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The Role of AMP-Activated Protein Kinase (AMPK) in Hypoxic Chemotransduction by the Carotid Body

Heidi Lynn Jordan
Wright State University

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THE ROLE OF AMP-ACTIVATED PROTEIN KINASE (AMPK) IN HYPOXIC CHEMOTRANSDUCTION BY THE CAROTID BODY

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

By
HEIDI LYNN JORDAN


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Wright State University
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SCHOOL OF GRADUATE STUDIES

May 24, 2012.

I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Heidi Lynn Jordan ENTITLED The Role of AMP-activated Protein Kinase (AMPK) in Hypoxic Chemotransduction by the Carotid Body BE ACCEPTED IN PARTIAL FULFILMENT OF THE DEGREE OF Doctor of Philosophy.

__________________________________________
Christopher N. Wyatt, Ph.D.
Dissertation Advisor

__________________________________________
Gerald Alter, Ph.D.
Director, Biomedical Sciences
Ph.D. Program

__________________________________________
Andrew Hsu, Ph.D.
Dean, School of Graduate Studies

Committee on Final Examination

__________________________________________
Christopher N. Wyatt, Ph.D.

__________________________________________
Robert W. Putnam, Ph.D.

__________________________________________
Mark M. Rich, Ph.D.

__________________________________________
Lynn K. Hartzler, Ph.D.

__________________________________________
David R. Cool, Ph.D.
ABSTRACT

Jordan, Heidi Lynn. PhD., Biomedical Sciences Ph.D. Program, Department of Neuroscience, Cell Biology and Physiology, Wright State University, 2012. The Role of AMP-Activated Protein Kinase (AMPK) in Hypoxic Chemotransduction by the Carotid Body.

The carotid bodies are small sensory organs located along the bifurcation of the carotid arteries. They detect changes in blood gases and relay this information to the brain to allow for initiation of appropriate respiratory and cardiovascular responses. A decrease in oxygen (hypoxia) sensed by the carotid body results in an increase in firing of the carotid sinus nerve and ultimately a change in one’s breathing pattern. An inability to respond to an acute hypoxic (low oxygen) episode via increased ventilation may result in death or lead to pathological or chronic conditions such as stroke and hypertension (Prabhakar et.al., 2005). The carotid bodies are therefore responsible for initiating the acute hypoxic ventilatory response (HVR) and blunting or attenuation of the HVR has been implicated in sudden infant death syndrome (SIDS) in ‘at risk’ infant groups (Calder et al., 1994; Horne et al., 2005; Gauda et. al., 2007).

The exact mechanism(s) responsible for hypoxic chemotransduction by the carotid body remains controversial and are the subject of intense investigation. There is evidence indicating the energy-sensing enzyme AMP-activated protein kinase (AMPK) may play a critical role in the transduction of an acute hypoxic stimulus by the carotid bodies (Evans et. al., 2005; Wyatt et.al., 2007). Global AMPK α1 and AMPK α2 subunit knockout mice were used to gain further insight into the role AMPK has in acute oxygen-sensing in whole animal and in isolated oxygen-sensing cells of mice carotid bodies. A two-chamber
plethysmography system was used to measure baseline breathing during normoxia (21% \(O_2\)) and the hypoxic ventilatory response to 8% oxygen. AMPK \(\alpha_1\) subunit knockout mice had a significant attenuation in percent change in breathing frequency during hypoxic exposure and AMPK \(\alpha_2\) knockouts also showed a significant decrease in percent change in minute ventilation. The plethysmography data shows AMPK\(\alpha\) subunits may be involved in baseline breathing and generation of acute hypoxic ventilatory responses. Isolated type I cells from global knockout mice were also used in calcium imaging experiments. Hypoxia induced \(Ca^{2+}\) signaling was inhibited by 85% in cells from AMPK \(\alpha_2\) knockout mice whereas AMPK \(\alpha_1\) KO mice showed a mixed response to hypoxia. The cellular data seem to show that a decrease in response to hypoxia may be due to an effect at the level of the carotid body. These findings provide further support for the hypothesis that AMPK regulates acute hypoxic chemotransduction and thereby energy supply at the whole body level.
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Background

Carotid Body

Carotid bodies (CB) are small-paired sensory organs, which can be found along the bifurcation of the carotid arteries (Figure 1). In 1926 Fernando De Castro discovered that the cell bodies of the carotid sinus nerve (CSN) lay within the petrosal sensory ganglion suggesting that the carotid body had sensory capabilities (De Castro, 1926). In 1928, De Castro went on to determine that the carotid body was indeed a sensory organ and not a gland, which he hypothesized was capable of detecting changes in the composition of blood (De Castro, 1928). From 1930-1933 Corneille Jean Francois Heymans, a Belgium physiologist, focused most of his time working on De Castro’s hypothesis of the CB being a chemoreceptor (De Castro, 2009). From these experiments, Heymans and collaborators were able to demonstrate that hyperventilation induced by a decrease in the partial pressure of oxygen in arterial blood (PaO$_2$) and pH or an increase in partial pressure of carbon dioxide in arterial blood (PaCO$_2$) was mediated via the carotid body (Heymans et.al.,1930). This work won Heymans the Nobel Prize in Physiology or Medicine in 1938 (De Castro, 2009).

It is now known that the carotid bodies are the primary peripheral arterial chemoreceptors (PAC) of mammals; they are responsible for sensing changes in the partial pressure of oxygen (PO$_2$), partial pressure of carbon dioxide (PCO$_2$), and pH and
Figure 1. Anatomical location of the carotid bodies.

The carotid bodies are small sensory organs located, bilaterally, at the bifurcation of the common carotid arteries and branch into the internal and external carotid arteries. The diagram indicates circulatory supply and the afferent nerve projections from the carotid bodies to the nucleus tractus solitarius.
Figure 1.
act to produce an increase in ventilatory response (Heymans et. al., 1930). Upon CSN resection, the hyperventilatory response ceased, thus illustrating the relationship between the CB and the hypoxia-mediated stimulation of the respiration rate (Heymans et. al., 1930). Carotid bodies are perfused with blood via one or more small arteries that originate near the branches of the carotid artery. Blood drains into the internal jugular vein by one or two veins originating from a dense vascular plexus located on the surface of the organ. Carotid bodies possess the highest blood flow relative to its size (1.5 and 2.0 liters100g⁻¹ min⁻¹) of any other organ in the body (Gonzalez et. al., 1994; Kumar & Bin-Jaliah, 2007). Blood flow to the carotid body is important in determining the PO₂ of the carotid body tissue. The carotid body is highly vascularized, thus it is able to quickly (within seconds) (Kumar, 2009) sense changes in arterial blood partial pressure of oxygen (PO₂), partial pressure of carbon dioxide (PCO₂) and pH. The ability of the carotid body to rapidly sense blood gas changes allows it to initiate appropriate physiological responses to the changes in an expedient manner (Barnett et. al., 1988). Carotid bodies sensory afferent innervations are from the carotid sinus nerve. Fibers from the afferent innervation ultimately project to the nucleus tractus solitarius, (NTS) of the medulla (Finley & Katz, 1992) and allow for initiation of respiratory and cardiovascular reflexes to ensure proper oxygenation to vital organs (Lahiri et. al., 2006). Two main cell types comprise the carotid body, glomus cells and sustentacular cells, which form clusters surrounded by a dense net of fenestrated capillaries (Gonzalez et. al., 1992). Glomus cells, also known as type I cells, are excitatory and originate from the neural crest, whereas sustentacular cells (type II cells) are similar to glial cells. Current research suggests type II cells in adult CB’s are functional and may be dormant stem cells which
proliferate in response to hypoxia and differentiate into new glomus cells (Pardal et al., 2007). By contrast, glomus cells are more complex, expressing a number of voltage- and ligand-gated ion channels in addition to transient receptor potential channels and background K⁺ channels. Combinations of voltage-gated Na⁺, Ca²⁺, and K⁺ channels are expressed and the level of expression varies among mammalian species. For example, rabbit glomus cells have relatively large voltage gated inward Na⁺ currents, as well as sustained Ca²⁺ currents (I_{Ca}) (Lopez-Barneo et al., 1988; Ureña et al., 1989; Duchen & Biscoe, 1992), whereas, in rat type I cells, the Na⁺ current is either absent completely (Peers & Green, 1991; Fieber & McCleskey, 1993) or is present only at low densities (Stea & Nurse, 1991). Due to the presence of voltage-gated channels found within glomus cells, most are excitable and able to elicit action potentials (Ureña et al., 1989) or calcium-dependent receptor potentials (Buckler & Vaughn-Jones, 1994).

Neurotransmitters (NT) found in the carotid body can be classified into two different categories, conventional and unconventional. Conventional NT’s are located within synaptic vesicles and their actions are mediated through activation of membrane-bound receptors often coupled to specific G-coupled proteins or ion channels. Transmitters found in this category include: acetylcholine (ACh), dopamine (DA), norepinephrine (NE), 5-hydroxytryptamine (5-HT), substance P (Sub. P), ATP and amino acids (Lahiri et al., 2006). Unconventional NT’s, which are not synaptically stored but instead are generated spontaneously through enzymatic reactions and express their biological actions via cytosolic enzymes or direct protein modifications. Examples of unconventional NT’s would include nitric oxide (NO) and carbon monoxide (CO) (Gonzalez et al., 1994; Lahiri et al., 2006; Prabhakar, 2006; Lopez-Barneo et al., 2008).
Release of neurotransmitters stimulates afferent nerve activity (i.e. ACh, Sub. P, Adenosine, and ATP) (Prabhakar et al., 1989; Zhang et al., 2000; Spyer et al., 2004) whereas some such as dopamine will inhibit nerve activity (Carroll et al., 2005; Prieto-Lloret et al., 2007). Studies have shown that hypoxia induces release of both stimulatory and inhibitory NT’s during afferent nerve activation (Ureña et al., 1989; Xu et al., 2003); therefore, the balance of excitatory to inhibitory NT’s released determines excitation.

**CO₂ and Acid Sensing**

The carotid body is excited by different “acidic stimuli,” such as: (A.) **Hypercapnic Acidosis:** rise in PCO₂ with a concomitant decrease in extracellular pH; (B). **Isohydric Hypercapnia:** proportional increase in both PCO₂ and extracellular [HCO₃⁻] with maintained extracellular pH; and (C). **Isocapnic Acidosis:** decrease in extracellular pH and [HCO₃⁻] at constant PCO₂. These “acidic stimuli” have been shown to elevate intracellular calcium, [Ca²⁺]ᵢ, in isolated rat type I cells resulting in decreased intracellular pH, (pHᵢ), suggesting intracellular acidosis is responsible for inducing a rise in [Ca²⁺]ᵢ (Buckler & Vaughan-Jones, 1994a). Decreases in pHᵢ cause the cells to depolarize via K⁺ channel inhibition, resulting in voltage-gated Ca²⁺ influx. Support for this model has been shown with evidence that acidosis inhibits whole-cell K⁺ currents in type I cells (Peers, 1990b; Stea et al., 1991; Buckler & Vaughan-Jones, 1993). Additionally, Peers and Green (1991) indicated that an increase in pHᵢ selectively inhibits Ca²⁺-activated K⁺ currents in CB type I cells of neonatal rats. Past studies have
suggested that a fall in pH\textsubscript{i} may lead to Ca\textsuperscript{2+} entry through activation of the Na\textsuperscript{+}-H\textsuperscript{+} exchanger, which would lead to a rise in intracellular Na\textsuperscript{+} ([Na\textsuperscript{+}]\textsubscript{i}) and Ca\textsuperscript{2+} entry via a Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger. However, in the absence of extracellular Na\textsuperscript{+}, [Na\textsuperscript{+}]\textsubscript{o}, there was no significant effect on resting levels of [Ca\textsuperscript{2+}]\textsubscript{i}. In addition, when [Na\textsuperscript{+}]\textsubscript{o} was removed and type I cells underwent depolarization/repolarization steps from -60mV to 0mV and back, recovery of [Ca\textsuperscript{2+}]\textsubscript{i} was indeed slower in the absence of extracellular Na\textsuperscript{+}. However, the effect was quite small and therefore indicates that if Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger is present it likely plays only a minor role in the homeostasis of [Ca\textsuperscript{2+}]\textsubscript{i}. Therefore, it seems as if the response of the carotid body to changes in pH and CO\textsubscript{2} is not dependent upon specialized mechanisms for proton-extrusion, instead it is mediated through changes in the relationship between pH\textsubscript{i} and pH\textsubscript{o} of type I cells (Buckler \textit{et. al.}, 1991).

In addition to the relationship between pH\textsubscript{i} and pH\textsubscript{o}, it is believed that TASK channels, also known as, acid (or alkali) sensitive K\textsuperscript{+}-channels of the tandem P-domain K\textsuperscript{+}-channel family, take part in CO\textsubscript{2}-sensing within the type I cells of the CB. These pH-sensitive K\textsuperscript{+}-channels are found in the brainstem neurons (Bayliss \textit{et. al.}, 2001; Duprat \textit{et. al.}, 1997) as well as the glomus cells of the carotid bodies (Buckler \textit{et. al.}, 2000). TASK channels belong to two main groups based upon structure and sequence homology, one group consists of TASK-1, TASK-3 and TASK-5 and the other TASK-2, TASK4/TALK2 and TALK 1 (Buckler \textit{et. al.}, 2007). Out of all the TASK channels, the CB seems to have ones most similar to that of TASK-1 and TASK-3. Buckler, \textit{et. al.}, (2000), has shown that in rat carotid body type I cells, brief pulses of isocapnic acidosis resulted in inhibition of the TASK-1 channels causing cell depolarization evoking a rapid increase in intracellular calcium levels. Oxygen sensitivity of the current was also
decreased by the acidosis. Further work done by Kim et. al., (2009) demonstrated that in rat carotid body type I cells, the predominant oxygen-sensitive TASK-like current is carried by a heteromultimer of TASK-1 and TASK-3. These data lend further support to data published by Buckler et. al., (2007) wherein channel activity of the TASK-like K⁺ channels were inhibited by reducing pH₀. Data published from Trapp et. al., (2008) using TASK-1⁻/⁻, TASK-3⁻/⁻, and TASK-1/3 double knockout mice, showed that TASK-1 and not TASK-3 channels play a role in cell depolarization, calcium influx, and neurotransmitter release during a hypercapnic event in mice carotid body type I cells.

**Oxygen Sensing**

Hypoxia stimulates neurotransmitter release from type I cells which leads to an increase in CSN firing rate and results in a change in ventilatory pattern until physiological PO₂ levels are re-established and maintained. Results from many studies suggest that hypoxic chemotransduction occurs through the inhibition of O₂-sensitive K⁺ channels present in the type I cells (Lopez-Barneo et. al., 1988; Peers, 1990; Stea & Nurse, 1991; Buckler, 1997). Potassium channel inhibition causes type I cells to depolarize allowing voltage-gated Ca²⁺ entry and NT release (Buckler & Vaughan-Jones, 1994b; Ureña et. al., 1994). The specific mechanism(s) by which K⁺ channels are inhibited via hypoxia in carotid body type I cells are still being examined and are the focus of this dissertation. While the precise oxygen-sensing mechanism(s) still remain to be fully defined, two main hypotheses have been proposed: the **Membrane** and the **Mitochondrial** hypotheses.
Membrane Hypothesis

According to the membrane hypothesis, hypoxia suppresses $K^+$ channels, resulting in depolarization of the membrane, $Ca^{2+}$ influx, and cytosolic $Ca^{2+}$ increase (Lopez-Barneo et al., 1988; Buckler & Vaughan-Jones, 1994b). If hypoxia is coupled to $K^+$ channel inhibition through a membrane-delimited mechanism, this should be evident and maintained in an excised patch of membrane. Necessary elements required for the channels to be maintained should be found in the removed portion. This has been demonstrated in certain experiments using adult rats (Riesco-Fagundo et al., 2001), whereas in other experiments using neonatal rats, hypoxic inhibition was no longer maintained after excision (Wyatt & Peers, 1995). If chemotransduction following a hypoxic exposure were truly a membrane-delimited process, then sensitivity to hypoxia should be maintained in the patch for a period of time following excision. Elements necessary for $O_2$-sensing need to be tightly adhered to the cell membrane otherwise they may be lost to the intracellular solution, resulting in attenuation of the cell’s oxygen-sensing ability (William & Buckler, 2004). Studies have illustrated ‘run down’ in activity of $O_2$-sensitive $K^+$ channel activity in rat type I cells when excised; however after ATP was added to the intracellular solution, channel activity recovered. Recovery suggests that intracellular factors were necessary for $O_2$-sensitive $K^+$ channel activity and the potential for metabolic regulation (William & Buckler, 2004; Varas et al., 2007). In favor of the “membrane” hypothesis, Williams et al., (2004) proposed that $O_2$-sensing via the $Ca^{2+}$-sensitive $K^+$ ($BK_{Ca}$) channels in the rat CB is mediated via $O_2$-sensitive dependent carbon monoxide production via heme oxygenase-2 (HO-2). Heme oxygenase-2 is an enzyme, which in the presence of nicotinamide adenine dinucleotide
phosphate (NADPH) and molecular oxygen (O$_2$), catalyzes the breakdown of cellular heme producing carbon monoxide (CO), iron, and biliverdin (BV) (Figure 2), which can be broken down further into bilirubin by BV reductase (Williams et. al., 2004). Evidence has emerged over the last 10-15 years suggesting that HO-2 may act as an O$_2$ sensor through the production of CO, which is a BK$_{Ca}$ channel activator (Lopez-Barneo et. al., 2008). It is believed that two K$^+$ channels, Twik-related acid sensing K$^+$ channels (TASK-like) and BK$_{Ca}$ channels, may play a role in the hypoxic depolarization of type I cells of the rat (Buckler, 1997). Work done by Riesco-Fagundo et.al., (2001) and Williams et. al., (2004) have shown that under normoxic conditions HO-2 in the presence of CO, elicits an excitatory response on nearby BK$_{Ca}$ channels assuring the channels remain open. While under hypoxia, no excitatory response is observed, likely because reduced CO levels combined with direct heme-dependent inhibition leads to K$^+$ channel closure, further supporting the role of HO-2 to K$^+$ channel activation.

As BK$_{Ca}$ channels are regulated by CO and/or NADPH cofactors, it is believed that HO-2 functions as an acute O$_2$-sensor in rat type I cells via regulation of BK$_{Ca}$ channel activity through the changing balance between intracellular concentrations of HO-2 and the production of CO. However, to date, no assessment has been made to outline the requirements necessary for hypoxic chemotransduction to occur within an intact rat carotid body (Williams et. al., 2004; Kemp, 2005; Lopez-Barneo et. al., 2008). The proposal of HO-2 acting as an O$_2$ sensor has been challenged by experiments conducted in HO-2 knockout (KO) mice. Oretga-Saenz et. al., (2006) have demonstrated that HO-2 KO mice show no impairment in their hypoxic chemotransduction capacity. Oxygen sensing in KO mice was not different from wild type (WT) mice when exposed
Figure 2. Proposed model for inhibition of BK$_{ca}$ channel during hypoxia.

Under normoxic conditions, cellular heme is broken down into biliverdin, iron, and carbon monoxide. Carbon monoxide is a (+) channel activator and makes certain the BK$_{ca}$ channel is open during normal oxygen levels. During hypoxic conditions, the availability of O$_2$ becomes limited and heme is no longer produced and CO production is greatly reduced. The reduction in CO along with possible direct heme-dependent inhibition (-) leads to channel closure (Modified from Kemp, 2005).
Figure 2.
to varying levels of hypoxia. No differences were detected in dose-response curves from glomus cells between KO and WT mice. These data suggest that the heme-oxygenase hypothesis cannot be fully supported, and the mechanisms outlined in the membrane hypothesis are not likely mediating O₂-sensing within type I cells of the carotid body. Even though BKCa channels in mice are not O₂-sensitive (Wyatt & Evans, 2007), the Oretga-Saenz data are still able to demonstrate that HO-2 does not play a role in hypoxic inhibition of oxygen-sensitive delayed rectifier Kv channels in type I cells of mice carotid bodies or indeed any K⁺ channels.

**Mitochondrial Hypothesis**

The mitochondrial hypothesis was first suggested after Heyman’s Nobel Prize winning experiments demonstrated a possible role for the mitochondria in hypoxic chemotransduction by the carotid body. Heyman’s use of potassium cyanide produced similar effects to that of hypoxia in the carotid body (Heymans et. al., 1931), and later other investigators demonstrated that cyanide inhibited the mitochondrial oxidase cytochrome C (Wainio & Greenless, 1960; Van Buren et. al., 1972). It is also known that physiological levels of hypoxia (20-60 mmHg) excite the carotid body (Lahiri et. al., 1993) whereas in other cells that do not monitor oxygen levels, such as the dorsal root ganglion, PO₂ levels must fall below 5 mmHg before oxidation of NADH is shown to accompany an increase in impulse discharge (Duchen & Biscoe 1992a, b; Jöbis, 1964). Thus, together with the fact that mitochondrial inhibitors and uncouplers increase carotid body activity, mimicking the effects of hypoxia (Krylov & Anichkov, 1968), these
studies have led to the hypothesis that O₂-sensing by the CB type I cells may be possible through a mitochondrial cytochrome that has an unusually low O₂-affinity (Mills & Jöbsis 1970, 1972).

Further support for a role of the mitochondria in O₂-sensing has been shown by Mulligan et. al., (1981), whose work with oligomycin, which acts by binding ATPase in such a way as to block the proton channel causing inhibition of oxidative phosphorylation of ADP, demonstrated that intact oxidative phosphorylation is necessary for O₂ chemoreception to occur. In addition, Mulligan’s work also reported that oligomycin stimulates the CB chemoreceptor afferents and inhibits their response to further changes in arterial PO₂. These data suggest that mitochondrial inhibitors not only mimic hypoxia but they stop further hypoxic effects at the cellular level. Buckler and Vaughan-Jones (1998) also demonstrated that both hypoxia and mitochondrial uncouplers inhibit background K⁺ currents suggesting that the same channels may be involved in eliciting a response to both stimuli. Additionally, Wyatt and Buckler (2004) showed that mitochondrial inhibitors such as rotenone, cyanide, myxothiazol and oligomycin mimic the effects of hypoxia by inhibiting background K⁺ currents, which led to membrane depolarization and voltage-gated calcium entry. Further work in this paper has also shown that in the presence of cyanide, rotenone or the mitochondrial uncoupler p-trifluorometoxyphenyl hydrazone (FCCP), further excitation of isolated carotid body type I cells during hypoxia was prevented. These results suggest that the mitochondria, does indeed play some role in O₂-sensing by type I cells of the carotid body.
**ATP in Hypoxic Chemotransduction**

Rat type I cells express background $K^+$ channels that have biophysical and pharmacological properties resembling the TASK group of tandem-p domain $K^+$ channels (Buckler, 1997; Buckler et al., 2000). The channels are active over a wide range of membrane potentials and are found to be the predominant $K^+$ conductance at the resting membrane potential of isolated type I cells. It is thought that modulation of the background $K^+$ currents within type I cells is an important event in the process of chemotransduction during hypoxia and/or acidosis. Background $K^+$ currents have also been shown to be sensitive to mitochondrial metabolic inhibitors (Wyatt & Buckler, 2004). Inhibiting cellular metabolism results in decreased background $K^+$ currents, which leads to membrane depolarization, voltage-gated $Ca^{2+}$ entry and NT release (Buckler & Vaughan-Jones, 1998; Wyatt & Buckler, 2004; Varas et al., 2007). Previous studies have also shown that the response of these channels to hypoxic stimuli is absent when mitochondrial function is inhibited, suggesting a strong relationship between $O_2$-sensing and energy metabolism. Williams and Buckler (2004) have shown that background $K^+$ channel activity in excised membrane patches is enhanced in the presence of millimolar levels of ATP. Further experimentation showed strong modulation of type I cell background $K^+$ currents in excised inside-out patches in the presence of MgATP as well as other nucleotides such as, GTP, UTP, AMP-PCP and ATP-$\gamma$-S; indicating a possible tie between energy metabolism and cell excitability that is mediated via alterations in cytosolic, or sub-membrane levels of ATP (Varas et al., 2007). This work is in agreement with previous studies which have illustrated that excitation of type I cells through inhibition of background $K^+$ channels, membrane depolarization, voltage-gated
Ca\textsuperscript{2+} entry and NT release is tied to mitochondrial metabolism (Williams & Buckler, 2004). It is possible there may be a link between changes in cytosolic ATP levels, the activity of background K\textsuperscript{+} channels and mitochondrial energy metabolism. However, it still remains to be determined if inhibition of mitochondrial energy metabolism results in significant changes in cytosolic ATP in type I cells (Wyatt & Buckler, 2004). Multiple nucleotides can activate background K\textsuperscript{+} channel activity (Varas et.al., 2007); therefore there may be other pathways that may also link mitochondrial energy metabolism to type I cell excitation.

Thus far it seems as if no consensus can be made regarding which hypothesis, the mitochondrial or membrane, actually plays a role in O\textsubscript{2}-sensing. However, recent work indicates that the “energy-sensing” enzyme, AMP-activated protein kinase (AMPK), may be a critically important kinase in the signal transduction cascade that couples the mitochondria to excitatory events in the type I cell plasma membrane (Evans et.al., 2005; Wyatt & Evans, 2007; Wyatt et.al., 2007; Evans et.al., 2009).

**AMPK Hypothesis**

Evidence suggests that physiological levels of hypoxia inhibits oxidative phosphorylation of mitochondria in type I cells (Mills & Jöbsis, 1970, 1972; Duchen & Biscoe, 1992a), which in turn causes an increase in the cellular ratio of ADP/ATP (Figure 3). Adenylate kinase converts two molecules of ADP to AMP and ATP to maintain ATP levels within the cell (Hardie & Hawley, 2001). The consequent increase in AMP/ATP (Hardie & Hawley, 2001) activates AMPK, which has been reported to
Figure 3. Proposed mechanism by which hypoxic inhibition of mitochondrial oxidative phosphorylation couples to type I cell depolarization and neurotransmitter release by activation of AMP-activated protein kinase (AMPK). Arrows in red indicate inhibition. Hypoxia (<60 mm Hg) inhibits oxidative phosphorylation of the mitochondria in type I cells, which leads to an increase in cellular ratios of ADP/ATP. Adenylate kinase converts two molecules of ADP into ATP and AMP in order to maintain ATP levels within the cell. Increased AMP/ATP ratio activates AMPK, which has been reported to inhibit oxygen sensitive potassium channels leading to depolarization of type I cells. Depolarization of the cells activates voltage-gated Ca\textsuperscript{2+} channels triggering an influx of calcium, release of neurotransmitters and an increase in the firing of the carotid sinus nerve.
Figure 3.

(Wyatt et al., 2007)
inhibit oxygen sensitive potassium channels causing a depolarization of type I cells (Evans et. al., 2005; Wyatt et. al., 2007; Ross et. al., 2011). The depolarization activates voltage-gated Ca^{2+} channels triggering calcium influx and the release of neurotransmitters, causing an increase firing of the carotid sinus nerve (Wyatt et. al., 2007). This cascade of events suggests that AMPK can couple hypoxic inhibition of oxidative phosphorylation to transduction of membrane-restricted events in type I cells, which lends support to the mitochondrial hypothesis. The cascade of events is the basic principle behind the AMPK transduction hypothesis (Figure 3). This dissertation will test the hypothesis that AMPK couples hypoxic inhibition of oxidative phosphorylation to mouse type I cell excitation and therefore plays a key role in hypoxic chemotransduction by the carotid body and thus the acute hypoxic ventilatory response.

AMPK α1 and AMPK α2 knockout mice (Jorgensen et.al., 2004; Viollet et.al., 2003b) will be used to examine this hypothesis.

**AMPK Structure and Function**

AMPK, is a serine/threonine protein kinase that forms a heterotrimeric complex made up of an α catalytic subunit and 2 regulatory subunits, β and γ (Hardie, 2007). Each subunit can occur as an alternate isoform that is encoded by 2 or 3 genes (α1, α2, β1, β2, γ1, γ2, and γ3) and there are at least 12 different heterotrimer combinations possible from the various isoforms (Hardie & Hawley, 2001). All 12 of the isoform combinations are capable of forming a complex (Hardie et. al., 2003) and diversity of
AMPK complexes occurs with alternative splicing and/or use of different promoters (Hardie, 2004).

The N-terminus of the α subunit contains a conventional kinase domain (Figure 4a), while the C-terminal end is necessary to form a complex with the β and γ subunits (Hardie, 2004). It appears there are distinct physiological differences between the α1 and α2 subunits (Violett et al., 2003b; Jorgensen et al., 2004). For example, it has been shown that mice lacking the α1 isoform of AMPK show no defect in glucose homeostasis, illustrated by a glucose tolerance test (B. Viollet and F Andreelli, unpublished data). The α1−/− mice also show a normal response to insulin and AICAR (Jorgensen et al., 2004). By contrast, mice lacking the AMPK α2 isoform exhibit glucose intolerance and reduced sensitivity to insulin as well as AICAR-resistant (Jorgensen et al., 2004; Viollet et al, 2003b). It has also been proposed that the AMPK α2 isoform but not the AMPK α1 isoform plays a major role in controlling skeletal muscle metabolism (Viollet et al., 2003b).

In all eukaryotes, the β subunit (Figure 4b) is made up of an N-terminal region that is followed by two regions, originally named the kinase interacting sequence (KIS) and association with SNF1 kinase (ASC) domains (Jiang & Carlson, 1997). It was first thought the KIS domain was necessary for interaction with the α subunit, while the ASC domain interacted with the γ subunit. However, it has been shown that only the ASC domain is necessary to give an active αβγ complex and the KIS domain is actually a glycogen-binding domain (GBD) (Hardie et al., 2003; Hardie, 2004). Each domain binds ATP with a lower affinity than AMP and the binding of ATP and AMP is mutually
Figure 4. Basic domain structure of the α, β, and γ subunits of AMP-activated protein kinase (AMPK). A. Schematic of AMPK α subunit indicating the kinase region, the site of phosphorylation by upstream kinases and the site for auxiliary subunit binding. B. Schematic of AMPK β subunit indicating possible glycogen binding domain and the site for αγ subunit binding. C. Schematic of AMPK γ subunit indicating the crystathionine β-synthase (CBS) regions that form the two AMP/ATP binding ‘Bateman’ domains (Modified from Hardie, et.al., 2003).
Figure 4.

A. \(\alpha\) Subunits

Upstream Kinases

\[ \text{N} \quad \text{KINASE} \quad \beta\gamma \text{ binding} \quad \text{C} \]

B. \(\beta\) Subunits

\[ \text{N} \quad \text{Glycogen-binding} \quad \alpha\gamma \text{ binding} \quad \text{C} \]

C. \(\gamma\) Subunits

\[ \text{N} \quad \text{CBS1} \quad \text{CBS2} \quad \text{CBS3} \quad \text{CBS4} \quad \text{C} \]

\text{AMP/ATP binding} \quad \text{AMP/ATP binding}
exclusive, thus when ATP levels are high activation of AMPK will not occur (Hardie, 2004, 2005). Activation of AMPK in mammals can occur via stressors such as hypoxia, heat shock, lack of nutrients, oxidative stress or metabolic poisoning (Hardie & Hawley, 2001; Hardie, 2004). Stress activation of AMPK results in a reduction in ATP consumption and promotes ATP production.

AMPK is allosterically activated via 5′-AMP (Figure 5) but must be phosphorylated by 1 or more upstream kinases, such as LKB1 or calmodulin-dependent kinase kinases β (CaMKKβ), at a threonine residue (Thr-172) within the α subunit kinase activation loop (Hardie et al., 2005). The most important AMPK upstream kinase appears to be LKB1 (Hawley et al., 2003) because it is constitutively active. Phosphorylation of the Thr-172 residue within the α subunit, results in more than a 100-fold increase in AMPK activation. Binding of AMP to the 2 Bateman domains of the AMPK γ subunit (Scott et al., 2004; Xiao et al., 2007) activates the kinase by inhibiting the de-phosphorylation of Thr-172 (Sanders et al., 2007), causing a switch to the active phosphorylated form. Further allosteric activation, of the phosphorylated AMPK, by up to 10-fold, occurs when AMP binds to the γ subunit, thus the combination of the two events leads to an increase in activation of about 1000-fold (Suter et al., 2006, Evans et al., 2009). ATP, AMP and ADP compete for the two sites on the γ subunit, and under normal physiological conditions ATP binds the inactive form of AMPK (Xiao et al., 2007). The bound inactive form is more abundant versus the AMP bound active form (Evans et al., 2009). The AMPK activation mechanisms ensure it is highly
Figure 5. Schematic illustrating activation of AMP-activated protein kinase (AMPK) via upstream kinases and AMP binding. AMPK is activated more than 100-fold through phosphorylation at the Thr-172 residue on the α subunit via an upstream kinase such as LKB1 or calcium/calmodulin-dependent protein kinase kinase beta (CamKKβ). LKB1 constitutively phosphorylates Thr-172 but binding of AMP to 2 exchangeable sites on the γ subunits of AMPK also activates the kinase through inhibiting the de-phosphorylation of Thr-172. AMPK is now switched to its phosphorylated form. Binding of the AMP to the γ subunits results in further allosteric activation of the phosphorylated kinase by up to 10-fold, thus between the two events there can be up to a 1000-fold increase in activation. Once AMPK is active it can phosphorylate its target protein. AMPK is turned off by increasing levels of ATP.
Figure 5.
metabolically sensitive and even small changes in the ADP/ATP ratio can allow for AMPK activation without a large decrease in ATP levels (Hardie & Hawley, 2001).

As previously mentioned, the most important upstream kinase, which plays a role in AMPK activation, is the tumor suppressor, LKB1 (Hawley et al., 2003). LKB1 is a 50 kDa serine/threonine kinase and inactivating mutations in LKB1 result in a dominantly inherited cancer in humans known as Peutz-Jeghers syndrome (Woods et al., 2003; Shaw et al., 2004). LKB1 exists as a heterotrimeric complex with two accessory subunits, Ste20-related adaptor protein-α (STRADα) and mouse protein 25-α (MO25α) (Hawley et al., 2003; Boudeau et al., 2004).

The two accessory proteins each have a closely related isoform, STRADβ and MO25β. STRAD has a kinase-like domain that is related to the Ste20 protein kinase, however it is classified as a pseudokinase because it lacks certain residues found in active protein kinases (Baas et al., 2003; Hawley et al., 2003). The complex, LKB1:STRAD:MO25, is only catalytically active when STRADα or STRADβ and MO25α or MO25β are available to form the heterotrimeric complex (Hawley et al., 2003; Boudeau et al., 2004). LKB1 appears to constitutively phosphorylate the Thr-172 residue thereby activating AMPK (Hawley et al., 2003; Shaw et al., 2004). Binding of STRAD to LKB1 is required for LKB1 to phosphorylate AMPK and is also required to anchor LKB1 to the cytoplasm. MO25 is necessary for stabilization of the LKB1:STRAD complex (Hardie, 2005).

In addition to stressors, AMPK in many cells can also be activated via a Ca²⁺-mediated pathway (Hawley et al., 2005). Stimuli, which lead to an increase in cytoplasmic Ca²⁺, result in the activation of the calmodulin-dependent protein kinase
(CaMKKβ). CaMKKβ is an upstream kinase of AMPK and activation of CaMKKβ activates AMPK by phosphorylating the Thr-172 residue independent of AMP (Hawley et. al., 2005). Work done by Woods et. al. (2005) provides strong evidence that intracellular Ca\(^{2+}\) acts as a second signaling pathway to activate AMPK. Instances that lead to an increase in calcium may result in ATP depletion because the Ca\(^{2+}\) pump(s) no longer have the energy needed to remove excess Ca\(^{2+}\) from the cytosol (Brini & Carafoli, 2009). Conditions that activate AMPK through an increase in the AMP:ATP ratio also lead to an increase in intracellular Ca\(^{2+}\), i.e. oxidative stress, mitochondrial uncouplers, and exercise in muscle (Patel et. al., 2001). As a result, it is therefore possible that activation of AMPK may be a consequence of both ATP and Ca\(^{2+}\) mediated effects involving both LKB1 and CaMKKβ (Woods et. al., 2005).

**Pharmacological Manipulation of AMPK**

Most data collected thus far with regard to the actions of AMPK has been obtained using pharmacological tools, such as the putative antagonist Compound C. However, it has been demonstrated that Compound C has off target effects, which are independent of AMPK inhibition (Emerling et. al., 2007). Compound C has been shown to not only inhibit AMPK but also many other protein kinases (Bain et. al., 2007). Many experiments have also used the AMPK agonist, 5-aminoimidazole-4-carboxamide ribose (AICAR). AICAR is taken up into the cells and converted to the AMP mimetic ZMP (AICAR monophosphate) and is able to activate AMPK without affecting the AMP:ATP ratio (Hardie et. al., 2003). However, recent experiments utilizing AICAR have shown
that it may act as an inhibitor of Complex I of the mitochondrial electron transport chain (Guigas et. al., 2007), which affects cellular energetics by decreasing the synthesis of ATP and thus affecting the AMP:ATP ratio. In addition, it is not always possible to differentiate between the specific actions of each of the catalytic subunit(s) through the use of drugs alone. Activation of both AMPK \( \alpha 1 \) and AMPK \( \alpha 2 \) subunits may occur when using AICAR, thus it is not possible to determine the action(s) of the specific subunit(s) (Viollet et.al, 2003a). Therefore, it is of great importance to be able to examine not only the role AMPK plays but also those subunit(s) combinations, which are being activated to get a better idea as to how the kinase functions in regards to the role it plays in hypoxia. In order to gain further insight into the role of AMPK \( \alpha 1 \) and AMPK \( \alpha 2 \) subunits, research in this dissertation will focus on global AMPK \( \alpha 1 \) and global AMPK \( \alpha 2 \) knockout mice as well as targeted AMPK \( \alpha 2 \) knockout mice.

**Summary of Oxygen Sensing**

To summarize, type I cells of the CB are the peripheral arterial chemoreceptors, which are activated not only by hypoxia, but also hypercapnia and extracellular acidosis. It has been known for over 70 years that the carotid body is an acute oxygen sensor and within the past 10-15 years or so it has been shown that the CB contains several classes of O\(_2\)-sensitive K\(^+\) channels. Inhibition of these channels leads to depolarization of type I cells, which allows entry of voltage-gated Ca\(^{2+}\) and NT release (Buckler & Vaughan-Jones, 1994a; Peers & Buckler, 1995; Lopez-Barneo et. al., 2001). However, the exact mechanism(s) which inhibits K\(^+\) channels during hypoxia in type I cells remains
controversial. Recently, AMP-activated protein kinase has been identified as a potentially critical kinase in the transduction of hypoxic stimuli in carotid body type I cells.

Clinical Relevance

Sudden infant death syndrome (SIDS) is the number one leading cause of death in infants from 1 month to 1 year of age (Gauda et. al., 2007). Carotid bodies are the key defense players in eliciting responses against hypoxia and hypercapnia. Therefore, possible exposure to oxygen extremes before birth may result in an ineffective response by the PAC’s when faced with a hypoxic or hypercapnic event. This may predispose a preterm infant to a higher risk of SIDS (Gauda et. al., 2007). Thus, basic research into the mechanisms behind hypoxic chemotransduction and how carotid bodies are able to respond to hypoxia may prove beneficial for successful therapies and/or strategies for treatment of this disorder. This information may also prove beneficial to other disorders, which are a result of prolonged or intermittent periods of hypoxia such as hypertension and sleep apnea (Prabhakar et. al., 2005).

Research laid out in this dissertation utilized global and targeted knockout mice to determine if and how the hypoxic ventilatory response (HVR) was altered when AMPK α1 or AMPK α2 subunits were knocked out. If AMPK is important in oxygen-sensing in the type I cells of the carotid body, knocking out of the catalytic subunit(s) may result in an attenuation or complete lack of ventilatory response to hypoxia.
**Hypothesis:** AMP-activated protein kinase (AMPK) couples hypoxic inhibition of oxidative phosphorylation to type I cell excitation and therefore plays a key role in hypoxic chemotransduction by the carotid body and thus the acute hypoxic ventilatory response (HVR).

**Specific Aims:**

**Specific Aim 1.** Generate global knockout AMPK α1 and AMPK α2 mice as well as generate mice with targeted AMPK α2 subunits knocked out in tyrosine hydroxylase (TH) containing tissues, including the carotid body.

**Specific Aim 2.** Test the hypothesis that AMPK is required for a complete hypoxic ventilatory response in mice using two-chamber plethysmography.

**Specific Aim 3.** Test the hypothesis whether knocking out of AMPK alpha subunits modulates calcium signaling in mouse carotid body type I cells.
Research Design and Methods

Global Knockout Mice

In the early 1980’s scientists, Martin Evan, Oliver Smithies and Mario Capecchi developed the generation of a genetic “knockout” mouse (Thomas & Capecchi, 1987; Doetschman et al., 1987; Waldman, 1992). This breakthrough technology was the first of its kind to allow replacement or disruption of a specific gene using an inactive or mutated allele. Knockout mice allow scientists to selectively knockout or disable a specific targeted gene in embryonic cells, which allowed them to study the effect of the knocked out gene. By the late 80’s, early 90’s, a “knock-in” mouse was finally developed in order to examine the effects of a mutated DNA sequence that is exchanged for an endogenous sequence without further disruption of the gene (Thomas et al., 1986; Capecchi, 1994). Knockout mice are valuable tools used today in various facets of biomedical and pharmaceutical research. The research in this dissertation employed the use of knockout mice by utilizing mice with either the AMPK α1 or α2 subunits knocked out throughout the entire mouse because simultaneous knockout of both subunits is embryonic lethal (Viollet, et al., 2003b). The global AMPK α1 knockout mouse (AMPKα1−/−) used in experiments is designated B6.129/Sv-Prkaa1tm1Sbj/Orl, it is a knock-in of beta-galactosidase/Neomycin (=GEO) gene fusion. In order to inactivate the AMPK α1 catalytic domain, a replacement vector was made with 7kb of ES-129-derived
genomic fragment and a selection cassette, which deleted part of the catalytic AMPKα1 subunit from amino acids 97-157 (Jorgensen et. al., 2004) (Figure 6). The global AMPK α2 KO mice (AMPK α2−/−) are designated B6.129/Sv-Prkaa2tm1.1Vio/Orl, and were obtained from a conditional knockout (alpha2 floxed mouse) crossed with an Ella-Cre deleter mouse. The AMPK α2 genomic clones were isolated after screening a mouse 129 strain genomic library (Stratagene, La Jolla, CA). The targeting construct was produced by flanking exon C, which encodes the AMPK α2 catalytic subunit with loxP sites for Cre recombinase and inserting a phosphoglycerol kinase (PGK) promoter-driven neomycin selection cassette that was flanked by an additional loxP site (Viollet et.al., 2003b) (Figure 7). The targeted construct was linearized and electroporated into 129/Sv MPI-I embryonic stems cells. Cells expanded from the targeted clones were injected into C57BL/6 mice. The heterozygous offspring were then bred with a “deleter” Ella-Cre transgenic mouse, in which Cre was expressed in the the germline, to produce AMPK α2+/- mice (Holzenberger et.al., 2000). These mice were used to confirm the role(s) AMPK α1 and/or AMPK α2 subunits play in response to hypoxia by the carotid body. The breeding plan for generating the global AMPK KO experimental animals is shown in Figure 8a. PCR was used to confirm correct genotype for both global AMPKα1 and AMPKα2 KO mice (Figure 8b). AMPK α1+/− and AMPK α2+/− mice were obtained from the European Mutant Mouse Archive (EMMA) with the kind permission of Dr. B. Viollet. These animals were bred onto a mixed C57BL/6J:129-1x background to provide homogeneity with all the other knockout mice used in the Wyatt laboratory. AMPK α1+/− and AMPK α2+/− mice were then bred to provide +/- mice and their +/+ controls.
**Figure 6. Generation of AMPKα1 Knockout Mice.** A schematic (not to scale) illustrating the genomic structure of the AMPK α1 wildtype allele (top), the AMPKα1 gene-targeting construct that contains a promoterless IRES-βGeo cassette (middle), and the targeted gene (bottom). E, EcoRI digestion; IRES, internal ribosomal entry site; βGeo, β-galactosidase and neomycin phosphotransferase fusion gene; pA, polyadenylation sequence; hatched vertical bar, introns.
Figure 6.
Figure 7. **Generation of AMPKα2 Knockout mice.** Schematic (not to scale) illustrating the genomic structure of the AMPK α2 wild-type allele, AMPK α2 gene-targeting construct, AMPK α2 targeted allele, and AMPK α2 null allele. Squares indicate loxP sites and H’s indicate HindIII restriction sites. C, exon encoding the AMPK α2 catalytic domain (amino acids 189-260).
Figure 7.
Figure 8. A. Global mouse breeding plan. Shown is the breeding plan to obtain global AMPK α1 and AMPK α2 knockout mice for use in plethysmography and cellular studies.

B. PCR bands for wild type, heterozygous and homozygous AMPK α1 and AMPK α2 knockout mice. Gel confirms correct genotype for AMPK α1 KO and AMPK α2 KO mice.
Figure 8. (A)

$F_0$

\[ \begin{array}{c}
\text{AMPK } \alpha_1 +/-
\end{array} \]

$X$

\[ \begin{array}{c}
\text{AMPK } \alpha_1 +/-
\end{array} \]

$F_1$

\[ \begin{array}{c}
+/+ \\
+/- \\
+/- \\
-/-
\end{array} \]

\[ \begin{array}{c}
\text{WT } 25\% \\
\text{Heterozygous } 50\% \\
\text{Homozygous KO } 25\%
\end{array} \]

\[ \begin{array}{c}
\text{Plethysmography & Cellular Studies} \\
\text{Breeding} \\
\text{Plethysmography & Cellular Studies}
\end{array} \]

Figure 8. (B)

![Image of gel electrophoresis results with base pair sizes and AMPKα1 and AMPKα2 bands]
Mice were tail clipped at 5 days of age and genotyped as follows (all primer sequences were obtained from Dr. B. Viollet) (Table 1).

AMPKα1 wildtype allele, forward: KO+ AGC GTA CCT GGT ATC TTA TAG G, reverse: KO- GGA CTT ATT ACT AAA CAG ACC TCT G (yields 329bp band for WT allele). AMPKα1 deleted allele, forward: GEO+ ACC AGA AGC GGT GCC GGA AAG CTG G, reverse: GEO- TGT AGT CGG TTT ATG CAG CAA CGA G (yields 466bp band for deleted allele). Tail DNA and primers were added to GoTaq mix (Promega) and PCR cycled as follows: 94°C, 4 min, 94°C, 45 sec, 35 cycles; 57°C, 45 sec, 35 cycles; 72°C, 60 sec, 35 cycles; 72°C, 7 min; 4°C hold.

AMPKα2 wildtype allele, forward: loxF1 GCT TAG CAC GTT ACC CTG GAT GG, reverse: loxR1 GTT ATC AGC CCA ACT AAT TAC AC (yields 200bp band for WT allele). AMPKα2 deleted allele, forward: same as for WT, reverse: koTneoR GCA TTG AAC CAC AGT CCT TCC TC (yields 600bp band for deleted allele). Tail DNA and primers were added to GoTaq mix (Promega) and PCR cycled as follows: 94°C, 4 min; 94°C, 20 sec, 35 cycles; 58°C, 20 sec, 35 cycles; 72°C, 30 sec, 35 cycles; 72°C, 7 min; 4°C hold.
Table 1. Primers used for genomic PCR of global AMPK α1, AMPK α2, and TH-targeted knockout mice shown with melting temperatures (TM) and amplicon size (SIZE).

<table>
<thead>
<tr>
<th>GENE</th>
<th>SEQUENCE</th>
<th>TM</th>
<th>SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPKα1 KO- AMPKα1 KO+</td>
<td>5’GGACCTTTACTAAACAGACCTTG 3’</td>
<td>94° C</td>
<td>329bp WT</td>
</tr>
<tr>
<td></td>
<td>5’ AGCACATACCTGTTATCTATTAGG 3’</td>
<td>94° C</td>
<td></td>
</tr>
<tr>
<td>AMPKα1 GEO+ AMPKα1 GEO-</td>
<td>5’ACCAGAAGCGGTGCCCGAAAAGCTG 3’</td>
<td>94° C</td>
<td>466bp AMPKα1+</td>
</tr>
<tr>
<td></td>
<td>5’TGTAGCTGGTTATGCAGCAACGAG 3’</td>
<td>94° C</td>
<td></td>
</tr>
<tr>
<td>TH-3 Reverse CRE-UD TH-5 Forward</td>
<td>5’ CTTTCCTTCTTTATTGAGAT 3’</td>
<td>92° C</td>
<td>290bp WT</td>
</tr>
<tr>
<td></td>
<td>5’ GATACCTGGCCTGGTCTG 3’</td>
<td>92° C</td>
<td>430bp TH-Cre+</td>
</tr>
<tr>
<td></td>
<td>5’ CACCCTGACCCAAGCAGCT 3’</td>
<td>92° C</td>
<td></td>
</tr>
<tr>
<td>AMPKα2 kOTneoR AMPKα2 LoxF1 AMPKα2 LoxR1</td>
<td>5’ GCATTGAAACCACAGTCTTCTTC 3’</td>
<td>94° C</td>
<td>200bp WT</td>
</tr>
<tr>
<td></td>
<td>5’ GCTTAGACAGTTACCTGGATGG 3’</td>
<td>94° C</td>
<td>600bp AMPKα2+</td>
</tr>
<tr>
<td></td>
<td>5’ GTTATCAGCCCAACTAATTACAC 3’</td>
<td>94° C</td>
<td></td>
</tr>
<tr>
<td>AMPKα2 LoxF1 AMPKα2 LoxR1</td>
<td>5’ GCTTAGACAGTTACCTGGATGG 3’</td>
<td>94° C</td>
<td>200bp WT</td>
</tr>
<tr>
<td></td>
<td>5’ GTTATCAGCCCAACTAATTACAC 3’</td>
<td>94° C</td>
<td>250bp floxed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200bp/250bp heterozygous</td>
</tr>
</tbody>
</table>
Targeted Knockout Mice

It is possible that globally knocking out AMPK α subunits may have an inhibitory effect on the hypoxic ventilatory response via a mechanism mediated by something other than the carotid body. Recent data reveal that AMPK has multiple ion channel targets (Dallas et al., 2009; Evans et al., 2009; Kreneisz et al., 2009) and by knocking out AMPK in the brain, it may affect the path that signals from the carotid body and integrates with the central mechanisms of ventilatory control. Thus, it is possible by knocking out AMPK there may be alterations in central neuronal firing such that it could directly modulate the mechanisms that pattern inspiration (Doi & Ramirez, 2008) in addition to any CB mediated actions. To minimize the possible central effects of knocking out AMPK, experiments focused on targeting AMPK α2 knockout to neurons containing tyrosine hydroxylase (TH). By targeting to dopaminergic and nor/adrenergic neurons, AMPK α2 subunits were deleted from the carotid body type I cells but were spared in much of the central processing hypoxic peripheral input (Gozal et al., 2000; Takakura et al., 2006). However, some central TH containing neurons can modulate the carotid body chemoreflex (Zhang & Mifflin, 2007; Viemari, 2008), thus caution would be needed when interpreting the data.

As mentioned previously, to minimize the possible central effects of knocking out AMPK, the experiments illustrated in this dissertation have focused on targeting AMPK α2 knockout to neurons containing tyrosine hydroxylase. Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of catecholamines (Lindeberg et al., 2004). Through targeting of dopaminergic and nor/adrenergic neurons, the AMPK α2 subunits should be knocked out in the carotid body type I cells but remain untouched in much of
the central processing of hypoxic peripheral input (Gozal et. al, 2000; Takakura et. al., 2006). One area of the brain that may be affected by the knockout of AMPK α2 and is believed to be involved in the hypoxic response includes the locus coeruleus (LC). The LC is a cluster of noradrenaline containing neurons found adjacent to the fourth ventricle in the pontine brainstem (Berridge and Waterhouse, 2003). It is the principle site for the production of noradrenaline and has many projections that innervate almost all regions within the central nervous system (CNS), which include the nuclei responsible for thermoregulation and cardiorespiratory function (Bockstaele et. al., 1999). Evidence suggests that excitation or inhibition of noradrenergic structures such as the LC can modulate the cardiorespiratory responses to peripheral chemoreceptor activation (Koshiya & Guyenet, 1994; Perez et.al., 1998). It is possible that by targeting AMPK knockout to TH containing neurons the activity of the LC is changed and, in theory, this could blunt the HVR (Zhang & Mifflin, 2007). However, work done by Biancardi et.al., (2010) used selective bilateral chemical lesions in the LC to demonstrate that under hypoxic conditions, chemical lesions of the LC had no affect on minute ventilation, mean arterial pressure, heart rate or core body temperature. Therefore they were able to show that the noradrenergic neurons of the LC do not play a role in cardiorespiratory control and thermoregulation during acute hypoxia.

Genetic manipulation through the use of the Cre-lox recombination system has proven to be extremely beneficial in elucidating gene function in many experimental organisms, especially mice (Le & Sauer, 2001). Through the use of the Cre-lox system it is now possible to generate knockout and targeted knockout animals without causing death. In many cases knocking out a certain gene can lead to embryonic death and thus
result in an inability to acquire adult animals to study the effect of the gene later in life (Sauer & Henderson, 1988; Holzenberger et al., 2000; Le & Sauer, 2001). The Cre-lox recombination makes it possible to study the effects of gene knockout in adult organisms as well as the effects of a tissue specific gene knockout. Cre recombinase is a single 38 kDa protein necessary for the catalyzation of DNA recombination between two 34 base pair loxP sites. Each loxP site contains two 13-basepair palindromes separated by an 8-basepair spacer that confers directionality to the site. The region of DNA that is flanked by the two loxP sites is said to be “floxed” (Le & Sauer, 2001). The loxP sites contain specific sites for Cre binding, which surround a specific DNA sequence where recombination may occur. Recombination occurs in cells that express Cre and contain loxP sites. Cre recombinase cuts the double stranded DNA at the specified loxP sites and the strands are then re-joined with DNA ligase (Sauer & Henderson, 1988). The sandwiching of DNA between the 2 loxP sites is termed “floxed,” which is a contraction of “flanked by lox.” The use of a conditional targeted gene knockout mouse model (Figure 9) has allowed for examination of the function of a specific gene in a targeted tissue without affecting the expression of the gene in non-targeted tissue (Lindeberg et al., 2004).

In order to effectively target knockout of AMPK α2 activity in the carotid body, mice conditional for the expression of floxed AMPK α2 were crossed with mice expressing Cre recombinase under a TH promoter (B6;129X-1TH^{cre}Te/Kieg), (Lindeberg et al., 2004). Mice conditional for AMPK α2 allows for the specific gene to be knocked out in a specific organ or tissue versus the entire mouse. AMPK α2 should be ablated in the catecholaminergic TH-expressing type I cells of the TH-Cre^{+/+}Floxed^{F/FI}
Figure 9. Schematic illustrating TH-targeted AMPKα2 knockout to the carotid body. The Cre mouse is under a tyrosine hydroxylase promoter and was crossed with a LoxP (Floxed) mouse. The floxed mouse has the gene of interest, AMPK α2, flanked on either side by a loxP site. When the TH-cre mouse is bred with the floxed mouse, cells with active Cre recombinase cuts the double stranded DNA at the specified loxP sites and the strands are then re-joined. Any cells containing tyrosine hydroxylase will have AMPK α2 knocked out including the carotid body. Cells lacking active Cre recombinase do not have AMPK α2 knocked out. Recombination occurs only in cells that express Cre and contain both loxP sites. Cre recombination allows one to study the knockout of a specific gene as well as the effects of a tissue specific gene knockout.
Figure 9.
mice (Figure 10). Lindeberg et al., (2004), has been able to show the TH-Cre (B6;129X-1TH<sup>tm(cre)Te/Kieg</sup>) mice can be successfully crossed with a β-galactosidase (LacZ) reporter strain wherein the reporter gene is activated via Cre recombination. The double heterozygous offspring showed good correlation between LacZ expression and TH-expressing cells illustrating efficient genomic recombination in the TH-expressing cells. Therefore, crossing the AMPK α2 floxed mice with the TH-Cre mice should remove AMPK α2 from TH-expressing cells including the carotid bodies. To increase the efficiency of the targeted knockout of AMPK α2, F<sub>2</sub> generation male TH-Cre<sup>+/+</sup> Floxed<sup>Fl/-</sup> mice (Figure 10) were crossed with female TH-Cre<sup>+/+</sup> Floxed<sup>Fl/-</sup> mice. The F<sub>1</sub> generation mice from this cross are shown in Figure 11. This genotype should provide knockout of the AMPK α2 subunits in the targeted TH-cells (Sakamoto et al., 2005). By using the refined breeding strategy illustrated in Figure 11, the percentage of viable experimental mice increased to 100% by giving mice that are all homozygous for TH-Cre and either homozygous or heterozygous for the flox gene. Mouse DNA genotyping via PCR was done on tail clippings at 5 days of age and genotyped using specific primers for the Cre transgene and floxed AMPK α2 (all primer sequences were obtained from Dr. B. Viollet (Figure 12, Table 1)).
**Figure 10. Targeted knockout breeding strategy.** Shown is the initial breeding plan to obtain TH-targeted AMPK α2 knockout in the carotid body type I cells. Crossing the heterozygous F₁ generation results in a wide range of possible F₂ genotypes.
Figure 10.

\[ \text{F}_0 \quad \text{TH-Cre}^{++} \quad \text{X} \quad \text{Fl}^{Fl/Fl} \quad \text{F}_1 \quad \text{X} \quad \text{TH-Cre}^{+/-} \text{Fl}^{Fl/-} \quad \text{F}_2 \]

\[ \begin{align*} \text{*TH-Cre}^{++} \text{Fl}^{Fl/Fl} & \quad \text{Successful Target} \\ \text{*TH-Cre}^{++} \text{Fl}^{Fl/-} & \quad \text{Successful Target} \\ \text{*TH-Cre}^{+/-} \text{Fl}^{Fl/Fl} & \quad \text{Same as F}_0 \text{ (i.e. control)} \\ \text{*TH-Cre}^{+/-} \text{Fl}^{Fl/-} & \quad \text{Same as F}_1 \text{ (i.e. control)} \\ \text{*TH-Cre}^{++} \text{Fl}^{-/-} & \quad \text{Same as F}_0 \text{ (i.e. control)} \\ \text{*TH-Cre}^{+/-} \text{Fl}^{-/-} & \quad \text{Wildtype (i.e. control)} \\ \text{*TH-Cre}^{+/-} \text{Fl}^{-/-} & \quad \text{Wildtype (i.e. control)} \end{align*} \]
Figure 11. Refined targeted breeding strategy. The targeted breeding strategy shown in Figure 10 will result in a low percent of TH-targeted AMPK α2 knockouts. Crossing the F2 TH-Cre<sup>+/−</sup>Flox<sup>Fl/−</sup> mice from Figure 10, will yield 100% experimentally viable progeny.
Figure 11.

\[
\begin{align*}
F_0 & \quad \text{TH-Cre}^{+/+}\text{Flx}^{FL-} & \quad X & \quad \text{TH-Cre}^{+/+}\text{Flx}^{FL-} \\
F_1 & \quad \text{TH-Cre}^{+/+}\text{Flx}^{FL/FL} & \quad \text{TH-Cre}^{+/+}\text{Flx}^{FL-} & \quad \text{TH-Cre}^{+/+}\text{Flx}^{+/+} \\
& \quad \downarrow & \quad \downarrow & \quad \downarrow \\
& \quad \text{Experimental} & \quad \text{Experimental} & \quad \text{Experimental}
\end{align*}
\]

All animals are useable for experimentation
Figure 12. PCR primer bands for TH-Cre and Floxed2. Gel confirms correct genotype for TH-targeted knockout mice.
Figure 12.
**General Methods:**

**Plethysmography**

Ventilatory in all wildtype, global AMPK α1 and AMPK α2 knockouts as well as TH-targeted AMPK α2 KO mice was measured using pressure plethysmography (Stunden *et al.*, 2001). All experiments were conducted at room temperature (22-24°C). Each mouse was placed into a 100cc plexiglass Respiromax plethysmography chamber **(Figure 13)** (Columbus Instruments, Columbus, OH). A tail rod moved the mouse forward in the chamber securing it and preventing it from backing up. Mice were set in a prone position allowing their snouts to emerge through an inflatable latex cuff into the head chamber. The cuff was inflated (maximum 20 P.S.I.) giving an airtight seal around the snout of the mouse. Pressure changes within the body chamber as a result of the animals’ respiration were measured through a transducer and this signal was digitized at 200Hz data points per second using a Respiratory Function program (Columbus Instruments, Columbus, OH). Information was displayed and stored in a computer for statistical and graphical analysis. Oxygen flow passing into the head chamber was controlled with a Pegas 4000 (MF) Gas Mixer and Gas Mixer System Software (Columbus Instruments, Columbus, OH). Respiration rates (RR) and tidal volume (V<sub>T</sub>) were measured in all wildtype, global and TH-targeted knockout mice using a Columbus Instruments Tidal Volume Meter.
Figure 13. **Mouse two-chamber plethysmography unit.** Schematic of Respiromax two-chamber plethysmography unit indicating position of mouse and separate face and body chambers.
Figure 13.

Mouse 2 chamber plethysmography unit
The head chamber was supplied with humidified 21% O₂ and 0% CO₂ gas mixture (balanced Nitrogen) at a flow rate of ~0.8L/min to establish a baseline recording for 3 minutes. To obtain peak acute hypoxic responses, oxygen levels were decreased to 12% or 8% for 3 minutes and then returned to 21% for a final 3 minutes. Respiration rates and tidal volume data were monitored, collected and analyzed using the Respiromax/tidal volume computer program, version 2.06. Data was taken from 40-50 seconds of stable breathing during the peak response of each change in O₂ level, 21%, 12%, 21% as well as 21%, 8%, 21%. In addition, data was taken from 40-50 seconds of stable breathing during exposure to 0%, 7%, 0% CO₂ at a flow rate of 1L/min for 2 minutes at each level. Data containing artifacts caused by mouse movement were excluded from analysis. All animals were awake during data acquisition and were weighed before testing (25-35 grams).

Isolation of Type I Cells

All cellular experiments were conducted using type I cells isolated and enzymatically dissociated from the carotid bodies of mice. The carotid bifurcations of the mouse were removed and the carotid bodies were isolated under a dissecting microscope. Isolated CB’s were placed in ice-cold oxygenated phosphate buffered saline (low Ca²⁺ and Mg²⁺). Carotid bodies were then placed into low Ca²⁺ and Mg²⁺ saline with 0.040% collagenase Type I (22µ/mg, Worthington Biochemical Corporation) and 0.025% trypsin Type I (10100µ/mg, Sigma) and put into a 37°C incubator for 20 minutes for dissociation. Carotid bodies were then teased apart using forceps and incubated for an
additional 7 minutes. Cells were then centrifuged at 730 rpm (200g) for 5 minutes and supernatant was removed. Cells were re-suspended in Hams F12 (Sigma) supplemented with 10% fetal bovine serum (Biowest) and then centrifuged for an additional 5 minutes. Type I cells were plated onto 10mm poly-d-lysine (Sigma) coated (0.1mg/ml) cover slips and incubated for at least 2 hours to allow the type I cells to settle and stick to the cover slips. Cells were used within 8 hours of isolation because cell viability greatly decreases after this point in time.

**Immunocytochemistry**

Isolated type I cells for AMPKα2 +/+ or -/- were processed separately using the same reagents. Cells were processed on the same day at the same time and every effort was made to treat the two groups of tissue identically. Coverslips with attached type I cells were fixed by immersion in methanol at -20° C for 20 minutes. Cells were then permeablized by 3x5min washes with 0.3% triton X-100 (Sigma) in phosphate buffered saline (PBS). Type I cells were given another 3x5min wash with blocking solution (1% donkey serum, 0.3% triton X-100 in PBS) to limit non-specific binding of the specific anti-AMPK α2 receptor antibodies. Anti-AMPK α2 1° antibodies (Kinasource, UK) were diluted 1:750 with blocking solution, added to the coverslips and incubated at 4° C for 16 hours. Cells were also stained with a rabbit anti-mouse tyrosine hydroxylase antibody (1:2000, Sigma) to assist identification of type I cells. Following incubation with the 1° antibodies, coverslips were washed 4x5min with blocking solution and then incubated for 1 hour, at room temperature in the dark with 1:200 dilution of Rhodamine
Red-X-conjugated affinipure donkey anti-rabbit IgG (Jackson Immunoresearch) and FITC-conjugated affinipure donkey anti-sheep IgG 2° antibody (Jackson Immunoresearch, 1:200). Coverslips were then washed for 5x5min with PBS before being inverted and attached to microscope slides with hard setting anti-fade mountant (2.4g Mowiol 4-88, 6g glycerol, 6ml H₂O, 12ml 0.2M Tris buffer pH 8.5, 2,5% diazobicyclo-octane) containing 4’, 6-diamidino-2-2-phenylindole dihydrochloride (DAPI, 1mg ml⁻¹) for visualization of type I cell nuclei. For controls, 1° antibody was omitted during this procedure. Images were acquired using a DeltaVision microscope system (Applied Precision) on an inverted Olympus IX71 microscope with an oil immersion, x63 magnification, 1.4 n.a. objective and Coolsnap HQ CCD camera (Photometrics). Multiple z-sections (focal depth 0.28 μm, z-step 0.25μm) were taken through individual cells. Images were deconvolved on-line via Softworx software (Applied Precision). Deconvolved images of these identically processed cells were then viewed using identical intensity parameters in Softworx and intensity line plots were constructed by appending a line across the center of the cell of interest. Pixel intensity was reported along the line and allowed comparisons between staining in AMPK α2⁺/⁺ and AMPK α2⁻/⁻ type I cells (Figure 14).

**Genomic Polymerase Chain Reaction**

Genomic DNA was isolated from 5-7 day old tail tips using DNeasy® tissue kit (Qiagen) according to manufacture’s instructions. Genomic polymerase chain reaction (genomic PCR) analysis was performed using 1.0 µl of genomic DNA, 6.7 µl double
Figure 14. AMPK α2 staining in mouse carotid body type I cells from wildtype and AMPK α2 knockout mice.  A. Staining in wildtype AMPK α2 mouse type I cell.  Green is AMPK α2 and blue is DAPI staining for the nucleus.  The white scale bar is 10 µm and the red line indicates the location of the intensity plot shown in panel C.  B. Reduced staining shown in AMPK α2 KO mice type I cells.  Green is AMPK α2 and blue is DAPI staining for nucleus.  The red line indicates the location of the intensity plot shown in panel C and D.  C and D. Intensity line plots from panels A and B respectively.  Data is represented in thousands of units.  Staining was done on AMPK α1 WT and KO mice type I cells but was not successful.  Lack of success in the AMPK α1 mice may have been a result of the antibodies not working properly.
Figure 14.
distilled water, 1.6 µl of 10 µM Primer TH-5, 1.6 µl of 10 µM Primer TH-3, 1.6 µl of 10 µM Primer Cre-UD and 12.5 µl 2X GoTaq Green™ (Promega) mix. Genomic PCR was performed as follows: a 5 minute denaturation step at 92°C, 35 cycles of 45 seconds at 92°C, 45 seconds of annealing at 56°C, and 1 minute extension at 72°C, followed by a final extension for 7 minutes at 72°C. Genomic PCR products were separated on a 1.5% agarose gel, and visualized using ethidium bromide and UV light. Images were captured using a **LAS-3000 Imager (Fuji)** and Image Reader software.

**Ca**\(^{2+}\) **Imaging**

Isolated type I cells were loaded with Fura-2AM (5µM, Invitrogen) in an extracellular solution containing (in mM): NaCl, 140; KCl, 4.5; CaCl\(_2\), 2.5; MgCl\(_2\), 1.0; glucose, 11.0; HEPES, 20.0; pH 7.4 with NaOH, for 30 minutes, at room temperature (22-24°C) in the dark. Fura-2AM, is a membrane permeable, dual wavelength, fluorescent dye. Once inside the type I cells, the –AM portion of the dye is cleaved off leaving behind the charged Fura-2, which is calcium-sensitive, and binds free Ca\(^{2+}\) resulting in a change in its fluorescent properties. Cover slips with Fura-2 attached loaded cells were washed with extracellular solution for 15 minutes before being mounted in a chamber (0.4 ml) and perfused at 8 ml/min\(^{-1}\) with a standard bicarbonate buffered solution (in mM; NaCl, 117; KCl, 4.5; CaCl\(_2\), 2.5; MgCl\(_2\), 1.0; glucose, 11.0; NaHCO\(_3\), 23.0) gassed with 5% CO\(_2\) balanced air (35-37°C, pH 7.4). Cells were visualized with a CFI super fluor 40x oil immersion objective. Fura-2 was excited with 50 ms exposures to 340nm and 380nm light at 0.2-0.5 Hz using a Lambda 10-3 filter.
wheel (Sutter) and emitted fluorescence measured at 470-550nm using a CoolSNAP HQ2 CCD camera. Neutral density filters of 0.7 optical densities were placed in the excitatory light path to reduce any photodamage to cells (Burlon et.al., 2009). Data acquisition and analysis was controlled using Metafluor 7.1.2. imaging software (Molecular Devices). Only cells that maintained a low resting intracellular Ca\(^{2+}\) level and had a strong and reversible increase in fluorescence ratio to 20% CO\(_2\) were used for further experiments. The robust response signifies a healthy cell, which has not been damaged by enzyme digestion (Burlon et. al., 2009; Thompson et. al., 2010; Thompson & Wyatt, 2011). Type I cells were then exposed to a hypoxic solution (10 mm Hg/10 torr) and the ratio of \([Ca]_i\) was measured. Calcium ratio data was reported as a ratio instead of absolute calcium values because the system could not be calibrated properly with ionomycin. In order to properly calibrate for absolute calcium the following equation would be used:

\[
[Ca] = K_d(S_f2/S_b2)(R_{exp}-R_{min}/R_{max}-R_{exp})
\]

where \(K_d\) is the affinity of Fura-2 for calcium, \(S_f2\) is the intensity at 380nm for calcium free, \(S_b2\) is intensity at 380nm for bound calcium, \(R_{exp}\) is measured experimental ratio, \(R_{min}\) is 0 Ca ratio and \(R_{max}\) is Ca saturated ratio. The oxygen tension reached in the bath was measured using an OxyMicro oxygen sensor (WPI). The level of hypoxia reached in the bath over one minute was 10 torr, which is a level shown to excite the carotid body type I cells (Figure 15).

**Metabolism**

Basal metabolic rates (BMR) were assessed on all wildtype, global AMPK \(\alpha1\) and AMPK \(\alpha2\) knockout mice. Oxygen consumption and carbon dioxide excretion were
Figure 15. **Recording Bath Oxygen Tension.** Figure illustrates the oxygen level reached in the bath for calcium-imaging experiments. The level of oxygen (~10 torr) reached excites the type I cells. Experiments took place at a bath temperature of 37° C.
Figure 15.
measured with open flow respirometry using oxygen and carbon dioxide gas analyzers (S-3A/I, Applied Technologies, Pittsburgh, PA, USA; CD-3A, Applied Technologies, Pittsburgh, PA USA, respectively). Flow rate (0.248-0.257 L/min) was controlled with a 4100 Series flow meter (TSI Inc., Shoreview, MN). All mouse weights were noted before trials began. $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$ were corrected as standard temperature and pressure under dry conditions (STPD). Mice were placed in a 14cm x 10.2cm x 7.6cm plastic metabolic chamber (Figure 16) and allowed to acclimate to their surroundings for 30 minutes before the experiment began. All metabolic experiments were run for 30 minutes and data were collected every 5 minutes unless there was a change of more than 0.05 in consumed oxygen or excreted carbon dioxide and then data were collected and the time point was noted. Chamber temperature and barometric pressure were measured for each trial and were on average 27 ºC and 736 mmHg, respectively. Oxygen and carbon dioxide analyzers were calibrated each day before experiments were run to ensure accurate readings.

**Data Analysis and Statistics**

Values in figures and text are reported as means ± S.E.M. Significance was determined where appropriate using unpaired Student’s t-Tests with P<0.05 defined as a significant difference. Further statistical guidance will be sought from the Wright State University Statistical Consulting Center.
Figure 16. Schematic illustrating the metabolic chamber set up. A. Metabolic chamber, B. Filter, C. Flow meter, D. Carbon dioxide analyzer with drying column, E. Oxygen analyzer, F. Suction pump
Figure 16.
Results

Plethysmography

Ventilatory Response of Global AMPK α1 and AMPK α2 Knockout and TH-targeted Knockout Mice to Hypoxia

It is well known that the carotid bodies respond to changes in the PO$_2$ of the blood and act to produce an increase in ventilatory response upon exposure to a hypoxic situation (Heymans et.al., 1930). A two-chamber plethysmography unit was used to determine the role of AMPK in the hypoxic response. Resting or baseline breathing rates were examined first then mice were exposed to a hypoxic challenge. Figure 17 shows the baseline breathing rates for all wildtype mice (n=9), global AMPK α1 knockout mice (n=20) and global AMPK α2 knockout mice (n=22). The frequency (f), or respiration rate, in breaths/minute was not significantly different between the WT (269.66±17.23 breaths/min) and global knockout mice (α1KO, 253.91±14.10; α2KO 273.51±14.73 breaths/min) at rest (Figure 17, i.). There was no difference in tidal volume (V$_T$, µl/g) between the WT (7.12±0.90 µl/g) and global knockout α1 (6.38±0.34 µl/g) or α2 (6.82±0.61 µl/g) mice at rest (Figure 17, ii). In addition, at rest, minute ventilation (V$_E$, ml/min/g) was slightly attenuated in the AMPK α1 knockout mice (1.56±0.08 ml/min/g) compared to the WT (1.91±0.24 ml/min/g) and AMPK α2 (1.84±0.15 ml/min/g) mice but the difference was not significant. Upon exposure to 12% oxygen, the percent change
Figure 17. Histograms showing baseline ventilatory responses in wildtype, AMPK α1 and AMPK α2 knockout mice. (i.) Resting Breathing, (ii.) Tidal Volume, (iii.) Minute ventilation at rest (WT, n= 9; AMPK α1−/−, n=20; AMPK α2−/−, n=22).
Figure 17.

Resting breathing
in frequency, which is the difference in respiration rate between 12% O\textsubscript{2} and 21% O\textsubscript{2} was not significantly different between the WT (8.62±2.04%) or α1 knockout mice (9.21±2.26%). The AMPK α2 knockout mice (2.37±2.76%) showed a reduced percent change in respiration rate but it was not significant due to wide variability (Figure 18, i). Due to wide variability the percent change in tidal volume, difference in tidal volume at 12% O\textsubscript{2} and 21% O\textsubscript{2}, showed no significant difference between the WT (2.32±8.29), global α1 knockout (-1.74±4.18%) or AMPK α2 KO mice (2.75±3.12%) (Figure 18, ii). Overall, there was no significant difference between the % increase in minute ventilation seen with the WT, (11.53±10.29%) global AMPK α1, (6.34±3.56%) and AMPK α2 knockout (4.99±3.69%) mice during a 12% oxygen challenge (Figure 18, iii).

In order to determine the acute ventilatory response to a more severe hypoxic challenge, baseline breathing rates were collected, then mice were exposed to 8% oxygen, which corresponds to 25,000 feet (7620 meters) and is an extreme level of hypoxia. Figure 19, illustrates the baseline breathing for the ventilatory response at rest in the control (n=21) and global AMPK α1 knockout (n=33) mice before exposure to 8% oxygen. The frequency in breaths/minute between the WT (249.22±8.06 breaths/min) and α1-/- mice (275.59±7.16 breaths/min) at rest was slightly elevated in the knockout mice but was not significant (Figure 19, i). Tidal volumes of the WT (9.1±0.52 µl/g) mice were slightly elevated at rest versus the AMPK α1 KO (6.4±0.34 µl/g) mice but were not significant (Figure 19, ii). Resting minute ventilation was significantly attenuated in the global AMPK α1 KO (1.73±0.08 ml/min/g; unpaired
Figure 18. Percent Increase in Breathing upon exposure to 12% O₂. (i.) Percent change in frequency (F), (ii.) Percent change in tidal volume (Vₜ), (iii.) Percent change in minute ventilation (Vₑ) in AMPK α1 and AMPK α2 KO mice when exposed to 12% oxygen compared to WT (WT, n= 9; AMPK α1⁻/−, n=20; AMPK α2⁻/−, n=22).
Figure 18.

Percent increase in breathing during 12% O₂
Figure 19. Histograms showing baseline ventilatory responses in wildtype and Global AMPK α1 Knockout Mice. A.(i.) Resting respiratory rate (F), (ii.) Tidal volume (VT) and (iii.) Significant decrease in resting minute ventilation (VE) in AMPK α1 KO mice compared to WT (p<0.0005) (WT, n=21; AMPK α1−/−, n=33).
Figure 19.

Resting Breathing
Student’s t-Test, p<0.005) when compared to the WT mice (2.25±0.12 ml/min/g) (Figure 19, iii). When the WT and global AMPK α1 were exposed to 8% O₂ the percent change in frequency of the AMPK α1⁻/⁻ mice (5.34±1.06%; unpaired Student’s t-Test, p<0.004) was significantly less compared to the WT mice (13.89±3.04%) (Figure 20, i). The percent change in tidal volume of the α1 KO (10.01±2.38%) mice was elevated compared to the WT (4.79±2.64%) mice but was not significant (Figure 20, ii). Finally, the percent change in minute ventilation at 8% O₂ in the WT (19.04±4.12%) mice was slightly elevated when compared to the knockout mice (15.73±2.54%) but was not significantly different (Figure 20, iii).

The AMPK α2 KO mice were also used in plethysmography experiments to determine their acute hypoxic ventilatory response. Figure 21 shows the baseline breathing rates at rest of the WT (n=21) and global AMPK α2 knockout mice (n=18). The respiration rate or frequency of the WT (249.22±8.06 breath/min) mice was slightly lower versus the AMPK α2⁻/⁻ mice at rest (308.84±9.74 breaths/min) but it was not significantly different (Figure 21, i). When comparing the tidal volume of the knockouts to the WT (9.1±0.52 µl/g) mice, the AMPK α2⁻/⁻ (5.3±0.39 µl/g; unpaired Student’s t Test, p<0.00001) mice had a significantly attenuated tidal volume vs. the WT (Figure 21, ii). In addition, there was a significant decrease in minute ventilation in the global AMPK α2 KO (1.62±0.09 ml/min/g; unpaired Student’s t test, p<0.0005) mice compared to the WT (2.25±0.12 ml/min/g) mice at rest (Figure 21, iii).

When the WT and AMPK α2 KO mice were exposed to 8% oxygen, there was a significant decrease in the percent change in respiration rate between the AMPK α2⁻/⁻
Figure 20. Percent change in ventilatory parameters in response to 8% hypoxia. (i.) Significant decrease in the percent change in respiratory rate in the AMPK α1−/− mice compared to wildtype (p<0.004), (ii.) Percent change in tidal volume, (iii.) Percent change in minute ventilation (WT, n=21; AMPK α1−/−, n=33).
Percent Increase in Breathing During 8% O₂

Figure 20.
Figure 21. Histograms showing baseline ventilatory responses in wildtype and Global AMPK α2 Knockout Mice. (i.) Resting Breathing, (ii.) Significant decrease in tidal volume in AMPK α2−/− mice compared to wildtype (p<0.0001), (iii.) Significant decrease in resting minute ventilation in AMPK α2 KO mice compared to WT (p<0.0005) (WT, n=21; AMPKα2−/−, n=18).
Figure 21.

**Resting breathing**

![Bar charts showing breaths per minute and volumes](image)
Figure 22. Percent change in ventilatory parameters in response to 8% hypoxia in WT and global AMPK α2 KO mice. (i.) Significant decrease in the percent change in respiration frequency in AMPK α2 KO mice compared to WT (p<0.04), (ii.) Percent change in tidal volume, (iii.) Significant attenuation (~80%) in % change in minute ventilation in AMPK α2−/− mice when exposed to 8% O2 compared to WT (p<0.005) (WT, n=21; AMPK α2−/−, n=18).
Figure 22.

Percent increase in breathing during 8% O₂
(6.09±1.53%; p<0.04) and the WT (13.89±3.04%) mice (Figure 22, i). The percent change in tidal volume observed in the AMPK α2−/− mice (-1.79±2.93%) decreased compared to the WT (4.79±2.64%) mice but was not significant (Figure 22, ii). Finally, upon exposure to 8% O2 there was a significant attenuation of % change in minute ventilation in the global AMPK α2 KO (3.80±2.62%; unpaired Student’s t-Test, p<0.005) mice by about 80% when compared to the WT (19.03±4.12%) mice (Figure 22, iii).

It is possible that knocking out AMPK globally may have an inhibitory effect on the HVR through a mechanism mediated by something other than the carotid body. In order to try and minimize possible central effects to knocking out AMPK globally, mice that potentially had knockout of AMPK in tissues containing tyrosine hydroxylase, which includes the carotid bodies were used. The AMPK α2 subunits in the carotid bodies of these targeted knockout mice should be deleted, thus sparing much of the central processing hypoxic peripheral input (Gozal et al., 2000; Takakura et al., 2006). The ventilatory response of WT (n=21), Floxed controls (FlFl/Fl; n=20), and AMPK α2 TH-targeted KO mice (THCre+/−FlFl/Fl; n=13) were examined to determine if a central effect of global AMPK α2 knockout was removed.

Figure 23 illustrates the baseline breathing rates for the WT, floxed controls and TH-targeted KO mice. There was no difference in the respiration rate between WT (249.22±8.29) mice compared to the flox control mice (259.7±7.30) at rest; however, the targeted KO (303.92±6.19) mice showed a significant increase in respiration rate compared to wildtype mice (249.22±8.29; unpaired Student’s t-Test, p<0.00004) at rest (Figure 23, i). When comparing the tidal volume at rest of the WT, flox controls, and
Figure 23. Histograms showing baseline ventilatory responses for wildtype, Flox control (Fl\textsuperscript{F/F}) and TH-Targeted knockout mice (ThCre\textsuperscript{+/- Fl\textsuperscript{F/F}}). (i.) Significant increase in resting breathing in TH-targeted KO mice compared to WT (p<0.0004), (ii.) Significant decrease in tidal volume in flox control mice and TH-targeted KO mice compared to WT mice (Flox\textsuperscript{F/F}, p<0.0068; TH-targeted, p<0.008), (iii.) Significant decrease in resting minute ventilation in flox control mice compared to WT (p<0.02) (WT, n=21; Flox\textsuperscript{F/F} n=20; THCre\textsuperscript{+/- Fl\textsuperscript{F/F}}, n=13).
Figure 23.

**Resting breathing**

![Graph showing resting breathing parameters for WT, ThCre, Fl^{111}, and Fl^{111} groups.](image)
targeted KO’s (Figure 23, ii), there was a significant decrease in the flox controls (7.4±0.31 µl/g; unpaired Student’s t-Test, p<0.0068) compared to the WT mice (9.1±0.52 µl/g) as well as a significant attenuation in the tidal volume of the targeted KO (6.98±0.48 µl/g; unpaired Student’s t-Test, p<0.008) when compared to wildtype mice at rest. The flox control mice also showed a significant decrease in resting minute ventilation (1.90±0.068 ml/min/g; unpaired Student’s t-Test, p<0.02) when compared to the WT mice (2.25±0.122 ml/min/g), but there was no significant difference between the WT and ThCre^{+/+}FliFl/Fl (2.12±0.153 ml/min/g) mice at rest (Figure 23, iii).

After exposure to 8% oxygen the flox controls (31.45±5.58%) showed no significant difference in the percent change in respiration rate compared to the wildtype mice (23.73±7.30%). There was a significant attenuation in the percent change in respiration rate in the TH-targeted KO (-2.81±12.23; unpaired Student’s t-Test, p<0.007) mice vs. the WT (31.45±5.58%) (Figure 24, i). Both the flox control mice (0.98±0.21%) and targeted KO (1.03±0.31%) mice showed a slightly elevated percent change in tidal volume when compared to the wildtype (0.33±0.22%) but neither were significantly different (Figure 24, ii). In addition, there was no significant difference in the percent change in minute ventilation between the flox controls (0.446±0.079%), ThCre^{+/+}FliFl/Fl (0.250±0.051%) and the wildtype (0.367±0.053%) mice (Figure 24, iii) when exposed to 8% oxygen.

Thus, during exposure to 12% O2 AMPKα1 and AMPK α2 mice did not show a significant difference in their hypoxic ventilatory response when compared to the wildtype mice. As a result the level of hypoxia was decreased to 8%. Baseline minute
Figure 24. Percent change in ventilatory parameters in response to 8% hypoxia in WT, TH-targeted KO (ThCre<sup>+/+</sup>F<sup>Fl/Fl</sup>) and Flox control (F<sup>Fl/Fl</sup>) mice. (i.) Significant decrease in the percent change in respiration frequency in TH-targeted KO mice (p<0.007), (ii.) Percent change in tidal volume, (iii.) Percent change in minute ventilation (V<sub>E</sub>) in flox controls and TH-targeted KO mice when exposed to 8% O<sub>2</sub> compared to WT (WT, n=21; Flox<sup>Fl/Fl</sup> n=20; THCre<sup>+/+</sup>F<sup>Fl/Fl</sup>, n=13).
Figure 24.

Percent increase in breathing during 8% O₂
ventilation rates for both AMPKα1 and AMPK α2 mice were significantly less compared to their wildtype counterparts. During exposure to 8% oxygen AMPKα1 and AMPK α2 KO mice showed a significant attenuation in the percent change in frequency, as well as α2 KO mice having a significant decrease in the percent change in minute ventilation (~80% less).

**Ventilatory Response of Global AMPK α1 and AMPK α2 Knockout and TH-targeted Knockout Mice to Hypercapnia**

It has been shown that increasing inspired CO₂ is a well established respiratory stimulus (Haldane & Priestly, 1905) mediated in part, by the carotid body (Gonzalez, *et al.*, 1994). It is believed that TASK channels play a role in the CO₂-sensing ability within the type I cells of the carotid body. Therefore the ventilatory response of the mice to hypercapnia was done as control experiments to illustrate that AMPK plays no role in how the type I cells of the carotid body sense changes in carbon dioxide. **Figure 25** illustrates the ventilatory response of the WT (n=15), global AMPK α1 (n=29), and AMPK α2 (n=22) knockout mice at resting breathing (0% CO₂). The AMPKα1 KO (222.23±11.00 breaths/min) mice have a slightly attenuated resting respiration rate compared to the WT (254.75±12.59 breaths/min) but was not significant, whereas the AMPK α2 KO mice have a significantly elevated resting respiration rate (291.1±11.94 breaths/min; unpaired Student’s t-Test, p<0.048) (**Figure 25, i**). There was no difference in tidal volume between the AMPK α1−/− (5.96±0.23 µl/g) mice compared to the wildtype (6.32±0.28 µl/g) at rest but the AMPK α2+− mice (4.4±0.25 µl/g; unpaired
Figure 25. Histograms showing hypercapnic ventilatory responses in wildtype, AMPK α1 KO and AMPK α2 KO mice. A. (i.) Significant increase in resting frequency in AMPK α2 KO mice compared to WT (p<0.048). (ii.) Significant decrease in tidal volume in AMPK α2 KO mice compared to WT at rest (p<0.00013). (iii.) Significant decrease in resting minute ventilation in AMPK α1 KO mice and AMPK α2 KO mice compared to WT at rest (α1−/−, p<0.004; α2−/−, p<0.01; WT, n=15; AMPK α1−/−, n=29; AMPK α2−/−, n=22).
Figure 25.

Resting breathing

<table>
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<th>$F$</th>
<th>$V_t$</th>
<th>$\dot{V} \text{O}_2$</th>
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<tr>
<td>WT</td>
<td></td>
<td>6.0</td>
<td>0.4</td>
</tr>
<tr>
<td>$\alpha_{1/-}$</td>
<td></td>
<td>7.0</td>
<td>0.6</td>
</tr>
<tr>
<td>$\alpha_{2/-}$</td>
<td></td>
<td>8.0</td>
<td>0.8</td>
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</table>

$^*$ $p<0.048$

$^*$ $p<0.000013$

$^*$ $p<0.004$

$^*$ $p<0.010$
Student’s t-Test, p<0.0001) had a significant decrease compared to the WT mice (Figure 25, ii). Both the global AMPK α1 KO (1.30±0.054; unpaired Student’s t-Test, p<0.004) mice and AMPK α2 KO (1.28±0.079; unpaired Student’s t-Test, p<0.010) mice had a significant decrease in minute ventilation at rest compared to the WT (1.60±0.09 ml/min/g) (Figure 25, iii). Upon exposure to 7% CO₂ the WT (20.06±6.13%) and AMPK α1⁻/⁻ (17.98±3.47%) mice did not show any significant difference in percent change in respiration rate (Figure 26, i). The AMPK α2⁻/⁻ (8.91±1.88%) mice showed a decrease in the percent change in respiration rate during 7% CO₂ but it was not significant (Figure 26, i). The percent change in tidal volume was not significantly different between the WT (10.36±3.31%), α1⁻/⁻ (8.41±2.44%) or α2⁻/⁻ (9.06±2.01%) mice during 7% CO₂ (Figure 26, ii). Both the AMPK α1 KO (26.82±3.29%) mice and AMPK α2 KO mice (18.42±2.38%) showed a slight decrease in percent change in minute ventilation (Figure 26, iii) compared to the WT mice (32.10±7.59%) when exposed to 7% CO₂ but it was not significant.
Figure 26. Percent change in ventilatory response when exposed to 7% CO$_2$ in WT, AMPK $\alpha$1 and AMPK $\alpha$2 KO mice. (i.) Percent change in frequency, (ii). Percent change in tidal volume, (iii). Percent change in minute ventilation in AMPK $\alpha$1 KO and AMPK $\alpha$2 KO mice compared to WT during 7% CO$_2$ (WT, n=15; $\alpha$1$^{-/-}$, n=29; $\alpha$2$^{-/-}$, n=22).
Percent increase in breathing during 7% CO₂
Metabolic rates for Wildtype, Global AMPK α1 KO and Global AMPK α2 KO Mice During Steady State.

Minute ventilation rates at rest in both global AMPK α1 and AMPK α2 knockout mice were significantly attenuated as a result, metabolic experiments were run in order to rule out a metabolic difference between the knockout and WT mice. Rates for mean oxygen consumption ($\dot{V}_{O_2}$), carbon dioxide excretion ($\dot{V}_{CO_2}$) and respiratory exchange ratio (R) for wildtype, AMPK α1 KO and AMPK α2 KO mice are shown in Table 2. There were no significant differences between oxygen consumption in the wildtype (82.34±6.62), AMPK α1 KO (80.70±7.28) and AMPK α2 KO (98.48±2.58) mice (Figure 27, A). AMPK α1 KO (62.87±2.60) and AMPK α2 KO (65.46±2.12) mice showed slightly elevated levels of $\dot{V}_{CO_2}$ compared to wildtype (61.73±3.14) but were not significant (Figure 27, B). The respiratory exchange ratio, which is the ratio of carbon dioxide produced/oxygen consumed, showed no significant difference between the wildtype (0.79±0.03), AMPK α1 (0.81±0.05) and AMPK α2 KO (0.66±0.02) mice (Figure 27, C).
Table 2. Mean $\dot{V}_O_2$, $\dot{V}_CO_2$ and Respiratory ratios (± standard error) for wildtype, AMPK $\alpha_1$ KO and AMPK $\alpha_2$ KO mice used in metabolism trials (WT, n=11; AMPK $\alpha_1^{-/-}=7$; AMPK $\alpha_2^{-/-}=8$).

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>$\dot{V}_O_2$ (ml/min/Kg)</th>
<th>$\dot{V}_CO_2$ (ml/min/Kg)</th>
<th>Respiratory Exchange Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>82.34±6.62</td>
<td>61.73±3.14</td>
<td>0.79±0.03</td>
</tr>
<tr>
<td>AMPK $\alpha_1$ KO</td>
<td>80.70±7.28</td>
<td>62.87±2.60</td>
<td>0.81±0.05</td>
</tr>
<tr>
<td>AMPK $\alpha_2$ KO</td>
<td>98.48±2.58</td>
<td>65.46±2.12</td>
<td>0.66±0.02</td>
</tr>
</tbody>
</table>
Figure 27. Metabolism Histograms showing metabolic rate in wildtype, AMPK α1\(^{-/}\) and AMPK α2\(^{-/}\) mice.  

A. No significant differences between mice in the rate of oxygen uptake (\(\dot{V}_{O_2}\)).  
B. No significant difference between mice in the amount of CO\(_2\) produced (\(\dot{V}_{CO_2}\)).  
C. No significant difference in mice in the ratio of carbon dioxide produced in the tissue to oxygen consumed (R). (WT, n=11; AMPK α1\(^{-/}\), n=7; AMPK α2\(^{-/}\), n=8).
Figure 27.

A. \( \text{VO}_2 \)

\[
\begin{array}{c}
\text{ml/min/Kg} \\
\hline
\text{WT} & \alpha 1/- & \alpha 2/-
\end{array}
\]

B. \( \text{VCO}_2 \)

\[
\begin{array}{c}
\text{ml/min/Kg} \\
\hline
\text{WT} & \alpha 1/- & \alpha 2/-
\end{array}
\]

C. Respiratory Exchange Ratio (R)

\[
\begin{array}{c}
\text{WT} & \alpha 1/- & \alpha 2/-
\end{array}
\]
Problems with the Targeted Knockout Mouse

To ensure that AMPK α2 knockout was targeted to TH-expressing tissue the adrenal medulla and cortex were removed from TH-targeted mice and examined for the presence of an AMPK signal. The adrenal medulla expresses tyrosine hydroxylase and therefore the AMPK signal in this tissue should be absent or knocked out in a TH-targeted KO mouse as Cre recombinase will be active. By contrast, the adrenal cortex tissue does not contain tyrosine hydroxylase and should give a +/+ AMPK signal in a targeted KO mouse. Figure 28 is a PCR gel showing the presence or absence of the AMPK signal in the medulla and cortex for a WT mouse, an AMPK alpha 2 global KO mouse and a TH-targeted KO mouse. The WT mouse shows a +/+ signal for AMPK in both the medulla and cortex, which is what one would expect. When examining the AMPK α2 global KO mouse, AMPK is not present in either the adrenal medulla or adrenal cortex and this is also to be expected since AMPK is globally knocked out throughout the entire mouse. In the targeted KO mouse there is a -/- signal in the medulla for AMPK (top band), which is to be expected because the medulla is TH-positive tissue. The bottom +/+ band in the medulla is a residual flox band because there are both TH positive and TH negative tissue in the medulla. For example, chromaffin cells are TH positive whereas blood vessels are TH negative. However, when looking at the TH-negative cortex the top band is also showing a -/- signal for AMPK (Figure 28, red circle), this should NOT occur because the cortex does not contain TH-positive tissue.

To ensure the reason for observing AMPK knockout in the adrenal cortex was not due to contamination with medulla fragments, adrenal cortex tissue was taken from outside the adrenal gland, which does not contact the medulla. The same results were
Looking for Presence of AMPK α2 Signal. The wildtype mouse (WT) shows the presence of AMPK (+/+), the global KO mouse (α2ko) has AMPK α2 (-/-) knocked out, and the targeted KO mouse (target ko) shows AMPK α2 (-/-) knocked out in both medulla and cortex because it is a global KO vs. a targeted KO. The targeted KO mouse also shows lack of AMPK in the cortex, which should not occur because the cortex does not contain tyrosine hydroxylase and should not be cut out in the targeted KO mouse.
Figure 28.
found each time: TH-negative cortex showed AMPK α2 knockout. This lack of AMPK signal in the cortex was the first indication there was a potential targeting problem with the targeted KO mice.

It was discovered that two main problems presented themselves with regard to the targeted KO mice (Figure 29). The first issue was a loss of the flox signal in the targeted KO mice. Primers designed to identify whether a mouse was +/+,-/+ or -/- for floxed AMPKα2 failed to produce an identifiable band. Figure 29 (issue 1) shows a single targeted knockout mouse that is +/+ for Cre but does not have a Flox signal. The lack of a flox signal could possibly be explained if somehow the animal had lost all AMPK α2. Indeed the AMPK α2 signal from the tail was -/- in this mouse. The fact that the AMPK signal is -/- and the flox signal is absent would indicate this mouse was a global knockout instead of a TH-targeted mouse. If this mouse was a true targeted KO mouse, AMPK would still be present because the tissue for the PCR was taken from the tail where no TH-positive tissue should be found.

There was also a second issue that arose with the targeted knockout mice. The ideal targeted knockout mouse is +/+ for Cre and +/+ for Flox, which appears to be the case for the single targeted KO mouse shown in Figure 29 (issue 2). However, when looking for the AMPK signal in this mouse it was absent which should not be the case because tail tissue does not contain tyrosine hydroxylase. Knockout of AMPK in the tail tissue should only occur if Cre was sending the knockout throughout the entire mouse versus just TH-positive tissue. It is possible that Cre is not targeting to
Figure 29. PCR gel illustrating the two main issues in regards to the TH-targeted knockout mice. **Issue 1:** Loss of flox signal in the targeted knockout mouse. DNA from tail tissue shows the mouse is +/+ for Cre but the flox signal is absent and the mouse is -/- for AMPK α2. Red circles indicate loss of flox signal and -/- band for AMPK α2. **Issue 2:** Gel seems to illustrate the preferred targeted knockout mouse genotype (+/+ for Cre, +/+ for Flox), but shows a negative signal for AMPK, which should not occur because the DNA is from tail tissue. Red circle indicates negative signal for AMPK α2.
Figure 29.

<table>
<thead>
<tr>
<th>Issue 1</th>
<th>Issue 2</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre</td>
<td>Fl</td>
<td>a2</td>
</tr>
<tr>
<td>Cre</td>
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<tr>
<td>Cre</td>
<td>Fl</td>
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</table>

Controls:

- +/-
- +/-

- +/-
- +/-
- +/-
just TH-positive tissues but to other tissue as well. AMPK should only be knocked out in TH-positive tissue if the cre is correctly targeted/expressed.

As a result of the two aforementioned genotyping issues (spontaneous global KO and mis-targeting of cre) the parents of the targeted knockout mice were examined to determine if the parent’s genotype could explain the unexpected results that were obtained. Figure 30 examines the presence or absence of Cre, Flox and AMPK in the father (F), mother (M) and offspring (O) of a single targeted knockout mouse. The father and mother were +/− for Cre and produced a +/+ Cre offspring. The parents were +/− for Flox but the flox signal was lost in the offspring, which prompted further examination of the father, mother and offspring for the presence of AMPK. Both parents were +/+ for AMPK and the offspring was −/− for AMPK α2, which is not genetically possible since both parents were +/+ for AMPK α2. It appears as if the offspring was a global knockout mouse instead of a targeted KO mouse. Mice that showed a loss of the flox signal were not used for any experiments, so no plethysmography or imaging data were collected from these mice.

Next, the parents of a mouse that appeared to be an ideal targeted AMPK KO were examined for the presence of Cre, Flox and AMPK. Upon examination of the PCR gel shown in Figure 31 both the father and mother were +/− for Cre and Flox which gave an offspring that was +/+ for Cre and +/+ for Flox. The offspring of this cross appeared to be an ideal targeted KO (ThCre^{+/+}Flox^{F/F}) and the mice were used in plethysmography experiments. However, when the presence of AMPK α2 was looked for, the offspring showed a negative signal while the parents were +/+ for AMPK α2. If
Figure 30. PCR gel showing the presence or absence of Cre (C), Flox (F) and AMPK α2 (A) in the Father (F), Mother (M) and Offspring (O) of a single TH-targeted knockout mouse in regards to Issue 1. Both father and mother are +/- for Cre and flox giving an offspring that is Cre<sup>+</sup><sup>-</sup> but lacks a flox signal. Both parents are +/- for AMPK but the offspring shows a -/- signal for AMPK indicating the offspring is a global knockout. The first red circle indicates a lack of flox signal in the offspring while the second circle indicates a lack of a negative signal in the parents for AMPK, making it difficult to explain the -/- AMPK in the offspring.
Figure 30.
Figure 3. PCR gel showing the presence or absence of Cre (C), Flox (F) and AMPK α2 (A) in the Father (F), Mother (M) and Offspring (O) of a single TH-targeted knockout mouse in regards to Issue 2: Both father and mother are +/- for Cre and flox giving an offspring that is Cre^{+/-}/Flox^{+/-}, which is the ideal genotype for the targeted KO. Both parents are +/- for AMPK α2 but the offspring shows a negative signal for AMPK, which if the offspring were a true targeted KO AMPK α2 should be present because tail tissue does not contain TH.
Figure 31.

<table>
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<tr>
<th></th>
<th>Cre</th>
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Legend: +/- denotes the presence or absence of a specific trait or condition.
this mouse was truly a targeted KO mouse an AMPK α2 signal should be seen because the tissue used for the PCR came from the tail. The tail does not contain tyrosine hydroxylase and thus the AMPK signal should be present whether the animal is a targeted knockout or a wild type.

It seems as if Cre was targeting not only to TH-tissue but was cutting out AMPK in other tissues as well. The presence of AMPK in these animals was not examined before using them for plethysmography experiments since they were +/- for Cre and +/- for flox, which lead me to believe they were indeed a targeted knockout. As a result of the genotyping issues that have come about, plethysmography data collected from the targeted animals is not reliable because it is not known if AMPK α2 was knocked out in tissues other than TH-containing tissue.

To reiterate, to check that the targeted mice were indeed targeting knockout properly, the adrenal medulla and adrenal cortex were screened for the presence of an AMPKα2 signal. Knockout of AMPK α2 was shown in the medulla, which is to be expected due to the presence of tyrosine hydroxylase. However, knockout was also seen in the cortex, which is an issue because the cortex does not contain TH-positive tissue. Another issue was the loss of the flox signal in the targeted knockout giving offspring that appeared to be global knockouts, which cannot be explained and thus these mice were not used in plethysmography or calcium imaging.

The final targeting issue came about when it appeared the perfect targeted knockout mouse had been generated because it was +/- for Cre and +/- for flox but showed knockout of AMPK α2 in tail tissue. Again, data from these mice were not
useable due to the uncertainty of whether or not AMPK α2 knockout had been targeted appropriately.

**Calcium Imaging**

**Type I Cells Response to Hypoxia from Global AMPK α1 and AMPK α2 Knockout Mice**

Functional carotid body type I cells are known to depolarize in response to hypercapnia or hypoxia leading to voltage-gated calcium entry (Buckler & Vaughan-Jones, 1993, 1994a,b) and neurotransmitter release. Isolated type I cells from wildtype and AMPKα1 KO mice were subject to hypercapnic (20% CO₂) and hypoxic (~10 torr) solutions and levels of intracellular calcium were measured. The AMPK α1 KO type I cells had a strong hypercapnic response but a mixed response to hypoxia. Figure 32(A.) illustrates a hypoxic response in an AMPK α1 KO type I cell whereas Figure 32(B.) shows a type I cell from an AMPK α1 KO mouse with little to no response to hypoxia.

Calcium imaging results from AMPK α2 KO mice type I cells show they respond nicely to hypercapnia (20% CO₂) but do not show a hypoxic (~10 torr) response as seen in the wildtype cells. Wildtype mice type I cells (Figure 33A) respond robustly to HC as well as hypoxia. The AMPK α2 KO type I cells have a slightly attenuated hypercapnic response about 28% less versus the wildtype cells (Figure 33B) and also show a significant decrease in hypoxic response. The lack of hypoxic response in the AMPK α2 KO type I cells is illustrated by a significantly reduced [Ca^{2+}], ratio (p<0.003) compared to the wildtype cells (Figure 33C).
**Figure 32. Ca\(^{2+}\) Imaging Results from Global AMPKα1 Knockout Mice** (A.) A global AMPK α1 KO mouse type I cell response to hypercapnia (HC) and hypoxia. (B.) Type I cell from a global AMPK α1\(^{-/-}\) mouse response to (HC) but little to no response to a hypoxic stimulus (WT, n=6; AMPK α1\(^{-/-}\), n=2).
Figure 32.

A.

B.
Figure 33. Ca\(^{2+}\) Imaging Results from Global AMPK α2 Knockout mice (A.)

Wildtype mice type I cells respond to hypercapnia (HC) and hypoxia. (B). Global AMPK α2\(^{-/-}\) mice respond to (HC) but have impaired ability to transduce a hypoxic stimulus compared to WT mice. (C.) Significant decrease in cells response to hypoxia in AMPK α2\(^{-/-}\) mice compared to WT mice (p<0.003) (WT, n=6; AMPK α2\(^{-/-}\), n=5).
Figure 3.

A. 2.5
Ratio $F_{\text{H}} / F_{\text{norm}}$

B. 1.4
Ratio $F_{\text{hyp}} / F_{\text{norm}}$

C. 0.9
$\Delta$ Ratio $F_{\text{H}} / F_{\text{norm}}$

Wildtype

$\alpha2\text{ko}$

p<0.03

WT $\alpha2\text{ko}$
DISCUSSION

AMPK and Pharmacological Tools

Previous work attempting to establish the role of AMPK has been largely pharmacological and many of these agents have proven to have some non-specific or off-target effects independent of AMPK, which suggests caution must be used if utilizing these compounds to examine the role of AMPK in hypoxic chemotransduction. For example, Emerling, et.al. (2007) have shown that Compound C, a known inhibitor of AMPK, was able to suppress the hypoxic activation of HIF-1 in cells lacking AMPK α1 or AMPK α2. These data also show Compound C inhibits respiration independent of AMPK illustrating an off-target effect. Furthermore, Compound C is known to be a more potent inhibitor of TWELVE other kinases besides AMPK (Bain et.al., 2007).

Caution should also be used when interpreting data collected using the AMPK agonist, AICAR as Guigas et.al. (2007) showed cellular respiration was inhibited in rat hepatocytes that lacked either AMPK α1 or AMPK α2 subunits when AICAR was used. The inhibition of respiration in these knockout mice by AICAR was mediated independent of AMPK α subunits. To get around the possible uncertainties observed with pharmacological tools, global knockout mice were utilized in this dissertation to characterize the role of specific AMPK α subunits to provide further concrete evidence
for AMPK’s role in the transduction of hypoxic signaling by the carotid body and the acute hypoxic ventilatory response.

Global Knockout Mice and Hypoxic Response to Plethysmography

Global knockout mice have been the foundation for research in this project. The ideal knockout mouse for these experiments would have been to knockout both AMPK\(\alpha_1\) and AMPK\(\alpha_2\) subunits in the same animal but this is embryonically lethal (Viollet et al., 2003b) and thus not feasible. A double knockout would have guaranteed that AMPK had no catalytic activity and would have allowed for examination of the role each alpha subunit may play. The double knockout model would have avoided the possibility that knocking out one of the subunits (i.e. \(\alpha_1\)) would result in compensation by the other (\(\alpha_2\)) and vice versa. A double KO mouse is not possible thus; mice used in the experiments had either the AMPK\(\alpha_1\) or AMPK\(\alpha_2\) subunit knocked out globally. The global knockout mice allowed for examination of the role the AMPK alpha subunit(s) played in response to a hypoxic challenge.

Upon exposure to acute hypoxia the carotid bodies become stimulated resulting in an increase in minute ventilation which peaks in the first few minutes (Powell et al., 1998; Smith et al., 2001). Minute ventilation then declines to a point that is still higher than at normoxia depending on the level of hypoxia. This decrease in minute ventilation is termed hypoxic ventilatory decline (HVD) (Figure 34). Hypoxic ventilatory decline generally occurs after a prolonged exposure to hypoxia (5-30 minutes) (Powell et al., 1998). In order to avoid HVD in the plethysmography experiments conducted in this
Figure 34. Hypoxic Ventilatory Decline (HVD). Time course of ventilatory response to hypoxia. Peak aHVR is the peak acute hypoxic ventilatory response, HVD is the hypoxic ventilatory decline.
Figure 34.
dissertation, all hypoxic challenges were limited to 3 minutes and plethysmography data were taken from the peak hypoxic response. All wildtype and global AMPK α1 and AMPK α2 knockout mice were first exposed to 12% oxygen to examine the role AMPK played in the hypoxic response (Figure 18). At 12% oxygen the global AMPK α2 KO mice showed a decrease in percent change in respiration rate as well as minute ventilation and the AMPK α1 KO mice showed a decrease in percent change in tidal volume and minute ventilation but none of these were significantly different from the WT responses. It appeared that 12% oxygen was not a sufficient stimulus to produce a significant hypoxic response between knockout and wildtype mice, thus the hypoxic challenge was switched to a more robust hypoxic challenge of 8% oxygen.

At rest, the AMPK α1 KO mice showed no difference in frequency or tidal volume when compared to wildtype (Figure 19), however, the AMPK α1 KO mice did show a significant attenuation (p<0.0005) in minute ventilation compared to WT mice. Upon exposure to 8% oxygen (Figure 20) the AMPK α1 KO mice had a significant decrease in the percent change in frequency of breathing (P<0.004). However, there was no significant difference in the percent change in tidal volume and no significant difference in percent change in minute ventilation in the AMPK α1 knockout when compared to WT. Even though there is no significant difference between the KO and WT with regard to % change in minute ventilation, it does appear that knocking out the AMPK α1 subunit may have some affect on the response to hypoxia, as there is a significant decrease in the frequency response of the KO mice to hypoxia.

In the AMPK α2 KO mice there was a slight increase in frequency of breathing at rest but it was not significant when compared with wildtype mice. In addition these KO
mice showed a significant decrease in tidal volume (p<0.00001) and minute ventilation (p<0.0005) at rest (Figure 2) compared to the wildtype mice. When the AMPK α2 KO animals were exposed to 8% oxygen there was a significant decrease in the percent change in frequency (p<0.04) as well as a significant attenuation in the percent change in minute ventilation (p<0.005). The % change in minute ventilation was about 80% less when compared to the WT mice at 8% oxygen (Figure 2), which demonstrates that knocking out AMPK α2 subunits affects the acute hypoxic respiratory response of mice. These data also lend further support that AMPK plays a role in the acute HVR.

The plethysmography data from the global knockout mice show a significant decrease in percent change in frequency of breathing during exposure to 8% oxygen in both the global AMPK α1 and AMPK α2 knockout mice, however there was no significant percent change in tidal volume. This observation warrants further discussion. There was also a significant decrease in the percent change in minute ventilation in the AMPK α2 KO mice. It has been reported in animals where carotid body resection has occurred the entire hypoxic ventilatory response is gone however, if there is tonic sinus afferent discharge from the carotid body a partial ventilatory response to hypoxia can take place (Smith et.al., 2010; Hill et.al., 2011). This partial ventilatory response is possible due to specialized neurons, which function as central oxygen-sensors and are found in the Pre-Bötzinger complex of the respiratory network of the brain stem (Hill et.al., 2011). As long as the carotid sinus nerve offers some afferent discharge, the Pre-Bötzinger is able to compensate for the loss of oxygen-sensing by the carotid bodies (Bissonnette, 2000; Hill et.al., 2011; Smith et.al., 2010; Curran et.al., 2000). Thus in terms of a hypoxic response, the carotid body is necessary for an increase in breathing
frequency but not tidal volume (Bissonnette, 2000; Hill et al., 2011). It appears as if the frequency response to hypoxia is under the control of the peripheral chemoreceptors whereas response to tidal volume can be mediated by central chemoreception, which could help explain the lack of significant tidal volume response reported in the aforementioned data.

However, a potential methodological issue must also be discussed. The range of tidal volume values reported in this dissertation is quite variable and a few appear to be less than tidal volume values reported in the literature (Trapp et al., 2008). Another possible explanation for the range of tidal volume values collected is the plethysmography equipment set up. If the head of the mouse was not properly held in place by the inflatable cuff air could possibly have leaked out at this point. Thus tidal volume values would be reduced but frequency readings would remain accurate. In order to address the potential issue with the tidal volume values in regards to equipment set up, use of an unrestrained whole body plethysmography unit might prove beneficial. Unrestrained whole body units would get rid of the animal head/body restraint issue and there is plenty of data in the literature to compare values. The only downfall to the unrestrained whole body unit is that it costs around $50-60,000, which at the time was not a feasible purchase.

**TH-targeted Knockout Mice and Hypoxic Response to 8% Oxygen**

In addition to the global knockout mice, mice were also used which targeted knockout of the AMPK α2 subunit to tyrosine hydroxylase containing tissue that
included the carotid body. Targeted knockout mice were used to try and minimize possible central effects of knocking out AMPK by focusing on targeting AMPK α2 knockout to neurons containing TH. The targeted knockout would also minimize peripheral effects that may have occurred in the global AMPK KO mice.

Knocking out AMPK subunits in the brain may affect the communication line between the carotid body and the central mechanisms of ventilatory control. Therefore, AMPK α2 subunits were deleted in TH-positive cells, which includes carotid body type I cells but was spared in much of the brain regions that process the hypoxic peripheral input (Gozal et al., 2000; Takakura et al., 2006). It has been postulated that potential sites for convergence between the carotid body and the brain stem include the nucleus tractus solitarii (NTS) and retrotrapezoid nucleus (RTN) (Blain et al., 2010). RTN neurons receive excitatory input from afferents of the carotid chemoreceptors which bypass the respiratory network and are believed to involve a glutamatergic neuron found in the NTS (Takakura et al., 2006). Neurons of the commissural NTS (commNTS) are activated by carotid body activity and innervate the RTN via projections that are mainly glutamatergic. Because these neurons are glutamatergic in nature and not tyrosine hydroxylase positive, these areas of the brain should not be affected in the targeted KO mice.

Some of the central TH neurons however can affect the chemoreflex of the carotid body (Zhang & Mifflin, 2007; Viemari, 2008). Certain central noradrenergic neural structures of the brain such as A1, A2, A5 and A6 cell groups, are activated upon stimulation of the peripheral chemoreceptors. These structures innervate and possibly modulate neurons found in the caudal nucleus tractus solitarius (cNTS), which is the
location of the first central projections from the carotid bodies (Zhang & Mifflin, 2007). A2 neurons specifically receive sensory input from the peripheral chemoreceptors, which suggests they play a role in modulating the respiratory chemo-reflex (Viemari, 2008). It is also believed that A6 neurons are among the earliest born neurons (Lauder & Bloom, 1974) and therefore may have some role in the maturation of neural processes including that of the respiratory network (Viemari, 2008).

Since these norepinephrine neurons express tyrosine hydroxylase and possibly affect the carotid body chemoreflex (Zhang & Mifflin, 2007; Viemari, 2008), data from the TH-targeted knockout mice must be interpreted with caution. Knock out of AMPK α2 subunits in these TH-positive neurons may affect the signal sent from the brain to the respiratory network once the carotid bodies are stimulated.

Data were collected from the TH-targeted KO mice in order to examine their hypoxic ventilatory response (Figure 24). When exposed to 8% oxygen, the TH-targeted KO mice (ThCre+/FlFl) had a significantly attenuated percent change in frequency (p<0.03) compared to the WT and flox control mice. However, there was no significant difference between the percent change in tidal volume and percent change in minute ventilation among the groups. There were some concerns raised with these data since the global AMPK α2 KO mice showed a significant attenuation in minute ventilation during hypoxia but the targeted knockout minute ventilation values were not significant. A possible explanation is the TH-targeting may have not worked properly. As mentioned earlier, issues arose in regards to the targeting of AMPK α2 KO in TH-containing tissues as to the actual location of the knockout. The mice appeared to be the perfect targeted knockout because they were +/+ for Cre and +/+ for flox but lacked the presence of
AMPK α2 in tail tissue (Figure 29, issue 2). The lack of AMPK α2 in the tail is problematic because the tail does not contain TH-positive cells, thus AMPK should not be knocked out. Knockout of AMPK in the tail would occur only if Cre was somehow sending the knockout throughout the entire mouse versus just the TH-positive cells.

Lindberg et al., (2004) were able to demonstrate that cre-mediated site-specific recombination was successful by using a marker gene along with the loxP sites to detect recombination. These data illustrate that cre recombinase does in fact work therefore something in our mouse cre recombinase system is not working properly. Thus, plethysmography data collected from the TH-targeted KO mice cannot be reliably interpreted, as it is now evident that these animals were not properly targeted knockout mice.

**Global AMPK Knockout Mice Hypoxic Response and Calcium-Imaging**

To provide further support to the attenuated response to hypoxia observed during plethysmography experiments in the AMPK global knockout mice, FURA-2 calcium imaging techniques (Wyatt & Buckler, 2004; Wyatt et al., 2007; Burlon et al., 2009) were used to assess the functional integrity of the isolated carotid body type I cells. Isolated type I cells from WT mice were shown to depolarize in response to hypercapnia and/or hypoxia which led to entry of voltage-gated calcium (Buckler & Vaughan-Jones, 1993, 1994a,b) and neurotransmitter release.

Type I cells from the global AMPK α1 KO mice exhibited a robust response to hypercapnia but showed a mixed response to hypoxia. Figure 32A, illustrates the
hypoxic response of a type I cell from an AMPK α1 KO mouse. A strong response to hypercapnia was observed in addition to a small hypoxic response, whereas in Figure 32B, another AMPK α1 KO type I cell also showed a response to hypercapnia but little to no response to hypoxia. Thus, it appears as if there is a mixed hypoxic response from the type I cells of the AMPK α1 KO mice. The variable Ca²⁺ imaging response from the α1 KO mice seems to coincide with the variable response also seen in the plethysmography data where the percent change in frequency response to hypoxia was significantly less than the wildtype but, percent change in tidal volume increased and percent change in minute ventilation to hypoxia decreased but not significantly. In order to gain a better understanding of the aforementioned mixed hypoxic response, an increase in cell numbers is required for the global AMPK α1 KO imaging experiments.

Calcium imaging data collected from type I cells of global AMPK α2 KO mice showed a strong response to hypercapnia (Figure 33B). The hypoxic response was significantly smaller in the AMPK α2 KO compared to the control type I cells. This decrease in hypoxic response is illustrated in Figure 33C by the significantly (p<0.03) lower ratiometric signal in the AMPK α2 KO type I cells compared to the WT cells. The significant attenuation of the hypoxic response in the global AMPK α2 KO type I cells confirms that the expression of the AMPK α2 subunit is necessary for a complete acute HVR and a complete response of the carotid body type I cells to hypoxia. The lack of response to a hypoxic stimulus in the global AMPK α2 KO mice carotid body type I cells appears to support the reduced HVR observed in the AMPK α2 KO mice observed during the plethysmography experiments. The data show not only a reduced hypoxic
ventilatory response at the whole animal level but also a reduced response at the cellular level.

The mixed response of the AMPK α1 KO mice shown in the Ca$^{2+}$ imaging data could be the result of AMPK α2 subunits compensating for the KO of α1 in some of the type I cells. The expression level of each subunit varies among cells (Li et.al., 2006), thus one subunit may be expressed in higher amounts versus the other allowing it to compensate for knockout of the other subunit. The AMPK α1 subunits may not be able to compensate for the loss of the AMPK α2 subunits, which may explain the lack of hypoxic response in the AMPK α2 KO mice. Since the animals are global AMPK knockout mice and AMPK is knocked out throughout the entire organism central effects may be greater in the AMPK α2 KO versus the AMPK α1 KO mice due to tissue specific subunit expression differences. Currently this is speculation and increasing (n) numbers are required to help strengthen this argument. Further data collection will hopefully result in a more consistent response to allow for a more solid explanation.

There appears to be almost a complete total ablation of the hypoxic response in AMPK α2 KO mice but not in AMPK α1 KO mice. Both the AMPK α1 and AMPK α2 subunits appear to phosphorylate similar targets. It is therefore unlikely that AMPK α1 and AMPK α2 have different targets in the type I cells so this cannot explain the observed differences with the knockout. However, the expression levels of each subunit may vary in the carotid body therefore giving different results. Expression of one subunit may be at a higher level and when it is knocked out this may have a larger effect on signal transduction than knocking out a subunit with lower expression levels.
Also, it is not known what the tissue specific subunit combinations are, so different isoforms of AMPK maybe expressed at different levels in the different KO mice. The various subunit combinations may target AMPK expression to different locations such that the AMPK α2 subunits may be targeted to the plasma membrane whereas the α1 is localized elsewhere. It has been reported in a variety of cell lines and skeletal muscle tissue (Salt et al., 1998; McGee et al., 2003) that a significantly greater portion of AMPK α2 is localized in the nucleus versus that of the α1 subunit. Therefore, knocking out one or the other subunit may result in a different response based upon subunit location and expression level. This may help to explain why there is complete knockout of the hypoxic response in the AMPK α2 global KO mice but a mixed response reported in the α1 KO mice. In order to quantify alpha subunit expression levels, a Western Blot would be an ideal tool, however, due to the small size of the carotid body very little tissue is available so a Western Blot would not be possible. The only alternative possible method is to use single-cell RT-PCR to examine the protein level expression of AMPK α1 and AMPK α2 subunits to determine if one subunit is being expressed at a higher level.

**AMPK and Multiple Targets**

One challenge in carotid body research is the interspecies heterogeneity of oxygen-sensitive ion channels. Many of the channels expressed in the carotid body are not oxygen sensitive in other tissues. The absence of oxygen sensing capabilities is due to a lack of the necessary pathway to sense changes in oxygen levels and inhibit the
potassium channel. Recent research has shown that AMPK has multiple ion channel targets (Dallas et al., 2009; Evans et al., 2009; Kréneisz et al., 2009). Expression of oxygen-sensitive potassium channels inhibited via activation of AMPK is species dependent and expression of these channels is also different among animals. For example it has been reported that mice have the following oxygen-sensitive K^+ channels: Kv2.1 (Ikematsu et al., 2011), Kv3 (Pérez-Garcia et al., 2004), and possibly TASK-1 (Trapp et al., 2008); whereas rats are shown to have BK_{Ca} (Peers, 1990; Wyatt & Peers, 1995), TASK1/3 (Buckler, 1997; Buckler et al., 2000; Kim et al., 2009) and possibly TREK (Kréneisz et al., 2009) channels. AMPK activation leads to phosphorylation of a target protein(s) and the aforementioned potassium channels have proteins that contain AMPK phosphorylation sites, thus more than one potassium channel maybe inhibited when AMPK is activated. As a result, it is possible to have multiple oxygen-sensitive channels in an animal. Knocking out or altering one of the channels may still result in retention of a hypoxic response because another AMPK targeted channel may be able to compensate for the loss (Oretega-Sáenz, et al., 2010). Thus, it appears there is evidence in favor of AMPK being promiscuous because it is able to interact with multiple ion channel targets in different species.

**Hydrogen Sulfide**

In addition to AMPK it has been suggested over the last year or two that hydrogen sulfide, a naturally occurring ‘gastotransmitter’, plays a role in carotid body oxygen sensing through redox modulation in type I cells (Li et al., 2010; Peng et al., 2010).
Hydrogen sulfide is produced endogenously in cells via the breakdown of cysteine and homocysteine by the enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) (Mustafa et al., 2009; Gadalla & Snyder, 2010). Cystathionine-β-synthase is the main H₂S producing enzyme found in the brain whereas, cystathionine-γ-lyase is found in the glomus cells of the carotid body (Peng et al., 2010; Li et al., 2010). Hypoxia increases the production of H₂S in the carotid body (Peng et al., 2010) via a stimulus dependent manner. The mechanism as to how H₂S actually stimulates the carotid body is not exactly known but it has been shown that excitation of chemoreceptors through exogenous H₂S can be blocked by removing external calcium (Li et al., 2010; Peng et al., 2010). Inhibition of BK₇Ca channels (Li et al., 2010) by H₂S has been reported but this alone is not likely to excite the type I cells because these potassium channels are not active under resting conditions (Buckler, 1997).

H₂S has been reported to excite carotid body type I cells through inhibition of a background (TASK) potassium current leading to cell depolarization and voltage-gated entry, which mirrors the response to hypoxia (Buckler, 2012). Concerns have arisen in regards to the concentrations of exogenous H₂S required to excite the chemoreceptors and if these levels mirror those found in vivo (Haouzi et al., 2011). Hydrogen sulfide is highly membrane permeable and in order to generate a [Ca²⁺], response levels of H₂S need to be greater than 2.5 µM and in some cases greater than 7.5 µM (Mathai et al., 2009). The extreme levels required to generate even a small response pose a problem for H₂S to be seen as an oxygen sensor because type I cells are not capable of producing H₂S at this level (Buckler, 2012).
H₂S is also a known cytochrome oxidase inhibitor (Erecinska & Wilson, 1981) and has been shown to inhibit mitochondrial function via NADH autofluorescence at concentrations of 2.5 µM (H₂S) and above (Buckler, 2012). It does not appear as if H₂S plays any significant role outside of inhibition of oxidative phosphorylation, as it does not have any further effect on channel activity in the presence of cyanide (Buckler, 2012). In addition, the extreme levels of hydrogen sulfide required to excite carotid body type I cells does not make H₂S a likely candidate for mediating hypoxia in isolated type I cells as they are not capable of generating such large amounts of H₂S.

This dissertation suggests that AMPK does indeed couple hypoxic inhibition of oxidative phosphorylation of type I cells and plays a key role in the acute hypoxic ventilatory response. The data show that AMPK α subunits are necessary for a complete hypoxic ventilatory response as well as carotid body type I cells response to hypoxia. This work is supported by preliminary data from collaborators, in which knock out of LKB1, an upstream kinase of AMPK (Figure 5), in type I cells also gives an attenuated hypoxic ventilatory response in mice (Evans, 2012). Thus, not only does knockout of AMPK α catalytic subunits affect the hypoxic response, but also knocking out an upstream kinase which activates AMPK results in an attenuated HVR. These data suggest that the LKB1-AMPK signaling pathway is required for a hypoxic response coupled by the carotid body to regulate oxygen and energy supply at the whole body level.
Summary of Findings

- During exposure to 8% oxygen, both AMPK α1 and AMPK α2 knockout mice have a significant decrease in percent change in frequency of breathing.
- AMPK α2 knockout mice also show a significant attenuation in percent change in minute ventilation during exposure to 8% oxygen.
- The plethysmography data show the AMPK α subunits maybe involved in baseline breathing and generation of an acute hypoxic response.
- Calcium imaging data show a decrease in the hypoxic response may be due to an effect at the level of the carotid body.
- Knocking out AMPK globally could have central effects, thus TH-targeted knockout mice could be useful genetic tools to minimize possible central effects of knocking out AMPK.
Future Studies

One methodological approach that might get around the problem of non-selective targeting of AMPK knockout is to block or knockdown its expression through RNA interference. RNA interference is a process where double stranded DNA (dsDNA) is able to regulate gene expression. Small interfering RNA (siRNA) is a double stranded RNA molecule that is 21 nucleotides in length and is complementary to an mRNA transcript (Fire et al., 1998). When siRNA is introduced into a cell one strand is processed into the RNA-induced silencing complex (RISC) where it binds the complementary mRNA (Hammond et al., 2000). The nuclease activity of RISC results in the complimentary mRNA being degraded by a member of the Argonaute (AGO) family (Hammond et al., 2001), which no longer allows for protein translation (Scherr et al., 2003; Doi et al., 2003). SiRNA is not useful for long-term studies as it is diluted upon cell division (Rao et al., 2009).

An alternative method is to use short hairpin RNAs (shRNAs) to knockdown a specific target protein. shRNA is a sequence of RNA containing a tight hairpin structure which is used to silence gene expression through RNAi (Bernstein et al., 2001). shRNA is transcribed from a DNA template and uses a vector introduced into cells via the U6 promoter ensuring shRNA is always expressed (Harborth et al., 2003; Elbashir et al., 2001). The vector allows for heritable gene silencing as it is passed onto daughter cells.
Generation of a novel shRNA, which simultaneously targets both the AMPK α1 and AMPK α2 subunits, has been developed in collaboration with Dr. Tom Brown’s lab, Wright State University. Tissue-specific knockdown of AMPK α1 and AMPK α2 was accomplished through an shRNA construct (Figure 35). The construct is under a U6 promoter that is disrupted by a loxP cassette. The loxP cassette contains a stop sequence that is flanked on either side by a loxP site. When the promoter contains this cassette it becomes non-functional and the shRNA is not expressed and knockdown of AMPK does not occur. A tyrosine hydroxylase tissue specific promoter was used to direct the expression of Cre recombinase with a Myc tag (pLv-MCS-THp-CreMyc). The use of Cre, along with the shRNA that has a disrupted promoter will restore the promoter so shRNA is made in only cells that express TH, which includes the carotid body. The final construct has been generated and is currently being put into lentivirus. Lentivirus will infect 2-8 cell mice/rat embryos to ensure a full body incorporation of lentiviral DNA. All cells within the embryo will contain the viral DNA and only those cells expressing TH will make the shRNA and have knockdown of AMPKα1 and AMPK α2. Once infected the embryos will be transplanted into a pseudopregnant mother. Tissue specific knockdown of both AMPKα1 and AMPK α2 should occur in the embryos only in TH positive tissue and should not result in lethality since it is knockdown and not total knockout of both AMPK subunits. These animals will then be used by the Wyatt lab to examine the whole animal hypoxic response via plethysmography as well as look at the carotid bodies to further examine the role AMPK has in oxygen sensing of the type I cells.
**Figure 35. shRNA Construct.** (A.) shRNA structure under control of a U6 promoter. (B.) shRNA structure with disrupted U6 promoter. (C). DNA Recombination catalyzed by cre recombinase at loxP sites located between U and Stop sequence and between the Stop sequence and 6P. The Stop sequence is removed following binding of cre recombinase at the loxP sites with construct illustrated in (B.). (D). Virus structure.

U6P: promoter; shRNA: short hairpin RNA; Stop: stop sequence; LoxP: loxP sites; U/6P: disrupted promoter; ThP: tyrosine hydroxylase promoter; Cre: cre recombinase; Myc tag: (pLv-MCS-Thp-CreMyc) TH tissue specific promoter to direct expression of cre recombinase with the Myc tag.
Figure 35.

A. U6p → shRNA → Knockdown

B. U → LoxP → STOP → LoxP → 6p → shRNA → No Knockdown

C. U → LoxP → 6p → shRNA → Knockdown

D. U → LoxP → STOP → LoxP → 6p → shRNA → ThP → Cre → Myc
Another alternative approach to targeting knockout of AMPK besides the TH-targeted knockout mice would be to use dopamine transporter (DAT) (Augood et al., 1993) cre mice to specifically target knockout of AMPK to dopaminergic neurons in cre recombinase expressing mice. In order to direct expression of cre recombinase to dopaminergic neurons, cre is inserted downstream of the endogenous promoter DAT (DAT-cre) (Zhuang, et al., 2005). DAT-cre mice would also allow for AMPK α2 subunits to be deleted in the carotid body type I cells but again be spared in most of the central processing of hypoxic peripheral input.
REFERENCES


**Appendix**

5-HT: 5-hydroxytryptamine
ACh: acetylcholine
ADP: adenosine diphosphate
AGO: Argonaute
AICAR: 5-aminoimidazole-4-carboxamide ribose α: alpha
AMP: adenosine monophosphate
AMPK: amp activated protein kinase
AMP-PCP: β,γ-methylenadenosine 5'-triphosphate ATP: adenosine-5'-triphosphate
ASC: association with SNF1 kinase
ATP-γ: adenosine-5'-O-(3-thiotriphosphate)
β: beta
BKCa: maxi K⁺ potassium channel
° C: degrees centigrade
Ca²⁺: calcium
[Ca²⁺]: intracellular calcium
CamKKβ: cam kinase kinase beta
CB: carotid body
CO: carbon monoxide
CO₂: carbon dioxide
commNTS: commissural nucleus tractus solitarius
cNTS: caudal nucleus tractus solitarius
CSN: carotid sinus nerve
CBS: cystathionine β synthase
CSE: cystathionine-γ-lyase
DA: dopamine
DAT: dopamine transporter
DNA: deoxyribonucleic acid
dsDNA: double stranded deoxyribonucleic acid
F: frequency
FCCP: \( p \)-trifluorometoxyphenyl hydrazone
Fl: flox
\( \gamma \): gamma
GBD: glycogen binding domain
g: gram
GTP: guanosine triphosphate
H\(^+\): hydrogen
HC: hypercapnia
HCO\(_3\)-: bicarbonate
Hif-1: hypoxia inducible factor 1
HO-2: heme oxygenase-2
H\(_2\)S: hydrogen sulfphide
HVD: hypoxic ventilatory decline
HVR: hypoxic ventilatory response
K\(^+\): potassium
kDa: kilodalton
KIS: kinase interaction sequence
KO: knockout
Kv: voltage-gated potassium channel
LKB1: liver kinase B1
L/min: liters per minute
Mg\(^{2+}\): magnesium
\( \mu \)M: micromolar
mM: millimolar
min: minute
MO25\( \alpha \): mouse protein 25-\( \alpha \)
mmHg: millimeters of mercury
ms: millisecond
mV: millivolt
N₂: nitrogen
Na⁺: sodium
NADH: nicotinamide adenine dinucleotide
NADPH: nicotinamide adenine dinucleotide phosphate
NE: norepinephrine
NO: nitric oxide
NT: neurotransmitter
NTS: nucleus tractus solitarius
O₂: oxygen
PAC: peripheral arterial chemoreceptor
PCR: polymerase chain reaction
PO₂: partial pressure of oxygen
PCO₂: partial pressure of carbon dioxide
pHᵢ: intracellular pH
pHₒ: extracellular pH
P.S.I.: pounds per square inch
R: respiratory ratio
RISC: ribonucleic acid induced silencing complex
RR: respiration rate
RTN: retrotrapezoid nucleus
RT-PCR: reverse transcriptase polymerase chain reaction
shRNA: short hairpin ribonucleic acid
SIDS: sudden infant death syndrome
siRNA: small interfering ribonucleic acid
STPD: standard temperature and pressure under dry conditions
STRADα: ste 20-related adaptor protein-α
Sub P: substance P
TASK: acid (or alkali) sensitive K+-channels of the tandem P-domain K+-channel family
TH: tyrosine hydroxylase
Thr-172: threonine 172
UTP: uridine triphosphate

\( \dot{V}_{\text{CO}_2} \): carbon dioxide excretion
\( V_E \): minute ventilation
\( V_{E/g} \): minute ventilation/gram

\( \dot{V}_{\text{O}_2} \): oxygen consumption
\( V_T \): tidal volume
WT: wildtype
ZMP: 5-aminoimidazole-4-carboxamide ribose monophosphate