are constants determined in separate calibration experiments (Golub, 2010).

When measuring the PO\(_2\) of the interstitial space, the phosphorescent oxygen probe can be bound to albumin to prevent it from penetrating the cell and vasculature. When using a thin skeletal muscle, the dissolved probe can be loaded into the interstitium by simple topical application. After sufficient time has passed to allow for diffusion of the probe into the interstitial space (30-60 min), the tissue will be ready for determination of PO\(_2\) by PQM (Nugent et al, 2015). Once PO\(_2\) readings can be taken from the muscle, a “stop-flow” approach can be used to find the rate of oxygen consumption based on the subsequent decrease in oxygen tension over time. When
blood flow is halted, the tissue will continue to consume the available dissolved oxygen and the PO$_2$ will fall until it reaches approximately 0 mmHg.

*Type 2 Diabetes Mellitus*

Type 2 diabetes mellitus (T2DM) is characterized by a variety of dysfunctions that are caused by insulin resistance and inadequate insulin secretion. This results in chronic hyperglycemia and an assortment of related issues. It is a major disease that affects approximately 9.3% of the U.S. population and is increasing in prevalence worldwide (CDC). The major risk factors for T2DM include physical inactivity, poor diet, obesity, and increasing age. β-cells of pancreatic islets are involved in the secretion of insulin, which promotes the absorption of glucose from the bloodstream into the liver, fat, and skeletal muscle cells (Akash, 2013). The disease is known to occur as a consequence of β-cell dysfunction and reduction in β-cell size, which affects the secretion of insulin.
Goto-Kakizaki Rats: T2DM Animal Model

In order to study the effect of T2DM on the PO$_2$ dependence of oxygen consumption, a suitable animal model for the disease is required. Goto-Kakizaki (GK) rats are considered to be one of the best subspecies developed for the study of spontaneous T2DM (Akash, 2013). They were developed from selective inbreeding of Wistar rats, which serve as the best control group when research is being performed with these G-K rats.

GK rats share many significant characteristics with human diabetic patients including hyperglycemia, reduced β-cell mass, insulin resistance, and impaired glucose-induced insulin secretion (Akash, M, 2013). In order to help differentiate the impact of obesity and T2DM on hyperglycemia, they were developed as a non-obese model. G-K rat body weight is typically 10-30% lower than their age-matched Wistar control (Akash, M, 2013). Approximately 3 weeks after birth, these rats suddenly develop the characteristics mentioned above that accurately model T2DM. Similar to humans, the disease symptoms progress as they age. In adulthood, the rats often exhibit renal damage and proteinuria, which can be compared to diabetic nephropathy in humans. Overall, Goto-Kakizaki rats are a suitable non-obese animal model to investigate type 2 diabetes mellitus.
Purpose of Study

This project was initiated in order to study the PO$_2$ dependence of oxygen consumption in healthy and diabetic rats. It has been previously demonstrated in related *in vivo* studies that the oxygen consumption rate by the mitochondria of skeletal muscle cells is dependent on variations in PO$_2$ over a much wider physiological range than previously proposed (Golub AS, Pittman RN, 2012). To further investigate this concept, we decided to see if this dependence would change in a diseased animal model. Type 2 diabetes mellitus has broad metabolic effects primarily characterized by hyperglycemia and can be modeled well with Goto-Kakizaki rats. We sought to determine whether there was a difference in the oxygen dependence of respiration in the spinotrapezius muscles of healthy and diabetic rats by analyzing the disappearance rate of oxygen, following flow arrest, using phosphorescence quenching microscopy.
Materials and Methods

Animals and Surgical Preparations

Male Wistar rats (ENVIGO, Madison, WI, weight = 295.4 ± 23.2 g, N=5) served as the control group and male Goto-Kakizaki rats (Taconic Farms, Derwood, MD, weight = 249.8 ± 15.0 g, N=5) were used to characterize the PO$_2$ dependence of oxygen consumption in diabetic animals. Animals were housed in plastic cages with 2 rats per cage under a 12-hour/12-hour light/dark cycle. All animals received equal quantities of rat chow and water, which was provided by the Division of Animal Resources. There was continuous ventilation in a climate-controlled vivarium maintained at 20-23 degrees Celsius.

To perform the intravital microscopic studies, rats first underwent a surgical procedure to exteriorize the spinotrapezius muscle. Each rat was initially anesthetized with a Ketamine/Acepromazine (72/3 mg/kg) solution by intraperitoneal injection. After the rat was sufficiently anesthetized (about 10 minutes post-injection established by toe pinch), it was shaved in the bilateral inguinal creases, ventral neck region, and an area from the shoulder blades extending along the spine covering the spinotrapezius muscle using clippers. A depilatory cream (Nair, Church and Dwight Co., Inc., Ewing, NJ) was applied to remove any remaining hair. The animal was then placed under a stereomicroscope on a heating pad, to maintain core body temperature at 37 °C, in preparation for surgery. Blood glucose readings
were taken from the paw of the back left foot before and after each experiment using an AlphaTrak Blood Glucose Monitoring System (Zoetis Inc., Kalamazoo, MI). At the conclusion of the experiment, the rat was euthanized with Euthasol (150 mg/kg).

**Femoral Vein Cannulation**

Initially, an incision was made along the right inguinal crease and the right femoral vein was isolated. An incision was made perpendicular to the vein about half the diameter of the vessel. Before this incision was made, a knot was secured just distally to arrest blood flow through the vein. A cannula consisted of 26 cm of polyethylene tubing (PE 90) connected to a 6 ml syringe containing Alfaxan (Jurox Inc., Kansas City, MO, alfaxalone 10 mg/mL) and a 10 ml syringe containing heparinized phosphate-buffered saline (PBS). The Alfaxan was continually infused at 0.1 mg/kg/min for the duration of the experiment. This infusion rate was adjusted as necessary to maintain appropriate surgical depth of anesthesia based on toe pinch reflex and heart rate. The cannula was secured to the animal’s leg using Transpore tape; and a cotton ball was taped over the incision to prevent drainage of any fluids. This cannula was also used to infuse Euthasol at the conclusion of the experiment to euthanize the animal.
Tracheostomy

Immediately following the femoral vein cannulation, the trachea was cannulated with polyethylene tubing (PE 240) to ensure a patent airway for the duration of the experiment. This incision was taped down and secured with a cotton ball. The animal breathed room air spontaneously.

Spinotrapezius Muscle Preparation

The spinotrapezius muscle was exteriorized following the procedures described by (Gray, 1973) and placed on a thermo-stabilized platform for intravital microscopy (Golub and Pittman, 2003). Initially, an incision was made longitudinally along the dorsal midline of the rat between the shoulder blades and extending down the spine to expose the underlying fascia. The fascia was removed carefully and a cauterizing pen was used to minimize bleeding. After the muscle was exposed, tissue hydration was maintained by topical application of PBS as needed. At this point, the muscle was carefully separated from the underlying fascia using curved scissors and placed on the platform. Surgical silk (size = 6-0) was sutured every 2 cm around the border of the muscle to aid with securing the muscle to the platform. Excessive stretching or manipulation of the muscle was avoided to prevent damage to any vessels.
Figure 1. Photograph of a spinotrapezius muscle prepared for intravital microscopy and PQM.
**Phosphorescent Probe Application**

Prior to mounting the heated animal platform to the microscope, 2 ml of the phosphorescent oxygen probe (Pd-meso-tetra-(4-carboxyphenyl)porphyrin (Oxygen Enterprises, Philadelphia, PA) conjugated to human serum albumin in PBS) was topically applied to the spinotrapezius muscle at a concentration of 10 mg/ml and allowed to diffuse into the muscle for 60 minutes. It can be assumed that the probe does not enter the muscle fibers because it was bound to albumin, which is too large to pass through the cell membrane. Any residual probe that entered the microvasculature would be immediately “washed out” by blood flow. Therefore, it was determined that measurements taken from exciting the probe that diffused into the tissue in this manner would be confined to the interstitial fluid space. After the probe was applied, the muscle was covered with a gas-impermeable film (Krehalon, CB-100; Krehalon Limited, Japan) to isolate it from the room air.

**Phosphorescence Quenching Microscopy**

After the probe was distributed within the interstitial space, the animal platform was mounted on an Axioplan-2 microscope (Zeiss, Germany) in preparation for phosphorescence quenching microscopy studies. To excite the probe, pulses from a laser were produced at a rate of 1 Hz. A photomultiplier tube collected the phosphorescence emitted from the excited probe and a colored-glass long-pass filter (OG 550, Edmund Optics) was in place to avoid extraneous excitation. A video camera
was also used to aid in visually selecting well-perfused sites in the muscle and ensure that the pneumatic tissue compression fully evacuated the RBC’s from the site. The phosphorescence signal collected by the PMT was transmitted through an amplifier to an analog-to-digital converter. This allowed the phosphorescence decay curves to be analyzed by custom data processing software using the National Instruments platform. A $P_O^2$ value was produced for each phosphorescence decay curve using the analysis software.

An additional consideration when measuring $P_O^2$ using this technique is the photoconsumption of oxygen. As described in the introduction, singlet oxygen is produced by the interaction of the probe and $O_2$. The singlet oxygen can react with organic molecules and is therefore “consumed” by the method. This proportional reduction in $P_O^2$ needs to be accounted for by calculating a coefficient (K) that is related to photoconsumption. This is accomplished by measuring $P_O^2$ at two different excitation frequencies using a 5 s x 15 s high/low pressure cycling method (Nugent et al, 2015). For a total of 100 seconds, a series of 5 second compressions each followed by 15 seconds with no compression, alternating between the high and low frequencies were measured. The $P_O^2$ changes were then compared to calculate the photoconsumption coefficient using the following formula:

\[
K = \frac{[(dP/n)1 - (dP/n)2]/[(FP0)2 - (FP0)1]}{}
\]
In Equation 3: $F$ is the flash rate for each measurement, $dP_n/dn$ is the rate of oxygen disappearance per flash at the given high or low frequency, $P_0$ is the steady state $PO_2$ at the given flash rate prior to pneumatic compression.

**Phosphorescence Decay Curve Analysis**

A non-linear fitting program was used for each phosphorescence decay curve that was based on the rectangular $P_{ISF}O_2$ distribution model (Golub et al., 1997). The fitting program was used to obtain a continuous line fit for each decay curve calculate the phosphorescence decay rate. The fitting equation used was as follows:

\[
I(t) = I_0 \exp\left[-\left(K_0 + K_q M\right)t\right] \sinh\left(K_q \delta t\right)/K_q \delta t + B
\]

in which $t$ is time in $\mu$s from the beginning of the decay, $I(t)$ is the phosphorescence decay curve in volts, $I_0$ is the magnitude of the phosphorescence signal at $t = 0$, $M$ is the average $PO_2$ (mmHg) in the region being measured, $\delta$ (mmHg) is the half-width of the uniform $PO_2$ distribution, and $B$ (volts), is the baseline offset of the amplifier. $K_0$ and $K_q$ serve as constants and have been determined from calibration experiments (Golub and Pittman, 2016) to be $K_0 = 1.53 \times 10^{-4}$ mmHg$^{-1}$ and $K_q = 4.3 \times 10^{-4}$ mmHg$^{-1}$.
Tissue Compression

An airbag mounted on the objective lens of the microscope was used to arrest blood flow in the spinotrapezius muscle. The airbag was rapidly inflated (<1s) to approximately 150 mmHg, which is higher than systolic pressure, causing blood flow to cease and extrude most of the red blood cells from that region. This was confirmed by visually inspecting the site using the video camera. After the tissue was compressed, a fall in $P_{O_2}$ was observed as the tissue consumed the remaining dissolved oxygen. For each animal, five well-perfused sites were chosen to measure $P_{O_2}$ during the compression cycle. Measurement sites were chosen based on specific criteria: it was required that the site was well perfused with continuous RBC flow and was not close to any large vessels.

To obtain a baseline $P_{O_2}$ for each site, the $P_{O_2}$ was measured for 10 seconds prior to compression. After the airbag was inflated, $P_{O_2}$ continued to be measured for an additional 60 seconds in order to observe the complete disappearance of oxygen. The tissue was allowed to rest with no compression or excitation for 5 minutes between measurements at each site.

Analysis of Oxygen Consumption

For each site, an oxygen disappearance curve (ODC) was observed as the $P_{O_2}$ decreased during compression. The rate of $P_{O_2}$ change after the blood flow had been
arrested with the airbag was related to several factors. First, the tissue consumed $O_2$ as a part of normal respiration. Additionally, consumption by the method itself (photoconsumption) and $O_2$ diffusion into the excitation region had to be accounted for. It was important to account for these factors in order to accurately calculate the $O_2$ consumed specifically by tissue respiration.

The ODC was used to calculate the oxygen consumption rate ($V_n$) at a given $PO_2$, which is necessary to determine the $PO_2$ dependence of oxygen consumption. The relationship between $V_n$ and $PO_2$ is described by the following formula:

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

in which $\frac{dP_n}{dn}$ is the rate of $O_2$ disappearance per flash, $K$ is the coefficient related to consumption by the PQM method, and $Z$ is the coefficient related to inward diffusion of $O_2$ into the excitation region. The area being compressed is large enough so that the passive inward diffusion of $O_2$ can be neglected.

**Statistics**

The values expressed in tables and the text are mean ± SE (N). A t-test was conducted in comparisons between G-K and Wistar results to test for significance. Statistical significance was assigned for $p<0.05$. 
Results

The goal of this study was to determine whether there was any difference in the oxygen dependence of oxygen consumption between diabetic and non-diabetic animals. These data were collected using the “flow arrest” approach described in the preceding Materials and Methods section.

Oxygen Disappearance Curves

Following each spinotrapezius muscle compression, an oxygen disappearance curve (ODC) was produced using the collected PO$_2$ data and flash number (proportional to time). These ODC's were corrected for consumption by the PQM method using Equation 3. During the first 10 seconds of collection, the muscle was not compressed and the baseline PO$_2$ was established. After 10 seconds, the airbag was inflated and the disappearance curve was created based on the decrease in PO$_2$. 
Figure 2. This is an example of a typical oxygen disappearance curve collected from a Wistar rat using the PQM method. The laser was flashed at 1 Hz, so a new PO$_2$ value was measured every second.
Figure 3: The oxygen disappearance curve above is a representative curve from a single site on the spinotrapezius muscle of a Goto-Kakizaki rat.
The oxygen disappearance curves were used to calculate VO\textsubscript{2} for each PO\textsubscript{2} value. Plots of VO\textsubscript{2} vs. PO\textsubscript{2} were created and these plots were then fit with Hill’s equation (Equation 6).

\begin{equation}
VO_2 = \frac{V_{max} \times P_n^a}{P_{50}^a + P_n^a}
\end{equation}

In Hill’s equation, P\textsubscript{n} represents the PO\textsubscript{2} at a given flash number in the ODC and VO\textsubscript{2}, V\textsubscript{max} is the maximal rate of respiration, P\textsubscript{50} is the PO\textsubscript{2} at half-maximal respiration, and a is the Hill coefficient.

After fitting the data for the VO\textsubscript{2} vs. PO\textsubscript{2} plots with Hill’s equation, the best-fit parameters, V\textsubscript{max}, P\textsubscript{50} and a, were obtained. The mean V\textsubscript{max} for the Wistar control rats was 131.9 ± 8.7 (SE) nl O\textsubscript{2}/(cm\textsuperscript{3} s). The mean P\textsubscript{50} in the control group was 8.0 ± 0.6 (SE) mmHg. The spontaneously diabetic Goto-Kakizaki rats had an average V\textsubscript{max} of 215.9 nl O\textsubscript{2}/(cm\textsuperscript{3} s) with a standard error of 23.0 and the mean P\textsubscript{50} was 8.5 mmHg with a standard error of 0.5.
Figure 4. This is a typical plot of VO\textsubscript{2} vs. PO\textsubscript{2} that is used to assess the oxygen dependence of respiration in rat spinotrapezius muscle. The above example is taken from a single site on the spinotrapezius muscle of a Wistar rat. The Hill parameters calculated by fitting these data were as follows: $V_{max} = 181.4$ nl O\textsubscript{2}/(cm\textsuperscript{3}s), $P_{50} = 6.5$ mmHg, $a = 2.3$, $r^2 = 0.99$.

See Appendix for full collection of VO\textsubscript{2} vs. PO\textsubscript{2} plots from Wistar rats.
$VO_2$ vs. $PO_2$ Plot – Goto-Kakizaki Rat

Figure 5. The above plot was derived from analyzing an oxygen disappearance curve measured from a Goto-Kakizaki rat. The Hill parameters calculated by fitting these data were as follows: $V_{\text{max}} = 243.5$ nl $O_2/(cm^3s)$, $P_{50} = 7.6$ mmHg, $a = 1.9$, $r^2 = 0.99$.

See Appendix for a full collection of $VO_2$ vs. $PO_2$ plots from Goto-Kakizaki rats.
Table 1: The table above shows the best-fit values for $V_{\text{max}}$, $P_{50}$, and $a$ (mean ± SE) for Wistar and Goto-Kakizaki rats. The maximal rate of oxygen consumption in the G-K rats was significantly higher than that of the Wistar controls.

(n) refers to number of curves

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$ (nl $O_2/(cm^3*s)$)</th>
<th>$P_{50}$ (mmHg)</th>
<th>$a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar (23)</td>
<td>131.9 ± 8.7</td>
<td>8.0 ± 0.6</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>G-K (14)</td>
<td>215.9 ± 23.0</td>
<td>8.5 ± 0.5</td>
<td>2.1 ± 0.1</td>
</tr>
</tbody>
</table>
Blood Glucose

The blood glucose level of each rat was measured using an AlphaTrak Blood Glucose Monitoring System (Zoetis Inc., Kalamazoo, MI) before and after each experiment. The readings can be considered non-fasting because the rats had continuous access to food in their cages and their eating habits were not carefully monitored. The blood glucose level for the Wistar control group was $223.3 \pm 29.9$ (SE) mg/dL. The Goto-Kakizaki rats had a blood glucose level of $580.5 \pm 42.0$ (SE) mg/dL.
Table 2. Mean non-fasting glucose levels in Wistar and Goto-Kakizaki rats.

<table>
<thead>
<tr>
<th>Rat Sub-strain</th>
<th>Wistar</th>
<th>Goto-Kakizaki</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Level (mg/dL)</td>
<td>223.3 ± 29.9</td>
<td>580.5 ± 42.0</td>
</tr>
</tbody>
</table>
Discussion

Major Findings

The major finding in this project was that the maximal rate of oxygen consumption in rat spinotrapezius muscle measured using phosphorescence quenching microscopy was significantly higher in Goto-Kakizaki rats compared to Wistar controls: Goto-Kakizaki, \( V_{\text{max}} = 215.9 \pm 23.0 \, \text{nl} \, O_2/(cm^3*s) \) and Wistar, \( V_{\text{max}} = 131.9 \pm 8.7 \, \text{nl} \, O_2/(cm^3*s) \). This finding indicates that, in an animal model for type 2 diabetes mellitus, a resting striated muscle has an elevated rate of oxygen consumption in the upper range of PO\(_2\). The PO\(_2\) at half maximal rate of consumption was not significantly different in Goto-Kakizaki and Wistar rats (Goto-Kakizaki, \( P_{50} = 8.5 \pm 1.9 \, \text{mmHg} \) and Wistar, \( P_{50} = 8.0 \pm 3.0 \, \text{mmHg} \)). Additionally, based on these results for \( P_{50} \), it is important to note that the PO\(_2\) dependence of oxygen consumption in rat spinotrapezius muscle does cover a wide range of physiological PO\(_2\) values in both Wistar and the diseased animal model. Previous studies on the sensing of oxygen by isolated cells and mitochondria (Wilson, 1985) have shown that mitochondria consume oxygen at a constant rate regardless of oxygen tension until PO\(_2\) falls below about 1 mmHg. The \( P_{50} \) values calculated in this study and previous studies on the spinotrapezius muscle of male Sprague-Dawley rats (Golub, A. S., & Pittman, R. N., 2003) suggest that the range of sensitivity to PO\(_2\) is much wider. Furthermore, the range is consistently wide in the spinotrapezius muscles of spontaneously T2DM rats and Wistar controls.
Oxygen Disappearance Curves

The oxygen disappearance curves (examples in Figures 2 and 3) collected from both the Wistar and G-K rats clearly display the previously observed behavior of the spinotrapezius muscle from Sprague-Dawley rats in terms of oxygen consumption over time. The 10 seconds of data collected before airbag inflation/tissue compression show the baseline PO$_2$. This PO$_2$ value represents the amount of oxygen that is maintained in equilibrium in the interstitial fluid by the inflow of oxygenated blood through the microcirculation and consumption by the tissue. On average, the G-K rats had a higher baseline PO$_2$ compared to the Wistar controls. At 10 seconds, when the airbag was inflated and blood flow was arrested, that equilibrium was disturbed. There was no longer oxygen bound to hemoglobin flowing into the area, so the PO$_2$ immediately began to decrease in proportion to the rate of oxygen consumption by the muscle. The initial slope of the line is near linear, indicating that the rate of consumption is not very dependent on PO$_2$ at those levels. As the oxygen tension continues to drop, due to continued consumption without replenishment of oxygen from the blood, the line begins to curve downward exponentially until it reaches approximately zero mmHg. The point at which the line stops behaving linearly marks a critical point at which the tissue consumption rate starts to decrease with decreasing PO$_2$ levels. The difference in the time at initial compression to the time the curve reaches approximately 0 mmHg represents the total amount of time
required for the tissue to consume all the available oxygen in the interstitial space. This ranged from 30-45 seconds in both Wistar and G-K rats.

\[ VO_2 \text{ vs. } PO_2 \text{ Plots} \]

Figures 4 and 5 display the plots of the \( PO_2 \) dependence of oxygen consumption for a Wistar and G-K rat, respectively. The data were fit with Hill's equation to provide a non-linear fit line and several key parameters. \( V_{\text{max}} \) is used to describe the maximum rate of consumption by the given muscle in the tissue region being measured. Another useful parameter for describing this relationship is \( P_{50} \), which is equal to the \( PO_2 \) at half-maximal consumption rate. A higher \( P_{50} \) indicates that the oxygen consumption rate is dependent on a wider range of \( PO_2 \) values. On average, the G-K rats had a higher, but non-significant, \( P_{50} \), compared to the Wistar rats.

\[ Explanation \ for \ Increased \ V_{\text{max}} \ in \ G-K \ rats \]

If the skeletal muscle of diabetic rats consumes oxygen at a higher average maximal rate, one explanation could be that there is an “uncoupling” of \( O_2 \) consumption and ATP production in the mitochondria that leads to less efficient production of ATP and a higher requirement for oxygen. In that case, a given cell would need to consume more oxygen in order to produce the same amount of ATP required for normal cellular processes. This may lead to a higher maximal rate of
oxygen consumption in skeletal muscle at resting conditions. The term “mitochondrial uncoupling” can be used to describe any situation in which the electron transport chain is not used to produce ATP or create proton gradients (Mookerjee SA, 2010). If a situation were to arise in which oxygen was still being consumed through oxidative phosphorylation, but the proton gradient was not being created as efficiently and ATP was not being produced at the same rate, the cell may have to compensate by increasing the rate of respiration. This would make up for the decreased energy production and also consume oxygen at a higher rate, leading to a higher $V_{\text{max}}$.

In related studies, it has been shown that the mitochondria in diabetic rats are smaller and fewer in number (Morino K, 2005). This would logically suggest that overall consumption of oxygen by mitochondria in a particular cell or tissue would be lower than that of a healthy control. In fact, it has been demonstrated that muscle oxidative phosphorylation in diabetic rats was impaired (Kelley DE, 2002, Sivitz, William I, 2016). This appears contradictory to the results of this study, because decreased oxidative phosphorylation should result in less oxygen being consumed by the cell and less ATP being produced.

As described before, the classic view of ATP production involves oxidative phosphorylation and anaerobic glycolysis as separate processes that are carried out independently based on oxygen availability. In physiologically oxygen-rich environments, the cell will choose to produce energy more efficiently through oxidative phosphorylation and conversely, using glycolysis with the production of
lactate in environments where oxygen is unavailable. This either/or scenario fits well with the classic view that mitochondria consume oxygen at a steady rate throughout most of the physiological range until PO₂ drops below about 1 mmHg. This would suggest that oxidative phosphorylation is the sole process producing ATP until PO₂ levels fall to nearly zero.

The in vivo PQM technique used in this project demonstrates that the mitochondria appear to consume oxygen at rates that are dependent on PO₂ over a much wider physiological range. Since the rate of consumption varies based on oxygen tension, it may be the case that oxidative phosphorylation and glycolysis work simultaneously at a ratio that is dependent on PO₂. In situations where PO₂ is decreasing, for example, the cell would increasingly shift to using glycolysis to produce some of its ATP. Furthermore, when oxygen is abundant, the cell would shift to a higher utilization of oxidative phosphorylation. This PO₂-dependent ratio of oxidative phosphorylation and glycolysis differs from the classic view, but is more consistent with the oxygen dependence of respiration described in this project and previous similar studies (Golub, 2012).

The differences in values for half maximal respiration reported in earlier studies (Chance B., 1988) and those reported by Golub and Pittman are likely due to technical limitations. In the older studies, in-vitro techniques were used that involved suspensions of mitochondria or cells to measure oxygen consumption. A variety of error sources were likely introduced that led to vastly different results. Those potential sources of error were dramatically reduced when the in-vivo technique was implemented.
Figure 6. This is an illustration of the possible relationship between oxidative phosphorylation and glycolysis based on variations in PO₂. In this diagram, if PO₂ falls, the utilization of oxidative phosphorylation proportionally decreases as glycolysis increases.
Limitations and Future Studies

Although blood glucose concentrations were measured before and after each experiment, these animals were not fasted and it was not possible to determine how recently they had consumed rat chow. A more controlled eating schedule prior to experimentation may provide more consistent blood glucose levels. To monitor blood glucose, a single glucose reading was taken with the hand held glucometer. A more continuous system to monitor glucose may provide interesting correlations in oxygen consumption and glucose levels.

The rate of blood flow in the muscle was not measured in this project. Since blood flow is directly related to oxygen delivery to muscles, measuring this variable could improve the understanding of oxygen consumption measurements.

Additionally, the muscles studied were at rest. Inducing contraction while measuring PO$_2$ could help show how consumption rates are affected by an actively contracting muscle in healthy and diabetic rats.

It is relevant to note that a relatively small number of muscles were studied. More trials would be beneficial to more strongly validate the results obtained in this project and help clarify the observed differences in G-K and Wistar oxygen consumption characteristics.
The PQM technique used in this study utilized a 1 Hz flash rate, which provided a PO$_2$ reading every second. In future studies, the frequency of the laser flash rate could be increased to provide improved PO$_2$ resolution of the curves describing the PO$_2$ dependence of VO$_2$. This would, of course, require a different correction for the magnitude of photo-consumption by the method. More accurate Hill parameters may come from an increased flash frequency. Additionally, one could study oxygen consumption rate in diseased rats of different ages. The symptoms of the G-K rats used in this study progress over time much like the disease progresses in human diabetic patients. It would be interesting to compare an older, more “end-stage” diabetic rat with higher rates of microvascular complications to a younger rat. Since this method for studying the PO$_2$ dependence of oxygen consumption is relatively new, it would also be beneficial to determine if other chronic diseases such as hypertension or obesity would have an effect on consumption rates.
Conclusion

In conclusion, this project utilized a method of measuring the PO$_2$ of the interstitial fluid in spinotrapezius muscles of diabetic and non-diabetic rats. By arresting blood flow, the rate of oxygen disappearance due to respiration was calculated and differences between diseased and control rats were studied. The maximum rate of consumption for G-K rats was significantly higher than that of the Wistar control rats. The P$_{50}$ for both rat sub-strains demonstrated oxygen consumption rates that depended on PO$_2$ over a wide physiological range. This elevated maximum rate of consumption in spontaneously diabetic rats should be investigated further to help better understand the effect of the disease on cellular respiration and on functional consequences of a mismatch between oxygen supply and demand.
References


Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, Neschen S, White MF.


Appendix

*Wistar VO₂ vs. PO₂ plots*

A – PO₂ (mmHg)

B- VO₂ (nl O₂/(cm³*s))
**Hill Equation**

\[ y = \frac{V_{max} \cdot x^n}{k^n + x^n} \]

**Results**

- **V_{max}:** 94.08 ± 1.85058
- **k:** 5.36901 ± 0.2803
- **n:** 2.1184 ± 0.1184

**Reduced Chi-Square:** 23.38056

**R-Square (COD):** 0.98425

**Adjusted R-Square:** 0.98366

---

**Graphs:**

Two graphs showing the Hill fit of B. Each graph has a scatter plot with the fitted line. The x-axis is labeled as 'A' and the y-axis as 'B'. The data points are represented as squares and the fitted line as a red line. The graphs are labeled as 'B' and 'Hill Fit of B'.
Hill Equation: $y = \frac{V_m a}{x^n \left(1 + \frac{x^n}{k^n}\right)}$

**Plot B**

- $V_m = 68.47308 \pm 2.08507$
- $a = 4.4531 \pm 0.39954$
- $n = 1.96056 \pm 0.22664$

**Reduced Chi-Sqr**: 41.76401

**R-Square (COD)**: 0.95731

**Adj. R-Square**: 0.95573

---

**Plot B**

- $V_m = 155.166 \pm 6.97413$
- $a = 14.97077 \pm 1.27448$
- $n = 1.48901 \pm 0.09317$

**Reduced Chi-Sqr**: 32.51617

**R-Square (COD)**: 0.98668

**Adj. R-Square**: 0.98621

---

**Plot B**

- $V_m = 103.96794 \pm 5.87032$
- $a = 14.89777 \pm 1.27448$
- $n = 1.49001 \pm 0.09317$

**Reduced Chi-Sqr**: 55.0571

**R-Square (COD)**: 0.99668

**Adj. R-Square**: 0.99621
Hill Equation: \( y = V_{\text{max}} x \cdot x^n / (k^n + x^n) \)

- \( V_{\text{max}} = 1.5 \times 10^{-6} \pm 3.4502 \) with 141 data points and goodness of fit 0.9857
- \( k = 1.54658 \pm 0.5602 \) with 141 data points and goodness of fit 0.9857
- \( n = 2.1414 \pm 0.1358 \) with 141 data points and goodness of fit 0.9857

Chi-Square: 26.2183

Reduced Chi-Square: 0.99576

Adjusted R-Square: 0.99517
B
Hill Fit of B

Model
EQUATION = \( y = \frac{V_m x^n}{k^n + x^n} \)

Plotted
\( B_{Vm} = 129.43056 \pm 5.50138 \)
\( k = 11.89494 \pm 1.00319 \)
\( n = 1.50591 \pm 0.10001 \)

Reduced Chi-Sq 29.6418
R-Square (COD) 0.98473
Adj. R-Square 0.98418

/OCC ANALYSIS 04062017/032317/

A

B

/OCC ANALYSIS 04062017/032317/

A
Hill Fit of B

Model: Hill

Equation: $y = \frac{V_{\text{max}} \cdot x^n}{k^n + x^n}$

<table>
<thead>
<tr>
<th>Model</th>
<th>180</th>
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<tbody>
<tr>
<td>Equation</td>
<td>$y = \frac{V_{\text{max}} \cdot x^n}{k^n + x^n}$</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>180.4150 ± 4.88094</td>
</tr>
<tr>
<td>$k$</td>
<td>10.23849 ± 0.34511</td>
</tr>
<tr>
<td>$n$</td>
<td>2.727 ± 0.07218</td>
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<tr>
<td>Reduced Chi-Sqr</td>
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<tr>
<td>R-Square (COD)</td>
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<tr>
<td>Adj. R-Square</td>
<td>0.99675</td>
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/ODC ANALYSIS 04062017/032517/
Hill Equation:

\[ y = \frac{y_{\text{max}} \cdot x^n}{k^n + x^n} \]

**Table:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>( y_{\text{max}} )</td>
<td>117.8 ± 2.65259</td>
</tr>
<tr>
<td>( k )</td>
<td>5.62562 ± 0.19174</td>
</tr>
<tr>
<td>( n )</td>
<td>2.38999 ± 0.11494</td>
</tr>
</tbody>
</table>

**Reduced Chi-Square:** 19.31022

**R-Square (COD):** 0.99246

**Adjusted R-Square:** 0.99218

**/ODC ANALYSIS 04062017/032517/**
Model 1 | Hill
Equation | y = \frac{V_m a x \cdot x^n}{(k^n + x^n)}
Fitted | V_m a x = 144.3094 \pm 2.5327 \times 10^2
slope = 5.4427 \pm 0.2236
\chi^2_{red} = 2.9193 \times 10^{-4}
Reduced Chi-Sqr | 26.3353
R-Square(COD) | 0.9913
Adj. R-Square | 0.9908

/ODC ANALYSIS 04062017/032517/

B

Hill Fit of B

A
Hill Equation $y = \frac{V_{\text{max}}x^n}{k^n + x^n}$

Plot

Reduced Chi-Sqr 25.512 07
R-Square (COD) 0.97618
Adj. R-Square 0.97523

/ODC ANALYSIS 04062017/032517/
Model | Ed
--- | ---
Equation | \(y = \frac{V_{max} \cdot x^n}{k^n + x^n}\)
B | 8
\(V_{max}\) | 7.65 (7.56 ± 0.49) E3
\(k\) | 4.5 (5.04 ± 0.50) E1
\(n\) | 1.9 (8.03 ± 0.80) E1
Reduced Chi-Sq | 0.975
R-Square(COD) | 0.97646
Adj. R-Square | 0.97555
Goto-Kakizaki VO$_2$ vs. PO$_2$ plots

A – PO$_2$ (mmHg)

B- VO$_2$ (nl O$_2$/(cm$^3$s))

/ODC ANALYSIS 04062017/030817/

B

Hill Fit of B

/ODC ANALYSIS 04062017/030817/

B

Hill Fit of B
Hill Fit of B

Model: Hill Equation
\[ y = \frac{V_m a x * x^n}{(k^n + x^n)} \]

Parameters:
- \( V_m a x = 122.96792 \pm 3.85645 \)
- \( a = 7.00874 \pm 3.58912 \)
- \( k = 1.98685 \pm 0.57970 \)
- Reduced Chi-Sqr = 0.97245
- R-Square(COD) = 0.97041
- Adj. R-Square = 0.97251

/ODC ANALYSIS 04062017/030917/
Hill Equation:

\[ y = \frac{V_{ma}x^*}{x^n + k^n} \]

- \( x^* = 97.7947 \pm 1.1885 \times 10^{-9} \)
- \( k = 1.12324 \pm 0.01393 \times 10^{-1} \)
- \( n = 2.3 \times 10^{-7} \)
- \( \text{Reduced Chi-Sqr} = 2.93 \times 10^{-7} \)
- \( R^2 = 0.96175 \)
- \( \text{Adj. R-Square} = 0.96056 \)

Chi-Square (COD): 67.54468

R-Square: 0.97651

Adj. R-Square: 0.97566
Hill Equation:

\[ y = \frac{V_{\text{max}} \times x^n}{k^n + x^n} \]

- **B**
- **Hill Fit of B**

**Data Table**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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<tr>
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<tr>
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<td><strong>Reduced Chi-Sqr</strong></td>
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<td><strong>R-Square (COD)</strong></td>
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<td><strong>Adj. R-Square</strong></td>
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- **A**
- **ODC ANALYSIS 04062017/032217**

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<table>
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<td><strong>Reduced Chi-Sqr</strong></td>
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<td><strong>Adj. R-Square</strong></td>
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</table>

- **A**
- **ODC ANALYSIS 04062017/032217**
Vita

Alexander Cameron Liles was born on June 18, 1991 in Newport News, Virginia. He graduated from Menchville High School in June, 2009. He received his Bachelor of Science from Virginia Polytechnic Institute and State University in May, 2013 and went on to receive a Master of Science in Physiology and Biophysics from Virginia Commonwealth University in May, 2017.