2017

Is Mitochondrial Development Impaired in Hyperoxic Rats and does this Underpin the Blunting of the Acute Hypoxic Ventilatory Response?

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IS MITOCHONDRIAL DEVELOPMENT IMPAIRED IN HYPEROXIC RATS AND
DOES THIS UNDERPIN THE BLUETING OF THE ACUTE HYPOXIC VENTILATORY
RESPONSE?

A thesis submitted in partial fulfillment
of the requirement for the degree of
Master of Science

By

TARIQ HASAN FAYYAD
B.S., University of Cincinnati, 2014

2017
Wright State University
WRIGHT STATE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

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APRIL 27, 2017

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Tariq Hasan Fayyad ENTITLED Is Mitochondrial Development Impaired in Hyperoxic Rats and does this Underpin the Blunting of the Acute Hypoxic Ventilatory Response? BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF Master of Science.

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Carotid body (CB) responses to hypoxia are low at birth and increase over time to mature responses. Using an in vitro rat CB-carotid sinus nerve (CSN) preparation, Kholwadwala and Donnelly (1992) demonstrated that the CSN activity in response to hypoxia increased from low levels to robust adult responses after two weeks. This time course of maturation was paralleled by an increase in TASK channel sensitivity to hypoxia in the O2-sensing Type I cells (Kim et al, 2011). Previous studies have indicated that a fall in Type I cell mitochondrial volume and an increase in the rate of oxidative phosphorylation may underpin the development of the hypoxic ventilatory response (Paulet et al, 2012).

Previously, the lab has seen a significant reduction in mitochondrial volume during development in normoxic reared rats. Our most recent studies addressed the phenomenon that hyperoxia delays the maturation of the acute hypoxic response (Bavis, RW et al, 2010) and that this may be via an effect on type I cell mitochondria. However, contrary to Paulet et al, our findings demonstrated that in control conditions mitochondrial volume did not fall during development. This made interpretations of the hyperoxia experiments impossible.
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I: INTRODUCTION

HISTORY

The carotid body was named in the late 1600's ascribed by Jakob Winslow (Fitzgerald, 1996). The “ganglion minutumm” was further discussed in a dissertation published in 1743 by Hartwing Wilhelm Ludwig Taube while having Albrecht von Haller as an instructor. The glomus carotid, now commonly referred to as the carotid body, was identified as “gland” in the 18th century by Hubert Luschka (McDonald, 1981). Henle, along with the Weber brothers, performed cardiac frequency experiments on the vagus nerve a century later. These experiments led to the foundation of future research for the fundamentals of cardio-respiratory reflexes. For over 50 years, the opinions suggested by Pagano and Siciliano in 1900 that the carotid-region is where the cardio-respiratory reflexes generated from were neglected. Heinrich Hering then proved this to be correct by demonstrating that stimulation of the carotid sinus provokes atrial hypotension and bradycardia. This proved that these reflexes are generated from the carotid region, which is individualistic from the central nervous system (CNS). (taken from review by De Castro, 2009(a)).

Simultaneously, Corneille Heymans and his father, Jean-Francois Heymans, made the remarkable discovery that hypertensive bradycardia is a reflex mechanism independent of the CNS. This finding led to increased interest in the field of carotid body research in Spain where Fernando de Castro and Santiago Ramón y Cajal began publishing fundamental anatomo-histological studies, which, subsequently shaped the entire field of carotid research. In 1926, de Castro published drawings attempting to
describe the intricate structure of the glomus caroticum now known as the carotid body (Figure 1). De Castro was able to show that the carotid body had multiple different cells and provided evidence supporting baro-receptor presence with the carotid sinus. (Fitzgerald and Lahiri, 1996)
Figure 1- Original drawings de Castro made representing the carotid region. (A) part of the glossopharyngeal nerve innervating onto the carotid body; (B) colored nuclei represent Glomic cells of a young human; (C) the innervations of the carotid body including glomeruli, carotid artery, sympathetic nerve, superior cervical ganglion, vagus nerve, intercarotid nerve, glossopharyngeal nerve, and sympathetic microganglion; (D) intercarotid nerve showing sympathetic microganglia. (taken from review by de Castro, 2009(b)).
Four years later, Heymans and colleagues demonstrated respiratory reflexes due to changes in arterial pressure. This series of experiments led to the recognition that different respiratory responses can be effected by the carotid body. Importantly, Heymans was able to demonstrate that hyperventilation is driven by the carotid bodies. The carotid arteries from dog I (see Figure 2) were perfused to the carotid arties of dog II by means of anastomoses. Following the procedure, dog I was exposed to hypoxic air which prompted dog II to hyperventilate. This was demonstrated by an abnormal rapid rate of breathing. This parabiosis experimentation with dogs (Figure 2) was innovative and the data obtained from it led to Corneille Heymans nomination for the Noble Prize in Physiology or Medicine in 1938, despite not receiving it until December 12th, 1945 due to World War II.
**Figure 2** - Corneille Heymans and father Jean-Francois Heymans perfused the carotid arteries from two dogs together. Dog A was given hypoxia gas whereas dog B was not, however due to the cross perfusion of the dogs’ blood supply, the carotid bodies of dog B were stimulated, causing hyperventilation. (A) dog given hypoxic conditions; (B) dog that underwent hyperventilation; (1) head which was isolated of dog B; (2) isolated body of dog B; (3) trachea; (4) right vagus nerve; (4’) left vagus nerve; (5 & 5’) external jugular veins attached; (6 & 6’) both common carotid attached to each other; (7 & 7’) recording femoral blood pressure; (8) representation of hyperventilation by respiratory movements of the isolated head. (Heymans, 1965).
ANATOMY

The carotid body is located at the bifurcation of the carotid arteries (Figure 3) on either side of the throat. These sensory organs are made up of clusters of chemoreceptors that evoke postsynaptic signaling due to changes in arterial blood gases. The carotid body stimulates the carotid sinus nerve (CSN). This projects to the glossopharyngeal nerve “ninth cranial nerve (CN IX)” and then to the petrosal ganglion (PG). Signals are then sent to the nucleus tractus solitarii (NTS) located within the brain stem. This subsequently causes respiratory changes to maintain blood gas homeostasis. (Finley & Katz, 1992; Gonzalez et al. 1994).

The tissue of the carotid body predominantly consists of two types of cells, type I and type II. Type I cells are the oxygen sensing cells, which are also referred to as glomus cells, and are derived from the neuroectoderm. Type II cells are sustentacular cells that support the glomus cells and are more glial like. Once arterial blood gas changes occur within the carotid artery, the glomus cells depolarize opening voltage gated calcium channels. The influx of Ca\(^{2+}\) initiates release of neurotransmitters sending signals to the CNS. (Prabhakar, 2000)
**Figure 3 – Schematic identifying the Carotid Body.** (A) The carotid body located between the internal carotid (IC) and external carotid (EC) arteries. The chemoreceptor organ is at the bifurcation of the common carotid (CC) artery. (B) The organization of cells within the carotid body. Type II cells contain type I cells which depolarize to release neurotransmitters postsynaptically. (Peers et al. 2010).
TYPE I CELL SIGNAL TRANSDUCTION

Glomus cells (type I) are the main place for sensory transduction in the CB. In response to acidity, hypoxia or hypercapnia, CB type I cells are depolarized by inhibition of K⁺ channels causing an increase in [Ca²⁺]ᵢ initiating neurotransmitter release which directly affects the CSN. Fundamentally, this is how blood gas homeostasis is corrected during acute perturbations. In rats two type of chemosensitive K⁺ channels are present in the type I cells: high-conductance Ca²⁺ activated K⁺ (BK) channels (Peers, 1990) and TASK-like K⁺ channels (Buckler, 1997). Two major hypotheses exist that address how K⁺ inhibition occurs at the level of the type I cells: A membrane hypothesis and a mitochondrial hypothesis.

MEMBRANE HYPOTHESIS

The direct consequence of hypoxia on peripheral chemoreceptor CB type I cells is depolarization of the cell membrane potential, which is due to inhibition of K⁺ channels (Lopez et al. 1989). This further initiates an increase in [Ca²⁺]ᵢ causing NT to be released postsynaptically (Figure 4). The type of K⁺ current inhibited is species specific. For example, in rat glomus cells hypoxia inhibits large-conductance Ca²⁺ activated K⁺ channels whereas in rabbit, transient K⁺ currents are inhibited (Prabhakar, 2000). Interestingly hypoxia caused inhibition of K⁺ currents in isolated membrane patches of rabbit cells, but not in rat cells suggesting a direct consequence of low oxygen on the channel protein in rabbits but not rats (Wyatt and Peers, 1995) (López-Barneo, 1996). Two major types of K⁺ channels will be discussed.
High conductance Ca$^{2+}$ dependent K$^+$ (BK) channels are fundamental to the type I cell response to hypoxia (Peers, 1990). Inhibition of BK channels open at resting membrane potential by hypoxia will cause type I cell depolarization. However conflicting data exists, type I BK channels are not sensitive to hypoxia in an outside-out patch clamp set up (Wyatt and Peers, 1995) whereas (Riesco-Fagundo et al. 2001) showed that in the same species using a whole-cell and an inside-out configuration of the patch-clamp technique that BK channels are sensitive to hypoxia. Importantly Wyatt and Peers showed that some other cytosolic factor is aiding in the process of channel inhibition. Arguments are ongoing as to who is correct (Wyatt, personal communication).

TASK-like channels were initially discovered to be voltage-insensitive (Buckler, 1997) and are another type of O$_2$ sensing K$^+$ channels in CB type I cells, also found open at resting membrane potential. These TASK-like channels had similar properties to TASK channels expressed elsewhere (Buckler et al. 2000). TASK-1, TASK-3 and a heteromer “TASK-1/TASK-3” are all expressed in type I cells. The majority of K$^+$ current is due to the heteromer (Kim et al. 2009). Hypoxia and mitochondrial inhibitors all inhibit TASK channel activity. More importantly, these channels are sensitive to mitochondrial activity. Therefore, mitochondrial activity is necessary for inhibition of TASK-like channels in type I cells (Buckler et al. 2006). This further initiates an increase in [Ca$^{2+}$]$_i$, causing neurotransmitters to be released (Wyatt et al. 2007).
MITOCHONDRIAL HYPOTHESIS

Oxidative phosphorylation allows mitochondria to produce ATP. In cellular respiration, oxygen serves as the final electron acceptor. This mechanism is fundamental for energy production during times of decreased or absent oxygen. The role that oxygen plays in cellular metabolism led to the development of the mitochondrial hypothesis of chemoreception in the early 1960s (Anichkov and Belen’iï, 1963). One of the reasons oxygen sensing occurs may simply be due to the low affinity mitochondria has for oxygen within its complexes (Mills and Jabsis, 1972). This low affinity that mitochondria has for oxygen further allows oxygen sensing to occur at the level of the CB. Researchers at the University College London showed that mitochondria’s response to hypoxia in type I cells are specialized by identifying that the mitochondrial membrane depolarizes at 60 mm Hg O₂. However, this does not occur until around 5 mm Hg O₂ in dorsal root ganglion cells of sensory neurons (Duchen and Biscoe, 1992). Thus, type I cell mitochondria are unusually sensitive to hypoxia.

Hypoxia’s effects can be similarly duplicated using pharmacological compounds, which inhibit the mitochondrial electron transport chain or ATP synthase. Cyanide is an example of a mitochondrial electron transport inhibitor, which was identified as a potent carotid body stimulant (Heymans et al. 1931). Oligomycin is an example of an ATP synthase inhibitor which also prevents the production of ATP (Wyatt and Buckler, 2004).

How CB type I cell K⁺ channels are inhibited by hypoxia is unknown but mitochondria may be mediating the inhibition. The signaling pathway is still unknown however, one possible hypothesis is that when ATP levels are decreased, K⁺ channels
are inhibited. This may suggest that ATP can regulate these TASK channels (Varas, Wyatt and Buckler, 2007). A second major theory is that when ATP levels are low AMP/ATP ratio are increased, therefore increasing the activation of AMPK (Evans, 2006). This then causes inhibition of K⁺ channels (Figure 4) further initiating an increase in [Ca²⁺], causing neurotransmitters to be released postsynaptically (Wyatt et al. 2007). However, recent studies have shown that that genetic deletion of AMPK does not alter the CB activation due to hypoxia, suggesting that deletion of AMPK is blocking the hypoxic ventilatory response at another point in the respiratory network. (Mahmoud et al. 2016)
**FIGURE 4 – Schematic identifying the two possible hypotheses of how the CB senses oxygen.** (A) Mitochondrial hypothesis; ATP levels may be inhibiting plasma membrane K⁺ channels to depolarize the cell. Also mitochondria (red circle arrow A is projecting to) may be the underlining factor for oxygen sensing in the CB. (B) Membrane hypothesis; TASK channels and BK channels are inhibited once the carotid body senses changes in O₂, CO₂ or pH. Once the cell is depolarized (represented by blocked K⁺ channels) Ca²⁺ channels open causing an increase in [Ca²⁺]. Neurotransmitters are then released (represented by small gray vesicles) onto the carotid sinus nerve. Signals are then sent to the CNS to correct the control of breathing to achieve homeostasis.
NEUROTRANSMITTERS

CB type I cells release a variety of neurotransmitters once \([\text{Ca}^{2+}]_i\) levels have increased. Once the neurotransmitters are released a majority of them act on the carotid sinus nerve. The following neurotransmitters are released from the CB: Acetylcholine (Metz 1969; Fitzgerald and Shirahata 1993; Nurse, 2005), Adenosine (Ribeiro and Walker, 1975; McQueen and Ribeiro, 1981; Shirahata et al. 2007), ATP (Lopez-Barneo et al. 1988; Zhang et al. 2000; Gauda, 2002), Dopamine (Mills et al. 1978; Alcayaga et al. 2016), GABA (Oomori et al. 1994; Fearon et al. 2003; Nurse, 2014), Histamine (Koerner et al. 2004; Del Rio et al. 2008) and Serotonin (Hellstrom and Koslow, 1975; Ramirez et al. 2012; Nurse, 2014).

DEVELOPMENT OF THE HYPOXIC RESPONSE

In 1985, rat CB type I cells were for the first time isolated and stimulated with hypoxia while calcium influx was measured. (Pietruschka, 1985). The maturation of CB type I cells between juvenile to mature state is critical for development of the hypoxic response. Noninvasive experiments, which recorded breathing patterns of rats pups, identified that within the first (2-3) days post birth, newborns demonstrate a small biphasic respiratory response to acute hypoxia. Whereas in adult rats, obvious hyperventilation was observed and measured (Eden & Hanson, 1986). By performing an in vitro CB-CSN experiment, maturation of rat CB was identified to be within the first and second week after birth. This was demonstrated by weak CSN firing within the first six days and high CSN firing post six days in response to hypoxia (Kholwadwala and Donnelly, 1992).
Eager to understand where the O$_2$ sensing occurred, a group of researchers looked at the hypoxic response in type I cells by measuring intracellular Ca$^{2+}$, pH and performing electrophysiology. The development of O$_2$ sensitivity observed in isolated type I cells paralleled Kholwadwala and Donnelly's findings in 1992 (Bamford et al. 1999). TASK-like channels were studied during juvenile and mature states and no biophysical or pharmacological differences in the TASK channel properties were found. However, in juvenile cells the channels were unresponsive to O$_2$ whereas channels in mature cells were O$_2$ sensitive (Kim et al. 2011). Therefore, the TASK-like channels are not intrinsically O$_2$ sensitive and the oxygen sensing mechanism that inhibit them must be developing over this timeframe.

**WYATT LAB RESULTS**

Previous studies in the Wyatt lab have demonstrated a significant reduction in mitochondrial volume from juvenile to mature rat CB type I cells. During development this fall in mitochondrial volume (Figure 5) was not paralleled by a fall in metabolic rate (Figure 6), indicating an increase in mitochondrial oxidative phosphorylation in mature cell mitochondria. These findings suggested that a change in mitochondrial volume during development may play a crucial role in the mechanism by which type I cells detect changes in O$_2$ (Paulet et al. 2012).
Figure 5 – Total cell volume and percent volume of mitochondria relative to cell size in juvenile and mature carotid body Type I cells. A. Bar chart showing differences in cell volume between juvenile and mature carotid body Type I cells (**P<0.00002). B. Bar chart showing differences in the percent of cell volume occupied by mitochondria between juvenile and mature carotid body Type I cells (**P<0.00001). Ci. 3D reconstruction of deconvolved Deltavision images for an example juvenile Type I cell (Mitotracker, orange; Hoechst 33342, blue; CellTracker, green). Cii. Imaris rendered 3D reconstruction of the cell in Ci. Ciii. 3D reconstruction of deconvolved DeltaVision images for an example of mature Type I cell (Mitotracker, orange; Hoechst 33342, blue; CellTracker, green). Civ. Imaris rendered 3D reconstruction of the cell in Ciii. Scale bar is 1.5 μm in all images.
Figure 6 – Comparison of resarufin production in Type I CB cells from juvenile and mature rats. Graph represents change in fluorescence in isolated Type I cells at room temperature in HEPES solution after addition of 10μM C_{12}-resazurin dye measured with 2-photon confocal microscopy. No significant differences in resarufin production were observed between juvenile (n = 140) and mature (n = 57) Type I cells despite the fall in mitochondrial volume in mature cells.
HYPEROXIA

The amount of glomus cells decrease within the carotid bodies when neonatal rats are reared in hyperoxic (60% O₂) environments (Wang and Bisgard, 2005). Hyperoxia has also been shown to blunt the development of the acute hypoxic ventilatory response (Bavis et al. 2010) at the level of the whole animal and single cell. However, the effect of hyperoxia on mitochondrial content of type I cells during the O₂ sensitivity developmental period has not been studied.

HYPOTHESIS

This thesis will test the hypothesis that type I cell mitochondrial development is impaired in hyperoxic rats between a juvenile and mature state and that may underpin the blunting of the acute hypoxic ventilatory response. To test this, we studied animals raised in hyperoxia and normoxia then observed measured changes in type I cell mitochondrial volume and intracellular Ca²⁺ between control and experimental groups.

SUMMARY

The CBs are located at the bifurcation of the carotid artery and are the primary peripheral chemoreceptor in the body. CB type I cells detect changes in O₂, CO₂ and pH within arterial blood. When alterations away from homeostasis are detected in arterial blood, type I cells depolarize initiating neurotransmitter release. This causes a cascade of signals to the CNS to correct the control of breathing in order to obtain homeostasis once again. To investigate if hyperoxia could blunt the hypoxic response via an effect on type I cell mitochondria, following experimental paradigm (Figure 7) was developed.
Every time-pregnant (E15) Sprague-Dawley rat was estimated to give birth to approximately 12-14 pups. Once the rat gave birth, one-third of the litter was used at days 7, 14 and 21. At each stage of the experiment the carotid bodies were obtained and digested. Half of the digested cells were used for immunohistochemistry and the remaining half was used for Ca$^{2+}$ imaging. This was done at least three times each (six pregnant rats) for control groups (21 % O$_2$) and experimental groups (40 – 50 % O$_2$). (Figure 7).
Figure 7 – Experimental timeline. At day 7, 14, and 21 CBs were obtained from neonatal and mature Sprague-Dawley rats under control (21 % O₂) or experimental (40 – 50 % O₂) conditions.
1 Rat gives birth = 12 - 14 pups

Day 7

Day 14

Day 21

21% Oxygen

≈ 12 pups

Ca²⁺ Imaging

Immunohistochemistry

≈ 4 pups

Repeating 3 times.

40 - 50% Oxygen

≈ 12 pups

Ca²⁺ Imaging

Immunohistochemistry

≈ 4 pups

Repeating 3 times.
II: MATERIALS AND METHODS

All experiments and handling of pregnant, neonatal and mature rats were approved and conducted under accordance with Wright State University's Institutional Animal Care and Use Committee (IACUC).

PREPARATION FOR NEONATAL PUPS PRIOR TO EXPERIMENTATION

In order to ensure that the rat cortisol levels have reduced to unstressed levels, the pregnant rats were delivered on embryonic day fifteen (E15). This ensured that the pregnant rat was given three days to reduce any stress and anxiety that may have occurred during transportation ensuring cortisol levels have decreased from arrival date. Post acclimatization, the pregnant rat was either placed into a hyperoxic chamber (experimental) or left in a cage outside of the chamber (control) prior to pregnancy and for the remainder of the experiment.

HYPEROXIC CHAMBER APPARATUS

A flow through hyperoxic (≈ 40-50 % O₂) chamber (20.5" x 20", BioSpherix) was built and used to house all experimental animals. As represented by Figure 8, pre-purified compressed Nitrogen was mixed with compressed Oxygen using a gas mixer (Union Carbide). The mixed gas was then bubbled in a jar filled with distilled water and then released within the chamber. The oxygen level was measured using an oxygen microsensor (World Precision Instruments) attached to a microsensor oxygen meter.
(World Precision Instruments). This digitally showed the oxygen level via OxyMicro software (V2.00, World Precision Instruments) with a one-second sampling rate to ensure hyperoxic conditions. Relative humidity ($\approx 40 - 70\%$) and temperature ($\approx 70 - 78$) within the chamber was monitored with a digital meter (ThermoPro). The hyperoxic chamber also contained a dehumidifier (Eva-Dry).
**Figure 8 - Schematic of the hyperoxic chamber** that housed all experimental animals with approximately 40-50 % O₂.
CAROTID BODY TYPE I CELL DISSECTION FROM NEONATAL AND MATURE RATS

Due to the failure to achieve successful cell culture lines of carotid body type I cells, isolated primary tissue is critical for execution of physiological experiments. The number of pups produced during pregnancy resulted in how many pups were used in each stage of the experiment. Approximately three to five juvenile neonatal Sprague-Dawley rats (6-8 day old) were taken from the hyperoxic chamber (∼ 40-50 % O₂) or the control cage (21% O₂, room air) and were individually placed in an anesthetic induction chamber (1/3rd of total litter size). This allowed the rats to succumb to anesthesia (4.5 % isoflurane in oxygen at 0.9L.min⁻¹).

Following unconsciousness, which was tested by a tail or foot pinch withdrawal reflex, the rat was taken from the induction chamber and placed supine on a surgical board underneath a dissecting microscope (Omâna, Japan) with all four limbs taped to the board. This was followed immediately by placing a nose cone on the rat which continued delivery of the anesthetic to the animal. To confirm depth of anesthesia prior to surgery another tail or foot pinch was performed.

Surgery began with an initial transverse incision along the plane of the clavicles, superior to the sternum exposing the subcutaneous fascia using Noyes spring loaded scissors (14 mm straight cutting edge by Fine Science Tools). Bi-laterally removing the skin with Dumont #5 straight forceps (0.08 x 0.04mm by Fine Science Tools) followed by another sagittal midline incision allowed for exposure of the lateral tracheal muscles along with the salivary glands. Scrupulous attention to the surgery was especially required here to prevent a puncture in any of the surrounding blood vessels.
On the peripheral sides of the trachea a triangle of muscle can be identified, this landmark is superior to the common carotid arterial bifurcation. The landmark is formed by omohyoid, sternomastoid and sternohyoid muscles. Under low magnification, the hypoglossal nerve is located and aided in dissection to the carotid bifurcation. Location of the occipital artery by further removal of connective tissue helps in reassurance of the carotid bifurcation. At this point, the carotid body is removed by cutting the occipital artery and removing it off of the internal carotid artery to complete the in vivo surgery.

Following immediate removal, the carotid body was placed in a 35mm x 10mm petri dish (Fisher Scientific) with 3mL of ice-cold Dulbecco's phosphate buffered saline (DPBS) without MgCl₂ and CaCl₂ (Sigma-Aldrich). Following the completion of surgery, the rats were promptly euthanized by decapitation. The bodies were disposed of according to Wright State University's Laboratory Animal Resources (LAR) protocol and specifications. This entire process was then repeated with mature rat pups at days (13-15) and (20-22) for the remaining experimental and control litters.

IMAGING CAROTID BODIES

Juvenile and mature CBs were immediately imaged post dissection in 35mm x 10mm petri dish (Fisher Scientific) with 3mL of ice-cold Dulbecco’s phosphate buffered saline (DPBS) without MgCl₂ and CaCl₂ (Sigma-Aldrich). The microscope (MVX10, Olympus) was manually set to 2.5x zoom for all images. Desired white light (B&B Microscopes, LTD) was achieved by manually setting the light source to a strength of approximately 5. Images were captured using a c mount camera (MicroFire™ A/R,
Optronics®) that displayed the images on the computer using software (Application 2.2, PictureFrame™).

DISSOCIATION OF TYPE I CELLS FROM NEONATAL RATS

Upon successful dissection of experimental or control rat CBs, the excess tissue around the dissected carotid bodies were removed punctiliously using Dumont #5 straight forceps and a dissection microscope (Omâna, Japan). The cleaned up carotid bodies post excess connective tissue removal were transferred to another petri dish (35mm x 10mm, Fisher Scientific) which contained 3mL of digestive enzyme solution (refer to solutions section, p.46) for a 16-minute incubation at 37ºC, 5% CO₂, 21% O₂, Bal N₂ (HERAcell 150i incubator, Thermo Scientific). The carotid bodies were then slowly teased and partially extracted away from connective tissue and then incubated one last time under the same conditions for 5 minutes before complete extraction. Upon successful extraction the carotid bodies along with the remainder of connective tissue were triturated using a Borosilicate glass pasture pipette (Fisher Scientific) with a fire-adjusted tip. The pasture pipettes were then coated with Sigmacote (Sigma-Aldrich) to act as a siliconizing reagent. They were then used to transfer the carotid bodies into the 15mL centrifuge tube (Fischer Scientific) where they were further triturated for a couple of minutes. Following trituration, the cells were centrifuged at 200 x g (770 rpm) for five minutes (GPR Centrifuge, Beckman). Supernatant was then removed and 1mL of growth medium (refer to solutions section, p.46) was added to the tube. The cells were gently triturated with the growth medium.
The cells were centrifuged at 200 x g (770 rpm) for another five minutes. The supernatant was then removed followed by one last trituration.

**DISSOCIATION OF TYPE I CELLS FROM MATURE RATS**

The dissociation for mature rat carotid bodies follows the aforementioned neonatal dissociation protocol except with alterations with the time of digestion. The time for digestion for mature rat CBs is increased due to more connective tissue in the (13-15) and (20-22) day old rats. The only alteration is that the first digestion is increased to 20 minutes and the second digestion time is increased to 6 minutes.

**IMMUNOHISTOCHEMISTRY FOR FIXED SLIDES**

After the carotid bodies were dissociated, 22mm x 2mm (1mm thick) cover slips (12-542-B, Fisher Scientific) were prepared for immunohistochemistry by first coating them with Poly-D-Lysine hydrobromide (Sigma) and washing twice with distilled autoclaved water. Once dried, 10-30μL of dissociated cells were placed on the center of the coverslip using a Sigmacoted (Sigma-Aldrich) borosilicate glass pasteur pipet (5 3/4th, Fisher Scientific). The coverslips were then incubated for a minimum of two hours at 37°C, 5% CO₂, 21% O₂, balance N₂ (HERAcell 150i incubator, Thermo Scientific) to allow the cells to adhere to the coverslip.

The coverslips which contain adhered cells with 10-30μL of growth medium were tipped off using Dumont #5 straight forceps (0.08 x 0.04mm by Fine Science Tools) and washed three times at 5 minutes each with 300μL of DPBS (Dulbecco’s
Phosphate Buffered Saline) with MgCl$_2$ and CaCl$_2$ (D8662, Sigma). 300μL of CellTracker™ Green CMFDA (Invitrogen) mixed in a cocktail (refer to solutions section, p.47) and placed on the washed coverslip in a dark area for 30 minutes at room temperature. CellTracker™ Green was then tipped off and the slides were washed three times with 4 % para-formaldehyde (Sigma) in 1x PBS (Sigma) for 5 minutes each. 300μL of a cocktail solution (refer to solutions section, p.47) containing Tom20 (sc-11415, D0616, Santa Cruz) was placed on the slides and incubated for 24 hours at 4°C. The slide was then washed four times at 5 minutes each using a triton BSA solution (refer to solutions section, p.47). 300μL of a secondary antibody cocktail (refer to solutions section, p.48) including Rhodamine Red™ (110471, Jackson ImmunoResearch) were placed on the slide and incubated for 2 hours at 4°C. The slide was then washed five times, 5 minutes each with DPBS with MgCl$_2$ and CaCl$_2$. Lastly, 10-30μL of Vectashield® with DAPI (Vectashield®, Vector Laboratories) were placed on 25mm x 75mm (1mm thick) slides (Micro slides, VWR International) and the coverslip then adhered to the slides via. Vectashield and were incubated for 15 minutes at 4°C in the dark. Finally nail polish was used to seal the border of coverslips on the slides.

IMMUNOHISTOCHEMISTRY FOR LIVE CELL SLIDES

After the carotid bodies were dissociated, a 35mm FluoroDish™ (World precision instruments) with a 23mm well diameter was prepared for immunohistochemistry by first coating it with a Poly-D-Lysine hydrobromide (Sigma) and washing it twice with distilled autoclaved water. Once dried, 10-30μL of dissociated
cells were placed on the center of the coverslip using a Sigmacoted (Sigma-Aldrich) borosilicate glass pasteur pipet (5 3/4th, Fisher Scientific). The coverslips were then incubated for a minimum of two hours at 37°C, 5% CO₂, 21% O₂, balance N₂ (HERAcell 150i incubator, Thermo Scientific) to allow the cells to adhere to the coverslip. All dyes were prepared in a standard HEPES buffered salt solution cocktail which consisted of CellTracker™ Green CMFDA (Invitrogen), Mitotracker® Red CMXRos (Invitrogen) and Hoechst 33342 (Invitrogen). Refer to solutions section, p.46-48.

IMAGING & DECONVOLUTION PROCESS

A confocal microscope (Observer.Z1, Zeiss) was used for imaging live and fixed cells. The images were taken from the inverted microscope with oil immersion at the magnification of 63x, a numerical aperture of 0.55 and a working distance of 26mm. Very high luminance with a mercury lamp (HXP 120 C, Zeiss), a halogen illuminator (HAL 100, Zeiss), a camera (AxioCam MRm, Zeiss) along with an optical sectioning (ApoTome.2, Zeiss) device allowed for high quality 3D images. The fluorescent stains used varied between live and fixed cell imaging. Table 1 identifies the different stains and wavelengths for each staining technique. The exposure time varied among channels and cells but were around 50-100ms per channel.
**Table 1 - Live vs. Fixed Cell Staining.** (A) For fixed cell imaging Rhodamine Red™, CellTracker™ Green CMFDA and Vectashield® with DAPI were used to stain isolated CB type I cells. (B) For live cell imaging Mitotracker® Red CMXRos, CellTracker™ Green CMFDA and Hoechst 33342 were used to stain isolated CB type I cells.
A) **Fluorescent Stain**  
- **Staining**  
  - Mitotracker® Red  
  - CellTracker™ Green  
  - Hoechst  
- **λ: Ex/Em**  
  - Mitochondria: 579/599 nm  
  - Cytoplasm: 492/517 nm  
  - Nucleus: 350/461 nm

B) **Fluorescent Stain**  
- **Staining**  
  - Rhodamine Red™  
  - CellTracker™ Green  
  - Vectashield® with DAPI  
- **λ: Ex/Em**  
  - Mitochondria: 570/590 nm  
  - Cytoplasm: 492/517 nm  
  - Nucleus: 360.460 nm
Once an isolated CB type I cell was acquired the range and number of Z-stack slices were set by obtaining first and last positions of the cell using the focus feature on the microscope. The interval per z-stack was calculated to be .24 µm using the standard depth of field equation $d_{tot} = \frac{\lambda \cdot n}{NA^2} + \frac{n}{M \cdot NA} e$ represented in Figure 9 by a gray box intersecting the cell. Lastly, using the same software (Zen 2 Blue Edition, Zen), the 3D images were deconvolved; an algorithmic process in which light is refocused onto each individual Z-stack slice to form a crisper image.
Figure 9 - Schematic of Obzerver.Z1 inverted microscope that allowed for imaging stained cells with the Z-step (.24µm). The numerical aperture was .55. All images were taken at 63x zoom.
Light source

Z-step
.24µm

Cell

Slide

Oil Immersion
1.4

N.A = .55

63X
IMARIS

Deconvolved images were then imported into IMARIS (8.0.0, Bitplane Scientific) software. The different stains were used to mask the individual volumes of the cytoplasm, mitochondria and nucleus. The masked areas were then individually used to recreate the cytoplasm, mitochondria and nucleus of type I cells which formed reconstructed 3D images. Cell volumes along with the percent of cell volume occupied by mitochondria were analyzed between experimental and control CB type I cells at different developmental ages of (6-8), (13-15) and (20-22) days old.

PREPARATION OF SLIDES FOR CALCIUM IMAGING

After the carotid bodies were dissociated, 15 mm cover slips (CS-15R, Warner Instruments) were prepared for calcium imaging by first coating them with Poly-D-Lysine hydrobromide (Sigma) and washing twice with distilled water. Once dried, 10-30 μL of dissociated cells were placed on the center of the coverslip using a Sigmacoted (Sigma-Aldrich) borosilicate glass pasteur pipet (5 3/4th, Fisher Scientific). The coverslips were then incubated for a minimum of two hours at 37°C, 5% CO₂, 21% O₂, balance N₂ (HERAcell 150i incubator, Thermo Scientific) to allow the cells to adhere to the coverslip.

CALCIUM IMAGING

In order to look at intercellular calcium of CB type I cells, the prepared slides were attached to the bottom of a perfusion chamber (RC -25F, Warner Instruments)
using high vacuum grease (Dow Corning®). This allows for a diamond shaped recording chamber to be filled with premade normoxic and hypoxic physiological solutions (refer to solutions section, p.46). The solutions were heated to 36±1°C with water baths (180 Series, Thermo Scientific) and were gravity fed through an in-line heater (SH-27F, Warner Instruments) which is controlled with a temperature controller (TC-344B, Warner Instruments). The desired heated solution flows into the recording chamber with a bath volume of approximately 500µL, subsequently being removed into a beaker with a peristaltic pump (RP-1, Rainin Dynamax) at 8mLminute⁻¹.

An inverted microscope was used to obtain visualization of cells (TE2000-U, Nikon) at 40x objective lens (1.30 oil, ∞/0.17, WD – 0.22, S Flour Nikon) with immersion oil (Type-F, Olympus). Digital imaging was performed using Metafluor imaging software (7.1.2, Molecular Devices). The loaded cells were exposed to 340/380 nM light for 50msec to achieve excitation at .2 Hz. Xenon arc lamp (Lamda-LS, Sutter Instruments) generates white light at 175 watts through a filter wheel (Lamda 10-3, Sutter Instruments) that has specific filters (Chroma) for 340nm and 380nm wavelengths. Neutral density filters (0.7 optical density, Chroma) prevents ultraviolet damage of cells by being placed over excitatory light. Once the light passes this filter it reflects off of a beamsplitter (400 DCLIP dichromic mirror, Chroma) which transmits light above 400nm and reflects light below 400nm. The aforementioned detailed Ca²⁺ imaging setup is also shown schematically in Figure 10.
**Figure 10 - Schematic of Ca\textsuperscript{2+} imaging** that allowed for analysis of intercellular Ca\textsuperscript{2+}.
SOLUTIONS

Digestive Enzyme Solution:
The digestive enzyme solution consisted of 4mg mL\(^{-1}\) collagenase type I, 220-240 u mg\(^{-1}\) (Worthington Biochemical Corporation) and 2 mg mL\(^{-1}\) trypsin type I (Sigma), 8000 - 1200 u mg\(^{-1}\) (bought at ≈ 10,000u mg\(^{-1}\)) dissolved in 9.00 mL of DPBS (Dulbecco’s phosphate buffered solution) without MgCl\(_2\) and CaCl\(_2\) and .65mL of DPBS with MgCl\(_2\) and CaCl\(_2\).

Growth medium solution:
Mix 9mL of nutrient mixture with F-12 Ham (Sigma) with 1mL of fetal bovine serum (biowest).

Saline Solutions:
Standard HEPES buffered salt solution, which aids and endorses the stability of live cells, was used during the majority of imaging to represent a normoxic environment for the cells. The HEPES solution was prepared by adding the following into 1L of distilled water. 140 mM NaCl (8.12g/L), 4.5 mM KCl (0.34g/L), 2.5 mM CaCl\(_2\) (2.5mL/L), 1mM MgCl\(_2\) (1mL/L), 11 mM glucose (1.98g/L), and 20 mM HEPES (4.76g/L). After all the contents have been thoroughly dissolved using a magnetic stir bar, the standard HEPES buffered salt solution was adjusted to a pH of 7.4 at 37°C using a pH meter (Thermo Electron Corporation). Adding small increments of NaOH at 37°C allowed the buffered solution to achieve the pH of 7.4. Hypoxic HEPES solution follows the previous procedure however is bubbled with pre-purified compressed Nitrogen for 20 minutes prior to use.
Dyes and Stains:
Fura-2, AM (F1221, ThermoFisher) is permeable through the cell membrane until it converts to Fura-2 by the cleavage of ester bonds, which further allows it to act as a calcium indicator upon excitation at 340nm and 380nm. Fura-2, AM was bought at 50µg with a molecular weight of 1001.86 g/mol. A stock solution of this was made to a 10mM solution of DMSO by adding 50µL of DMSO (Dimethyl sulfoxide - SIGMA) to the 50 µg of fura-2, AM at 37°C. The cells were then loaded with 5µM Fura-2,AM by taking 15µL of the created stock solution and added it to 3mL of nutrient mixture with F-12 Ham (Sigma).

CellTracker™ Green CMFDA (Invitrogen) which freely passes across the membrane of cells and fluoresces the cytoplasm of living cells by exciting at 492nm and emitting at 517nm. It was bought at 50µg with a molecular weight of 454.86 g/mol. A stock solution of this was made to a 10mM solution of DMSO by adding 11µL of DMSO to the 50 µg of CellTracker Green at 37°C. For fixed cells 1 µL of stock solution was added to 300 µL of DPBS with MgCl$_2$ and CaCl$_2$ per slide. For live cell imaging, 1 µL of stock solution was added to the loading cocktail.

Tom20 (sc-11415, D0616, Santa Cruz) is a rabbit polyclonal IgG that was diluted 1:200 with a Triton BSA solution.

Triton BSA. 0.5g of 1 % BSA (Sigma) was mixed with 50mL of 0.3% Triton X-100 wash solution (Sigma).
Rhodamine Red™ (Jackson ImmunoResearch) is a secondary IgG donkey anti-rabbit polyclonal antibody diluted 1:200 with Triton BSA.

Mitotracker® Red CMXRos (Invitrogen) passively diffuses in the cell and acts as a mitochondrion selective probe. It was bought at 50µg with the molecular weight of 531.52 g/mol. A stock solution of this was made to a 1mM solution of DMSO by adding 94µL of DMSO to the 50µg of Mitotracker. 1µL of the Mitotracker stock solution was added into 1mL of HEPES at 37°C with a pH of 7.4. 100 µL of that dilution was added to into 0.95 mL of HEPES at 37°C with a pH of 7.4 which is the loading cocktail.

Hoechst 33342 (Invitrogen) was bought as 100 mg with a molecular weight of 615.99 g/mol, which is a DNA binding stain with selectivity for Adenine and Thymine. A stock solution was achieved by weighing out .001g (Denver Instrument) and adding it to 1 mL of DPBS with Ca²⁺ and Mg²⁺, 2 µL of the stock solution was added to the loading cocktail.
III: RESULTS

IMMUNOHISTOCHEMISTRY AND IMARIS RENDERED IMAGES

All techniques and protocols were performed carefully to ensure viability of cells. Prior to Z-stack imaging, cells had to meet strict visual criteria in order to be chosen for imaging. For cells to be chosen a single CB type I cell must have been isolated from other cells. A cell must have an intact cytoplasm, nucleus and mitochondria. Upon visual accomplishment of meeting the desired criteria, Z-stack images were acquired. These images were deconvolved and then IMARIS reconstructed. Statistical analysis was performed by doing unpaired student’s t-tests.

NORMOXIC AND HYPEROXIC CYTOPLASMIC VOLUMES OF JUVENILE AND MATURE CELLS

The total cell volume average of juvenile normoxic rat CB type I cells were calculated to be \(471 \pm 57.81 \, \mu m^3\) while juvenile hyperoxic cells were calculated to be \(338 \pm 43.69 \, \mu m^3\). Figure 11(Bi) and (Biii) show fluorescent cytoplasmic staining. Figure 11(Bii) and (Biv) show IMARIS rendered images. These difference were not statistically significant (\(P=.1355\)).

Cell volumes between mature cells also did not vary. The total cell volume average of mature normoxic rat CB type I cells were calculated to be \(444 \pm 32.4 \, \mu m^3\) while mature hyperoxic cells were calculated to be \(385 \pm 30.04 \, \mu m^3\). Fluorescent cytoplasmic staining’s are shown in Figure 12(Bi) and (Biii). IMARIS rendered images
are shown in Figure 12(Bii) and (Biv). These difference were not statistically significant (P=.2540).
Figure 11 – Immunohistochemistry and IMARIS Rendered images of Juvenile
normoxic and hyperoxic cell volumes. (A) Normoxic and hyperoxic rat CB type I cells
during a juvenile age (≈ 7 days old). The normoxic group had a n=6, where the hyperoxic
group had an n=4. These differences were not statistically significant P=.1355. (B)
Fluorescent cytoplasmic staining. Deconvolved images of a juvenile CB type I cell reared in
normoxia (Bi) and hyperoxia (Biii). IMARIS rendered image of a juvenile CB type I cell
reared in normoxia (Bii) and hyperoxia (Biv).
A. Cell Volume during a Juvenile State

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell Volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>~500</td>
</tr>
<tr>
<td>Hyperoxia</td>
<td>~400</td>
</tr>
</tbody>
</table>

B. Deconvolved IMARIS Rendered

- i: Juvenile Normoxic Cells
- ii: Juvenile Hyperoxic Cells
- iii: Juvenile Normoxic Cells
- iv: Juvenile Hyperoxic Cells
Figure 12 - Immunohistochemistry and IMARIS Rendered images of Mature normoxic and hyperoxic cell volumes. (A) Cell volumes of normoxic and hyperoxic rat CB type I cells during a mature age (≈ 14-21 days old). The normoxic group had a n=8, where the hyperoxic group had an n=18. These differences were not statistically significant P=.2540. (B) Fluorescent cytoplasmic staining. Deconvolved images of a mature cytoplasmic CB type I cell reared in normoxia (Bi) and hyperoxia (Biii). IMARIS rendered images of a mature cytoplasmic CB type I cell reared in normoxia (Bii) and hyperoxia (Biv).
A. Cell Volume during a Mature State

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hyperoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Volume (mm$^3$)</td>
<td>600</td>
<td>400</td>
</tr>
</tbody>
</table>

B. Deconvolved IMARIS Rendered

i. Mature Normoxic Cells

ii. Mature Hyperoxic Cells

iii. Deconvolved

iv. IMARIS Rendered
NORMOXIC AND HYPEROXIC NUCLEAR VOLUMES OF JUVENILE AND MATURE CELLS

The total nuclear volume of juvenile normoxic rat CB type I cells were calculated to be $224 \pm 33.18 \, \mu m^3$ while juvenile hyperoxic cells were calculated to be $162 \pm 21.88 \, \mu m^3$. Figure 13(Bi) and (Biii) show fluorescent nuclear staining. Figure 13(Bii) and (Biv) show IMARIS rendered images. These difference were not statistically significant ($P=.2044$).

Nuclear volumes between mature cells varied significantly ($P=.0142$). The total nuclear volume average of mature normoxic rat CB type I cells were calculated to be $183 \pm 10.65 \, \mu m^3$ while mature hyperoxic cells were calculated to be $133 \pm 11.48 \, \mu m^3$. The difference in fluorescent cytoplasmic staining were shown in Figure 14(Bi) and (Biii). The differences were also shown by IMARIS rendered images in Figure 14(Bii) and (Biv).
Figure 13 - Immunohistochemistry and IMARIS Rendered images of juvenile normoxic and hyperoxic nuclear volumes. (A) Nuclear volumes between normoxic and hyperoxic rat CB type I cells during a juvenile age (≈ 7 days old). The normoxic group had a n=6, where the hyperoxic group had an n=4. These differences were not statistically significant P=.2044. (B) Fluorescent nuclear staining. Nuclear deconvolved images of a juvenile nuclear CB type I cell reared in normoxia (Bi) and hyperoxia (Biii). IMARIS rendered images of a nuclear juvenile CB type I cell reared in normoxia (Bii) and hyperoxia (Biv).
A. Nuclear Volume at a Juvenile State

B. Deconvolved IMARIS Rendered

i. Juvenile Normoxic Cells

ii. Juvenile Hyperoxic Cells

iii. Juvenile Normoxic Cells

iv. Juvenile Hyperoxic Cells
Figure 14 - Immunohistochemistry and IMARIS Rendered images of Mature normoxic and hyperoxic nuclear volumes. (A) Nuclear volumes between normoxic and hyperoxic rat CB type I cells during a mature age (≈ 14-21 days old). The normoxic group had a n=8, where the hyperoxic group had an n=18. These differences were statistically significant P=.0142. (B) Fluorescent nuclear staining. Deconvolved images of a mature nuclear CB type I cell reared in normoxia (Bi) and hyperoxia (Biii). IMARIS rendered images of a mature nuclear CB type I cell reared in normoxia (Bii) and hyperoxia (Biv).
A. Nuclear Volume at a Mature State

B. Deconvolved IMARIS Rendered

Matric Normoxic Cells

Mature Hyperoxic Cells
NORMOXIC AND HYPEROXIC MITOCHONDRIAL VOLUMES OF JUVENILE AND MATURE CELLS

Juvenile normoxic cells had a significantly greater (P=.0090) mitochondrial volume. The average mitochondrial volume of juvenile normoxic rat CB type I cells were calculated to be $186 \pm 25.79 \mu m^3$ while juvenile hyperoxic cells were calculated to be $74 \pm 7.08 \mu m^3$. The difference in fluorescent mitochondrial staining is shown in Figure 15(Bi) and (Biii). The difference is also shown by IMARIS rendered images in Figure 15(Bii) and (Biv). The percent of cell volume occupied by mitochondria differed significantly (P=.0038). The percent of cell volume occupied by mitochondria of juvenile normoxic cells were calculated to be $39 \pm 3.35 \%$ while the percent for juvenile hyperoxic cells were calculated to be $22 \pm 1.77 \%$ as shown in Figure 15(B).

The total mitochondrial volume of mature normoxic cells were calculated to be $149 \pm 17.18 \mu m^3$ while mature hyperoxic cells were calculated to be $123 \pm 16.08 \mu m^3$. The fluorescent mitochondrial staining is shown in Figure 16(Bi) and (Biii). Figure 16(Bii) and (Biv) show IMARIS rendered images. These difference were not statistically significant (P=.3547).

The percent of cell volume occupied by mitochondria reared in normoxia were not statistically significant (P=.1070) between juvenile and mature cells. The percent of cell volume occupied by mitochondria in juvenile cells reared in normoxia were calculated to be $39 \pm 3.35 \%$ whereas the mature normoxic cells were calculated to be $32 \pm 2.22 \%$ (Figure 17A). The percent of cell volume occupied by mitochondria at a mature state had no significance (P=.8372) between normoxic and hyperoxic cells. The percent cell volume occupied by mitochondria in a mature state under normoxic air
were calculated to be $32 \pm 2.22 \%$ while the mature hyperoxic cells were calculated to be $31 \pm 3.18 \%$ as shown in Figure 17B. Most importantly, no statistical significance was shown between juvenile and mature cells reared in normoxia vs. hyperoxia.
Figure 15 - Immunohistochemistry and IMARIS Rendered images of juvenile normoxic and hyperoxic mitochondrial volumes. (A) Mitochondrial volume between normoxic and hyperoxic rat CB type I cells during a juvenile age (≈ 7 days old). The normoxic group had a n=6, where the hyperoxic group had an n=4. These differences were statistically significant P=.0090. (B) Percent of cell volume occupied by mitochondria at a juvenile state (≈ 7 days old) between normoxic and hyperoxic rat CB type I cells. The normoxic group had a n=6, where the hyperoxic group had an n=4. These differences were statistically significant P=.0038. (C) Fluorescent mitochondrial staining. Deconvolved images of a juvenile CB type I cell reared in normoxia (Ci) and hyperoxia (Ciii). IMARIS rendered images of a juvenile CB type I cell reared in normoxia (Cii) and hyperoxia (Civ).
A. Mitochondrial Volume at a Juvenile State

B. Percent of Cell Volume occupied by Mitochondria at a Juvenile State

C. Deconvolved IMARIS Rendered

Juvenile Normoxic Cells

Juvenile Hyperoxic Cells
Figure 16 - Immunohistochemistry and IMARIS Rendered images of Mature normoxic and hyperoxic mitochondrial volumes. (A) Mitochondrial volumes between normoxic and hyperoxic rat CB type I cells during a mature age (~14-21 days old). The normoxic group had a n=8, where the hyperoxic group had an n=18. These differences were not statistically significant P=.3547. (B) Fluorescent mitochondrial staining. Deconvolved images of a mature CB type I cell reared in normoxia (Bi) and hyperoxia (Biii). IMARIS rendered images of a juvenile CB type I cell reared in normoxia (Bii) and hyperoxia (Biv).
A. Mitochondrial Volume at a Mature State

B. Deconvolved IMARIS Rendered

i

ii

iii

iv
Figure 17 – Percent of cell volume occupied by mitochondria. (A) Percent of cell volume occupied by mitochondria reared in normoxia between rat CB type I cells during a juvenile age (≈ 7 days old) and a mature age (≈ 14-21 days old). The juvenile group had a n=6 where the mature group had an n=8. These differences were not statistically significant P=.1070. (B) Percent of cell volume occupied by mitochondrial at a mature state (≈ 14-21 days old) between normoxic and hyperoxic rat CB type I cells. The normoxic group had a n=8, where the hyperoxic group had an n=18. These differences were not statistically significant P=.8373.
A. Percent of Cell Volume occupied by Mitochondria reared in Normoxia

B. Percent of Cell Volume occupied by Mitochondria at a Mature State
CALCIUM IMAGING TRACE

Juvenile and mature CB type I cells were isolated for \( \text{Ca}^{2+} \) imaging along different developmental stages. An example trace for a juvenile cell reared in hyperoxia is shown (Figure 18). All cells were challenged with a hypoxic stimulus (refer to solutions section, p.46) for five minutes upon successful excitation (\( \text{K}^+ \) spike). \( \text{Ca}^{2+} \) imaging was performed in an attempt to correlate mitochondrial development with the development of the acute hypoxic ventilatory response. Due to the low number of successful excitation in the isolated CB type I cells, no statistical analysis was performed.
Figure 18 – Example of a Ca$^{2+}$ imaging trace testing the hypoxic response. Juvenile CB type I cells reared in hyperoxia had a very minimal hypoxic response if any at all.
Ca$^{2+}$ trace for a juvenile cell reared in a hyperoxic state
IV: DISCUSSION

MAIN FINDING

The main result of this thesis determined that using fixed cells, mitochondrial volumes did not fall with maturation. This finding was in contrast to previous studies the lab had demonstrated, a significant reduction in mitochondrial volume with maturation. The fall in mitochondrial volume previously observed in rat CB type I cells (Figure 19), was not paralleled by a fall in metabolic rate (Figure 20) indicating an increase in the rate of individual mitochondrial oxidative phosphorylation. These previous findings suggested that a change in mitochondrial volume may play a crucial role in the mechanism by which type I cells detect changes in O₂ (Paulet et al. 2012; Bavis et al. 2010). Possible reasons for the differences between the data presented in this thesis and those of Paulet et al. will now be discussed.
Figure 19 – Total cell volume and percent volume of mitochondria relative to cell size in juvenile and mature carotid body Type I cells. 

A. Bar chart showing differences in cell volume between juvenile and mature carotid body Type I cells (***P<0.00002). 
B. Bar chart showing differences in the percent of cell volume occupied by mitochondria between juvenile and mature carotid body Type I cells (***P<0.00001). 
Ci. 3D reconstruction of deconvolved Deltavision images for an example juvenile Type I cell (Mitotracker, orange; Hoechst 33342, blue; CellTracker, green). 
Cii. Imaris rendered 3D reconstruction of the cell in Ci. 
Ciii. 3D reconstruction of deconvolved DeltaVision images for an example of mature Type I cell (Mitotracker, orange; Hoechst 33342, blue; CellTracker, green). 
Civ. Imaris rendered 3D reconstruction of the cell in Ciii. Scale bar is 1.5 μm in all images.
Figure 20 – Comparison of resarufin production in Type I CB cells from juvenile and mature rats. Graph represents change in fluorescence in isolated Type I cells at room temperature in HEPES solution after addition of 10μM C₁₂-resazurin dye measured with 2-photon confocal microscopy. No significant differences in resarufin production were observed between juvenile (n = 140) and mature (n = 57) Type I cells despite the fall in mitochondrial volume in mature cells.
RATIONALE FOR MAIN FINDING

Two mitochondrial staining techniques were used. Both techniques differ greatly, the methodology behind each staining process is further discussed as a potential reason for obtaining different results with each technique. First, the mitochondrial stain for live cell imaging was Mitotracker®, which stained mitochondria via mitochondrial membrane potential. The membrane potential for healthy mitochondria is approximately -160mV (Duchen and Biscoe, 1992). Therefore, the positively charged Mitotracker® will be attracted to the mitochondria very strongly (Figure 21a). An ideal working concentration to obtain nicely stained mitochondria was difficult to achieve in our studies, therefore a fixed cell approach was attempted.

To do this, the digested CB type I cells were dissociated and fixed. Mitochondria were stained using a Tom20 antibody. Tom20, a rabbit polyclonal IgG antibody, binds to mitochondrial membrane bound proteins (Figure 21b). Rhodamine Red™, a secondary IgG donkey anti-rabbit polyclonal antibody, then was used to complete the staining. Staining with Tom20 showed a much higher percentage of cell volume occupied by mitochondria (39 ± 3.35 %) than those observed by Paulet et al. (20.69 ± 2.39%). A possible reason for this could be the binding sites e.g. (all dead mitochondria and proteins) that Tom20 would bind to, whereas with Mitotracker® dysfunctional and dead mitochondria were not stained as they would not have a negative mitochondrial membrane potential.
Figure 21 – Methodological Approaches between Live and Fixed staining of CB type I cells. (A) Live cell imaging of mitochondria using Mitotracker® which is drawn by the charge attraction of mitochondria aids as a mitochondrion selective probe by exciting at 579nm and emitting at 599 nm. (B) Fixed cell imaging of mitochondria uses Tom20 which is a rabbit polyclonal IgG that binds to mitochondrial surface proteins then Rhodamine Red™ which is a secondary IgG donkey anti-rabbit polyclonal is bound to the receptor and excites at 570nm while emitting at 590nm.
A. Mitochondrial Live Cell Staining

Mitochondrial Membrane Potential ≈ -160 mV

B. Mitochondrial Fixed Cell Staining

Step 1
Tom20 , rabbit polyclonal IgG antibody.

Step 2
Rhodamine Red™, secondary IgG donkey anti-rabbit polyclonal antibody

Membrane Protein

Fixed Mitochondria
CONCLUSION

According to the data presented in this thesis mitochondrial volume of CB type I cells do not decrease as animals mature. Thus, interpretations of the hyperoxia data in this thesis is difficult. The hypothesis was that the mitochondrial development would be impaired in rats reared in hyperoxia between a juvenile and mature state. If proven true, this may underpin the blunting of the acute hypoxic ventilatory response. However, the lab has previously seen a significant reduction in mitochondrial volume during development in normoxic reared rats. In order to determine whether changes in mitochondria may play a factor in the oxygen sensitivity of the carotid body, this thesis would need to be repeated entirely with live cell imaging. This would allow the study to focus on healthy type I cells from juvenile and mature rats raised in normoxic and hyperoxic conditions. Indeed, as the present data is inconsistent with Paulet et al. we cannot be sure which method is giving the correct information without repeating the work.
V: REFERENCES


Pietruschka, F. (1985). Calcium influx in cultured carotid body cells is stimulated by acetylcholine and hypoxia. *Brain research, 347*(1), 140-143.


