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Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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INTERMITTENT HYPOXIA AFFECTS LEPTIN AND LEPTIN RECEPTOR SIGNALING IN THE RAT CAROTID BODY

By

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Master of Science

The School of Graduate and Postdoctoral Studies

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Abstract

Carotid bodies contain blood oxygen-sensing cells expressing leptin and leptin receptor isoforms. Whether leptin is involved in chemoreception in the carotid body is unknown. Experiments were completed to investigate leptin signaling in carotid body glomus cells during intermittent hypoxia (IH). Rats were subjected to IH for 8 hours/day for 1, 7, 95 days or to systemic leptin injections. Immunohistochemical and Western bot analysis were used to localize leptin, its receptors and downstream signaling proteins in glomus cells. IH resulted in increased circulating and local leptin, increased activation of STAT3 and Fra-1 expression suggesting an activation of a leptin signaling system within these cells. Captopril treatment prior to IH eliminated the increased plasma and carotid body leptin in IH suggesting angiotensin II may exert an effect on leptin in glomus cells. The data suggest that leptin acting in the carotid body activates glomus cells and may play a modulatory role in the chemoreflex.

Key words: carotid body, leptin, leptin receptor, intermittent hypoxia

Co-Authorship Statement

The work presented in chapters 2, 4 and 5 received contributions from my colleague Jason Moreau. Jason was involved in sharing responsibilities in the animal intermittent hypoxic exposure protocol, including commencement and cessation of the protocol as well as animal sacrifice. Jason also was integral in gathering some of the data from both the leptin and angiotensin II enzyme immune assays, as well as administration of the angiotensin converting enzyme inhibitor captopril to the animals used in the study. The effort and support Jason has shown throughout these studies is gratefully acknowledged.

Acknowledgments

They say you can take the student away from Western but you'll never take Western away from the student. I was skeptical of this statement but almost seven years later, I think it may just prove to be accurate. The time has come for me to leave this place, but no matter how much time passes, the place will not leave me. I have met some extraordinary people in my time at Western and have created friendships that will last a lifetime.

First and foremost I should thank Dr. Ciriello for weathering the storm and helping me through the biggest learning experience of my life. In my short time we were successful in publishing multiple manuscripts in an exciting and novel field and for that I am thankful. A tremendous amount of gratitude goes to my colleague and friend Jason Moreau. He was a valuable asset for my project not only for his technical skills but his intellectual ability as well. Jay was also critical in helping me maintain my sanity while going through the process of grad school in the department of Physiology and Pharmacology. Additional thanks go to my fellow labmates Megan Migchels and Waseem Iqbal.

Time not spent in the lab was dispersed among the best friends a person could ask for. Whether it was skipping out early to go golfing, spent on a train to Toronto or on a plane down south, the leisure time was as critical to my learning experience as was the stress sustained in the lab and I have countless people to thank for spending this time with me.

Second to none, I owe my parents a great deal for continuing their support past the legal obligation of 18 years.

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List of Abbreviations

ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ANGII	Angiotensin II
ATP	Adenosine triphosphate
AT_1R	Angiotensin type-1 receptor
AT_2R	Angiotensin type-2 receptor
BMI	Body mass index
Ca^{2+}	Calcium
CIH	Chronic intermittent hypoxia
CPAP	Continuous positive airway pressure
CNS	Central nervous system
CO_2	Carbon dioxide
CSN	Carotid sinus nerve
ERK1/2	Extracellular-signal-regulated kinase 1/2
Fra-1	fos-related antigen-1
IH	Intermittent hypoxia
JAK	Janus Kinase
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
NG	Nodose ganglion

NO	Nitric Oxide
NTS	Nucleus of the solitary tract
O_2	Oxygen
OB-R	Obesity receptor
OB-Rb	Obesity receptor B
OB-R$_{100}$	Shortform obesity receptors
OSA	Obstructive sleep apnea
pERK1/2	Phosphorylated extracellular-signal-regulated kinase 1/2
PG	Petrosal ganglion
PO_2	Blood oxygen partial pressure
pSTAT3	phosphorylated signal transducer and activator of transcription 3
RAS	Renin-angiotensin system
SNA	Sympathetic nerve activity
SOCS3	Supressor of cytokine signaling 3
STAT3	Signal transducer and activator of transcription 3
TH	Tyrosine hydroxylase
WAT	White adipose tissue

Chapter 1

1   Literature Review

1.1   Obstructive Sleep Apnea

Obstructive sleep apnea (OSA) is a common type of sleep-disorder characterized by brief but repeated obstruction of the upper airway (Dempsey et al., 2010, 2012; Mannarino et al., 2012). These upper airway obstructions may be a partial blockage (hypopnea) or may result in a fully compromised airway occlusion (apnea), causing loud snoring, sleep fragmentation and excessive daytime sleepiness (Mannarino et al., 2012). Obstructions may occur due to both anatomic and neuromuscular influences. The human pharynx is not supported by the skeletal system which is prone to collapse during negative pressure (i.e. inhalation) and soft tissue build up, such as adipose tissue (Mannarino et al., 2012). A previous study has found that a reduction in BMI in patients with OSA resulted in a decrease in upper airway collapsibility events by over half (Schwartz et al., 1991). Not surprisingly, a narrow airway is generally more predisposed to obstruction than a larger one (Schwab et al., 1995). Additionally, abnormal activity of tonic and phasic dilator muscles can result in recurring airway obstructions (van Lunteren and Strohl, 1986).

OSA occurrence varies with differing populations and within age groups. OSA is more prevalent in men than in women with an estimated 4% of men and 2% of women aged 50 or over suffering from symptomatic OSA (Strollo & Rogers; 1996). However, this population-based evaluation may be grossly underestimated as a patient must undergo a formal sleep study in order for the diagnosis to be made. Additionally, many

sufferers are asymptomatic and may not seek medical attention. The male pattern of body fat distribution may account for the increased prevalence in men, particularly deposition in the trunk and neck area. In the middle-aged population, the numbers may be as high as 20-30% of the population suffering from OSA while not presenting symptoms (Young et al., 1997). To be diagnosed with OSA syndrome, one must experience 5 or more apnea/hypopnea events per hour of sleep in the presence of accompanying symptoms including excessive daytime sleepiness, fatigue or impaired cognition, or experience 15 apnea/hypopnea events per hour of sleep irrespective of additional symptoms (Park et al., 2011).

Although OSA is not uncommon in children, it is usually due to adenotonsillar hypertrophy (Marcus & Loughlin, 1996). Perhaps the single most important factor affecting OSA risk is body weight. Weight gain of 10% increases the risk of developing OSA by six-fold (Peppard et al., 2000a) and a 10% loss of body weight is estimated to decrease the apnea/hypopnea index (events/hour) by 26% (Peppard et al., 2000a). Within the obese population (BMI $\geq$ 30) (Wolk et al., 2003), approximately 40% experience significant OSA and approximately 70% of OSA patients are obese (Vgontzas et al., 1994), daunting numbers considering the obesity epidemic occurring in North America and estimates suggesting 41% of Americans will be obese by 2015 (Nejat et al., 2009).

1.1.1 Cardiovascular Consequences of OSA

The chronic effects of OSA are more severe than the fragmented sleep and daytime sleepiness. There are severe cardiovascular complications associated with the condition. OSA has been correlated with major cardiovascular disorders including hypertension (Moller et al., 2003; Peppard et al., 2000b), stroke (Redline et al., 2010),

atherosclerosis (Friedlander et al., 1999) and heart failure (Shahar et al., 2001) among others. Indeed when compared with non-OSA counterparts, OSA patients present with increased blood pressure during both night and daytime hours (Moller at al., 2003; Peppard et al., 2000b).

1.1.2 Hormonal Changes Associated with OSA

During normal daytime breathing, sympathetic nerve activity (SNA) is elevated in OSA patients (Somers et al., 1995; Leuenberger et al., 1995) which may be a contributing factor for many of these cardiovascular complications. Increased SNA may contribute to renin-angiotensin system activation, increasing circulating angiotensin II levels which have been positively correlated with daytime blood pressure (Moller et al., 2003). OSA syndrome also brings with it additional hormonal changes which may cause or exacerbate the cardiovascular morbidities previously described. For example, compared to BMI-matched controls, OSA patients experience increases in plasma leptin concentrations (Phillips et al., 2000; Harsch et al., 2003), as well as increased circulating catecholamines (Eisenberg et al., 1990; Carlson et al., 1993).

1.1.3 Treatment of OSA

Despite the severity of the complications associated with sleep apnea, there are treatments which reduce or abolish these deleterious effects. Unfortunately treatment of OSA is often overlooked due to lack of awareness of the condition, method of diagnosis and method of treatment. The primary treatment of OSA is continuous positive airway pressure (CPAP). CPAP consists of the patient wearing a mask during sleep, which exerts positive pressure on the upper airway, maintaining the integrity of the upper airway during sleep and thus encouraging proper airflow. OSA patients using the CPAP method

have marked reductions in SNA and blood pressure (Somers et al., 1995; Moller et al., 2003). CPAP treatment also reduces signs of atherosclerosis in patients suffering from OSA (Drager et al., 2007), which may also serve to further reduce risk of stroke and myocardial infarction. Furthermore, use of the CPAP mask reduces circulating leptin levels even with no change in patient BMI (Cuhadaroglu et al., 2009; Harsch et al., 2003) and catecholamines (Drager et al., 2007). Although the benefits certainly outweigh the costs, CPAP prescription and compliance is limited by the unwillingness of patients to attend sleep clinics for proper diagnosis, and the discomfort associated with sleeping while wearing the apparatus.

1.1.4 Intermittent Hypoxia

When a person experiences closure of the airway, as found in OSA, he/she experiences a decrease in blood oxygen tension as a result of the respiratory cessation. This decreased oxygen state is known as hypoxia and within OSA the patient experiences intermittent hypoxia (IH), repeated episodes of reduced arterial oxygen (Strollo and Rogers, 1996). Although a patient will experience fragmented sleep as a result of micro arousals restoring airway tone, IH is a major physiological insult associated with OSA and thus further study is needed to fully understand the effects of OSA and the mechanisms through which the comorbidities and mortalities develop. Recently, human subjects were used in the study of IH. When exposed to 4 weeks of nocturnal IH, human subjects presented with increased muscle SNA and increased vascular resistance (Gilmartin et al., 2010) consistent with some of the symptoms of OSA. Most studies of IH involve a rodent model, developed by Fletcher et al. (1992a). In this model, animals are exposed to cycling levels of oxygen during their night cycles, mimicking the IH

during sleep experienced by OSA patients. The mechanism of signaling IH to blood pressure responses was investigated by carotid sinus nerve (CSN) denervation and it was found the blood pressure responses to IH in this model were abolished in animals receiving the denervation but not in sham controls (Fletcher et al., 1992b). From this study, it becomes clear the peripheral carotid chemoreceptor reflex is vital to the hypertensive changes caused by IH in the animal model.

1.2   The Carotid Body Chemoreceptor Reflex

1.2.1 Central and Peripheral Chemosensitive Regions

Mechanisms of oxygen-sensing are critical to an organism's survival, especially when levels deviate from homeostatic norms. In cases of decreased oxygen, it is imperative cardiorespiratory parameters be altered to ensure proper oxygen (O_2) saturation reaches vital organs including increasing mean arterial pressure (MAP) and respiration rate. Multiple types of chemoreceptors exist to monitor blood O_2 and carbon dioxide (CO_2) levels via pH to keep these values within homeostatic ranges.

1.2.1.1   Ventrolateral Medulla

Central chemoreceptors, localized to the ventrolateral surfaces of the medulla (Schlaefke, 1981), are believed to play an integral role in CO_2 monitoring through pH changes of brain interstitial fluid (Feldman et al., 2003; Fencl et al., 1966). In fact, a very small increase in CO_2 above normal values results in rapid stimulation of respiratory drive (Feldman et al., 2003).

1.2.1.2   The Aortic Body

The aortic bodies are thought to be very similar to carotid body chemoreceptors however very little is known about how these organs function (Piskuric & Nurse, 2012). Additionally, previous studies have indicated a lack of functional aortic body chemoreceptors in the rat (Sapru and Krieger, 1977). Studies on carotid bodies frequently indicate a greater involvement in O_2 sensing and are considered the primary sensors of blood oxygen partial pressure (PO_2) (Feldman et al., 2003; Piskuric and Nurse, 2012). Due to previous IH studies using CSN denervation by Fletcher et al. (1992b) and the apparent lack of function of aortic chemoreceptors in the rat (Sapru and Krieger, 1977) this study will focus on carotid body chemoreceptors.

1.2.2 The Carotid Body

The concept of the carotid body was confirmed by Heymans & Bouckaert in 1930, who performed a variety of experiments on anesthetized dogs based on previous findings that electrical stimulation of the carotid bifurcation region caused a reflex stimulation of respiratory centres (Hering, 1927). The investigators found that occlusion of the common carotid arteries produced reflex increases in blood pressure and respiration however, occlusion of the common vertebral arteries did not affect blood pressure or respiration (Heymans & Bouckaert; 1930). Upon carotid sinus denervation, the reflex changes were abolished. These early studies suggested changes in blood pressure and respiration are due to reflexes originating from the carotid sinus.

1.2.2.1   Anatomy

The carotid bodies are small, ovoid organs about 0.6 mm long x 0.4 mm wide in the rat (Sapru and Krieger, 1977). These organs are located bilaterally at the bifurcation

of the carotid artery into the internal and external branches, an area of high blood-flow perfect for sampling of arterial blood. Not surprisingly, the carotid body is highly vascularized and has been suggested to be the highest perfused organ per unit body weight in an organism (McDonald, 1981). The carotid body receives blood supply from a small branch of the external carotid artery and signals the CSN (McDonald, 1981), which projects from the carotid body to the petrosal ganglion (PG) and joins the glossopharyngeal nerve before it enters the cranium.

The carotid body is composed of lobules or clusters consisting of two cell types: chemoreceptor type-I glomus cells (or chief cells) closely supported by type-II glial-like cells. Type-I glomus cells are small (8-15μm) cells arranged in clusters containing cytoplasmic vesicles (McDonald, 1981). These cells have morphological features, including gap junctions (Abudara et al., 1999) that support a great deal of intercellular communication. The lobule-like organization would suggest autocrine and paracrine signaling likely influences their responses. Additionally, coupling, both electrical and dye, between cultured cells and tissue slices has been demonstrated (Jiang and Eyzaguirre, 2003). Since almost all type-I cells are dopaminergic, the majority of type-I cells express tyrosine hydroxylase (TH), the enzyme involved in conversion of L-arginine to L-dihydroxyphenylalanine which is a precursor for dopamine. Due to the high expression of TH in type-I cells, this enzyme is frequently used in immunohistochemical studies to identify type-I cells (Bolme et al., 1977).

Type-II cells are present in carotid bodies in much lower numbers than type-I cells however they are distributed in tight proximity to the glomus cell clusters. It has been suggested for every type-II cell in the carotid body there are 4 type-I cells

(McDonald, 1981). Type-II cells, which lack vesicles, are elongated in shape and are typically found surrounding clusters of type-I cells. Type-II cells were thought to 'support' type-I cells however the suggestion of type-II cells acting as stem cell precursors for type-I cells has arisen (Pardal et al., 2007) as well as playing a potential role in paracrine signaling (Xu et al., 2003). Although not directly oxygen-sensing, data indicate that these type-II cells may play a much larger role than originally thought.

1.2.2.2   Afferent Projections of Carotid Body Chemoreceptors

The carotid body is heavily penetrated by nerve afferents of the CSN, a branch of the glossopharyngeal nerve, cranial nerve IX. Many of these nerves end in direct apposition to clusters of type-I cells. The somas of the CSN are located in the PG. During chemoexcitation, carotid body afferent impulses travel along the CSN to the nucleus of the solitary tract (NTS), a dorsal brainstem site of autonomic integration, a pathway known as the peripheral or carotid chemoreceptor reflex (Ciriello et al., 1981). Here, carotid body afferent signals are integrated with neural circuitry involved in blood pressure and respiration regulation. As one would expect, the carotid body signal due to low PO_2 traverses the CSN, arrives in the NTS and affects autonomic outflow resulting in increased respiration and blood pressure to ensure normal PO_2 levels are restored and enough O_2 is delivered to vital areas of the organism (Gonzalez et al., 1994; Fitzgerald et al., 2009).

1.2.2.3   Efferent Innervation of Carotid Body Chemoreceptors

The carotid body is a front-line responder to chemical stimuli and thus requires afferent innervation for conveying these stimuli to the central nervous system (CNS). The

carotid body also possesses efferent innervation, a lesser studied area of carotid body signaling but important nonetheless.

The carotid body receives parasympathetic innervation from a plexus of neuronal nitric-oxide synthase-positive nerve fibers located along the CSN and glossopharyngeal nerve (Wang et al., 1993, 1995). These nerve fibers are distinct from the afferent CSN innervating the afferent aspect of the carotid body. Data indicates these parasympathetic nerves act by releasing nitric oxide (NO) onto type-I cells. NO influence on carotid body cells is well characterized and results in inhibition and thus reduced CSN activity (Wang et al., 1995; Kline et al., 1998; Prabhakar, 1999). Although the exact conditions in which this efferent pathway is activated are not known, it has been discovered that adenosine triphosphate (ATP) may activate these neurons by acting through P2X receptors (Campanucci et al., 2006).

The role of sympathetic innervation of the carotid body is controversial, with studies supporting either an inhibitory action (O'Regan, 1981) or a potentiation of carotid body activity (Mitchell and McCloskey 1974; O'Regan, 1981). Indeed evidence seems to point to an inhibitory role as sympathectomy has been shown to potentiate carotid body activity (Hatcher et al., 1978) although others have shown no effect of sympathetic denervation on chemoreceptor activity to brief hypoxic tests (Davies et al., 1982; McQueen et al., 1989). In a previous experiment it was found that sympathetic activity to the carotid body from the superior cervical ganglion occurred only during sustained hypoxia of over 5 minutes (Cherniak et al., 1992), indicating an inhibition of the chemoreflex by efferent sympathetic activity may only occur during prolonged hypoxia, potentially acting as a brake on an overactive system.

1.2.2.4 Type-I Cells of the Carotid Body

Type-I cells exhibit a complex and dynamic phenotype including expression of a variety of cell surface receptors and potential signaling molecules involved in chemotransduction. No transmitter has been identified as the peptide responsible for all carotid body signaling; rather each plays its own role in modulating carotid body signaling and thus transduction of chemoreceptor information to the CNS via the PG and CSN (Figure 1.1).
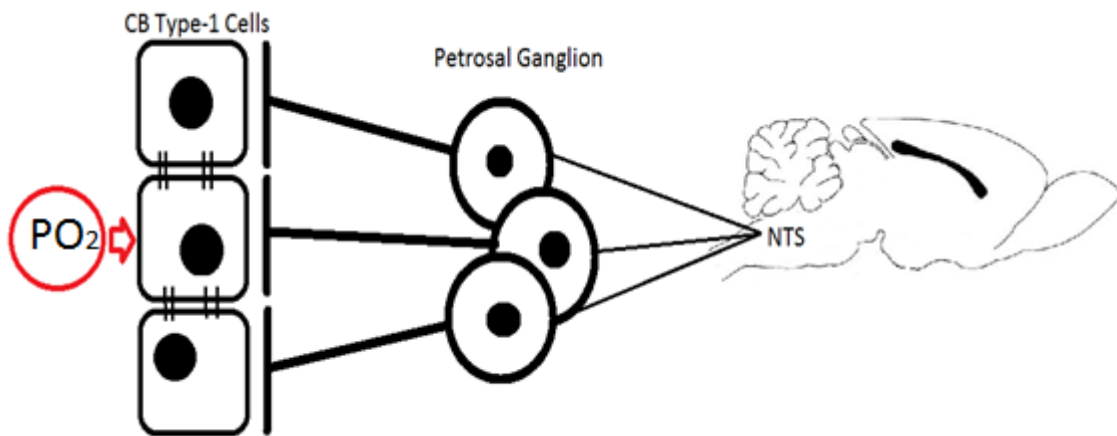
Figure 1.1. Schematic diagram of the afferent carotid body chemoreceptor reflex. PO_2 - partial pressure of oxygen in the blood; CB: Carotid body; NTS: Nucleus of the solitary tract.

Potential signaling molecules expressed by carotid bodies, and which display some type of modulation of carotid body signaling, include dopamine, histamine, adenosine, adenosine triphosphate (ATP) and acetylcholine (ACh).

During chemoexcitation, dopamine is released from type-I cells following increases in intracellular Ca^{2+} (Gonzalez et al., 1994; Iturriaga et al., 1996). The D2 dopamine receptor is the principal form expressed in type-I cells (Gauda, 2002).

Dopamine signaling is considered to exert inhibitory action on the chemoreflex by acting at pre- and post-synaptic sites. In the cat, basal and hypoxia-mediated CSN activity is increased upon dopamine receptor antagonist administration (Gonzalez et al., 1994; Iturriaga & Alcayaga, 2004; Shirahata et al., 2007). It is therefore believed dopamine is involved in regulating a negative feedback within carotid bodies inhibiting neurotransmitter release.

Similarly, histamine is present and released by type-I cells in response to hypoxic stimuli (Koerner et al., 2004). Additional analysis revealed the presence of histamine receptor mRNA for the H1, H2 and an isoform of H3 within carotid bodies (Koerner et al., 2004). Experimental evidence indicates histamine may be a local modulator of glomus cell activity, most likely acting in an autocrine/paracrine fashion. Histamine administration to both perfused and superfused cat carotid bodies resulted in increased frequency of discharge of CSN, whereas application to isolated PG did not alter frequency of discharge of CSN (Del Rio et al., 2008) which may implicate histamine as a modulator of carotid body activity rather than a CSN signaling molecule.

Adenosine also possesses the potential to modulate the chemoreceptor reflex, most likely through autocrine/paracrine influence. It is well known type-I cells possess adenosine A2A and A2B receptors (Conde et al., 2009; Gauda, 2002; Kobayashi et al., 2000) though the evidence for A1 receptors remains argumentative. Hypoxia also evokes adenosine release from type-I cells (Conde & Monteiro, 2004). Adenosine signaling through A2A receptors acts to decrease inward calcium (Ca^{2+}) currents into the type-I cells (Kobayashi et al., 2000), indicating adenosine, like dopamine, most likely acts as an inhibitor of chemoreflex signaling. However, it is well known that exogenous application

of adenosine serves to increase CSN activity in cats (McQueen & Ribeiro, 1981; McQueen & Ribeiro, 1983; Runold et al., 1990). Additional studies however have displayed opposing results to the Kobayashi (2000) study. In fact, the study by Xu et al. (2006) has indicated adenosine causes depolarization of type-I cells and results in increased intracellular Ca^{2+} concentrations via the A2A receptor, indicating adenosine may in fact potentiate the chemoreflex.

The cases of ATP and ACh are slightly different than the previously discussed signaling peptides. ATP and ACh show the potential for signaling not only pre-synaptically in autocrine/paracrine fashion but also post-synaptically. That is, ATP and ACh show evidence of release from type-I cells and subsequently signaling directly to the CSN and may be involved in transduction from chemical signal to electrical impulse.

ATP is commonly believed to be a critical excitatory signaler from type-I cells to the CSN. This notion is strongly supported by studies using co-cultures of type-I cells juxtaposed with PG neurons. In this situation, *in vitro*, functional interactions between type-I cells and PG neurons were re-created (Zhang et al., 2000; Zhong et al., 1997). Purinergic receptor (P2X) blockers were shown to inhibit the hypoxia-evoked postsynaptic responses of PG neurons in these culture conditions (Zhang et al., 2000). Consistent with these results, PG neurons are known to express two isoforms of the purinergic receptors, $P2X_2$ and $P2X_3$ (Zhang et al., 2000). Not only are these receptors present in PG neurons, they are localized to afferent nerve terminals with the carotid body of the rat (Prasad et al., 2001). Further support for ATP signaling post-synaptically arises from the fact ATP is released from type-I cells exposed to hypoxic conditions *in vitro* (Buttigieg & Nurse, 2004).

ACh also presents a compelling argument to be considered as a main player in chemoreceptor excitatory responses, though not as convincing as ATP. Early studies employing nicotinic blockers show partial inhibition of chemosensory discharge of the CSN in an isolated cat or rat CSN/carotid body preparation (Fitzgerald, 2000; Iturriaga & Alcayaga, 2004; Zhang et al., 2000; He et al., 2005). Indeed evidence suggests ACh itself is not responsible for all excitatory carotid body signaling but a combination of ACh and ATP. In co-culture preparations of PG neurons and type-I cells, application of both nicotinic and purinergic receptor antagonists is required to completely eliminate postsynaptic responses (Zhang et al., 2000; Zhang & Nurse 2004; Nurse, 2005). Additionally, those data also suggest ATP is co-released with ACh, supporting a dual transmitter system. HPLC studies *in vitro* have indicated chemosensory stimuli do in fact promote ACh release from both cat and rabbit carotid bodies (Fitzgerald, 2000; Kim et al., 2004). There is a good body of evidence implicating ACh as an excitatory transmitter directly on PG neurons via nicotinic ACh receptors (Zhong & Nurse, 1997; Iturriaga & Alcayaga, 2004; Fitzgerald, 2000) in addition to modulating type-I cells themselves as type-I cells also express multiple forms of nicotinic ACh receptors (Fitzgerald, 2000; Shirahata et al., 2007; Conde & Monteiro, 2006) suggesting an additional role in autocrine/paracrine signaling.

Finally, the renin-angiotensin system (RAS) has been shown to exist in carotid body cells (Lam & Leung, 2003). Evidence suggests a local RAS exists within the carotid body, specifically by the presence of the precursor angiotensinogen and the enzyme required for conversion to angiotensin II (ANGII), angiotensin-converting enzyme (ACE) (Lam & Leung, 2003). Additionally, up-regulation of both angiotensinogen and ACE has

been reported following hypoxia (Lam & Leung, 2003). ANGII action at the carotid body serves to modulate CSN activity, specifically to increase discharge in a concentration-dependent manner, an effect abolished in the presence of losartan (Allen, 1998; Leung et al., 2000), a specific angiotensin type-1 receptors (AT_1R) blocker. Furthermore, ANGII acting through the AT_1R has also been implicated in sensitization of the peripheral chemoreceptor reflex following exposure to chronic IH (CIH) (Marcus et al., 2010). Previous studies have shown that ANGII can modify carotid body afferent discharge within the CSN in an *ex vivo* carotid body preparation (Allen, 1998; Leung et al., 2000). Furthermore, studies have found a high density of ANGII receptors, specifically AT_1R, within carotid body cells (Allen, 1998), and interestingly, chronic hypoxic and CIH exposure were found to up-regulate AT_1R expression (Leung et al., 2000; Marcus et al., 2010). ANGII signaling in the carotid body was found to increase chemosensitivity of the chemoreceptor reflex induced by CIH (Marcus et al., 2010). Following CIH exposure, acute apnea serves to increase SNA to a higher value than in a control, non-CIH exposed animal. This effect is not evident in the presence of losartan, an AT_1R blocker (Marcus et al., 2010). Additionally, IH was found to increase circulating levels of ANGII (Prabhakar & Kumar, 2010).

Taken together, these data suggest that carotid body chemo transmission is an intricate and complex signaling process involving a multitude of signaling molecules working towards inhibition or excitation of the chemoreflex pathway. Additionally it is difficult to pinpoint, and probably unlikely that, one transmitter is solely responsible for direct excitation of the CSN. Additional signaling molecules exist and recent evidence

would suggest the list of carotid body modulators is far from complete. For full review of potential transmiters released from carotid body cells see Nurse, 2010.

1.3 Leptin

Coleman (1973) was the first to identify the presence of a circulating element involved in regulation of body weight. The original studies described that the *ob*/*ob* mouse, presenting with obesity, was due to a lack of this signaling element and that the obesity observed in the *db*/*db* mouse was a result of reduced sensitivity to the element. This circulating factor, leptin, is a 16 kDa peptide encoded by the *obese* (*ob*) gene in mice and rats and the *LEP* homolog gene in humans (Zhang et al., 1994). The protein is highly conserved across species and shows characteristics of the class I cytokine superfamily (Zhang et al., 1997). Leptin is produced primarily by adipocytes and plasma levels of leptin are proportional to body fat content (Frederich et al., 1995; Maffei et al., 1995; Lonnquist et al., 1995), providing the brain with an afferent signal proportional to body fat levels. Leptin also regulates food intake, functioning as a satiety signal, as well as increasing energy expenditure via hypothalamic action (Ahima et al., 1996; Jacob et al., 1997). In lean subjects, leptin tends to circulate at low levels (approximately 5-10 ng/ml) either attached to binding proteins or in a free-form state (Sinha et al., 1996), however these levels increase with adiposity. Interestingly, once body fat reaches 30%, circulating leptin levels increase almost exponentially and levels as high as 98 ng/ml have been observed (Ostlund et al., 1996). Circulating leptin may access the CNS through the blood-brain barrier via a saturable and unidirectional system (Banks et al., 1996) which is believed to involve binding to a short-form leptin receptor (Bjorbaek et al., 1998). Alternatively, leptin may also exert CNS signaling by production within the brain

(Wiesner et al., 1999) however the mechanism of action (i.e. autocrine/paracrine or otherwise) is currently unknown. Acting centrally, leptin promotes weight loss via promoting satiation and energy expenditure. One mechanism by which energy expenditure may be increased is by increasing sympathetic nerve activity (SNA) (Mark et al., 2009; Rahmouni & Morgan, 2007).

Although these metabolic effects are considered the traditional roles of leptin signaling, leptin may elicit a variety of effects within an organism by signaling either centrally or directly within a target tissue. Leptin is also involved in other processes including, but not limited to, modulating inflammatory and immune functions, reproductive, neuroendocrine, angiogenesis, fatty acid metabolism, glucose homeostasis, survival and proliferation, plasticity, sensory nerve input, autonomic outflow and respiration (Bjorbaek & Kahn, 2004; Lago et al., 2007; Malendowicz et al., 2007; O'Donnell et al., 1999, 2000; Ribatti et al., 2007; Tankersley et al., 1998; Tang, 2008). Leptin action on a target tissue may be mediated via circulating leptin and thus modulated by factors affecting leptin production and release from adipocytes or by leptin production directly from or near the tissue of action as leptin has shown its' presence not only in adipocytes but also within bone, mammary gland, stomach, placenta and recently carotid body glomus cells (Bado et al., 1998; Hoggard et al., 1997, 1998; Porzionato et al., 2011).

1.3.1 Leptin Production

Leptin gene expression and secretion are influenced by many factors, the primary influence being body adiposity, specifically white adipose tissue (WAT). Increases in gene expression and secretion are brought about by overfeeding, consuming a high-fat

diet, insulin, glucocorticoids and cytokines whereas decreases are manifested by fasting, testosterone, thyroid hormone as well as cold exposure (Coleman and Hermann, 1999; Fried et al., 2000). Additionally, the sympathetic nervous system and ANGII have shown evidence of modulating the production and secretion of leptin.

Activation of the sympathetic nervous system can decrease the amount of WAT and thus leptin production. Indeed denervation studies in the hamster have suggested sympathetic innervation directly affects WAT as an increase in fat pad mass was observed in the denervated side compared to the fat pad with intact sympathetic innervation (Youngstrom & Bartness, 1998). Fasting is known to increase SNA to WAT, noticed by increased noradrenaline turnover in WAT, in order to stimulate lipolysis and liberate fatty acids from energy stores for use as energy (Migliorini et al., 1997) which would thus decrease circulating leptin levels. It has also been shown using cell cultures that catecholamines inhibit leptin production in cultured adipocytes (Hardie et al., 1996). Eliminating peripheral sympathetic innervation chemically can be done by administration of 6-hydroxydopamine. Using this method, leptin levels are increased during fasting (Sivitz et al., 1999). These data would suggest that sympathetic activity affects leptin production and may well have an effect on leptin production in areas receiving sympathetic inervation besides adipose tissue.

Another method through which leptin production may be mediated is through ANGII action. It is well-known that the RAS is responsible for producing the vasoactive hormone ANGII, a hormone increased in the circulation by sympathetic outflow such as that observed by activation of the peripheral chemoreflex (Prabhakar & Kumar, 2010). The glomus cells of the carotid body express a myriad of signaling receptors and the

AT_1R is no exception. It has been suggested that ANGII acts through the AT_1R in type-I cells (Allen, 1998), exposing yet another potential modulating hormone exerting an influence on the peripheral chemoreflex. Evidence indicates the AT_1R may mediate Ca^{2+} release from intracellular stores in type-I cells (Fung et al., 2001) and may therefore be involved in transmitter release. Furthermore, ANGII effectively increases the release of leptin from cultured cardiomyocytes and promotes *de novo* synthesis (Rajapurohitam et al., 2006) which may play a similar role within type-I cells of the carotid body.

1.3.2 Leptin Receptors

The obese receptor (OB-R), a member of the class I cytokine-receptor family, possesses an extra-cellular ligand-binding domain, a transmembrane domain and a cytoplasmic signaling domain. Leptin signals through these receptors of which there are six known isoforms (a-f) as a result of alternative mRNA splicing from a single gene (Lee at al., 1996; Wang et al., 1996) with the exception of OB-Re. The differences in these isoforms lie in the cytoplasmic domain. The short isoforms, OB-Ra,c,d and f possess shorter cytoplasmic terminals whereas the OB-Re is a secreted and circulating form of the OB-R and lacks a transmembrane domain (Cottrell & Mercer, 2012). Some of these short form receptors serve known functions whereas others remain unknown. OB-Ra functions in transport of leptin across the blood-brain barrier (Golden et al., 1997; Bjorbaek et al., 1998) and OB-Re binds circulating leptin (Gavrilova et al., 1997; Yang et al., 2004).

The OB-Rb, often referred to as the long-form leptin receptor due to its extended intracellular cytoplasmic domain has been implicated in transducing the majority of leptin's central signaling effects within the hypothalamus and the NTS (Elmquist et al.,

1997; Huo et al., 2006, 2007; Wang et al., 1998). Only the OB-Rb is considered capable of full intracellular signal transduction (Baumann et al., 1996) and thus is the focus of leptin signaling. In fact, in terms of body weight regulation the OB-Rb is critical as no phenotypic differences were found between mouse models lacking all OB-R isoforms, *db/db* mice lacking only the OB-Rb and *ob/ob* leptin-deficient mice (Chua et al., 1996; Tartaglia, 1997).

Upon leptin binding to the ligand-binding domain of the OB-Rb, the receptors act primarily via the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway although other pathways including the mitogen-activated protein kinase (MAPK) cascade have been known to be stimulated by leptin signaling (Banks et al., 2000; Elmquist et al., 1997; Fruhbeck, 2006; Hou et al., 2006, 2007; Wang et al., 1998). Briefly, leptin binding results in JAK2 recruitment and activation which phosphorylates intracellular tyrosine residues on the OB-Rb. These phosphorylated tyrosine residues allow association of additional intracellular proteins with phosphotyrosine binding domains which the STAT3 protein possesses. These STAT3 proteins are phosphorylated into the active pSTAT3 form which then dimerize and translocate to the nucleus where they influence gene transcription (Vaisse et al., 1996) including transcription of the negative feedback of this signaling cascade suppressor of cytokine signaling 3 (SOCS3) (Banks et al., 2000). See Figure 1.2 for representative schematic diagram.
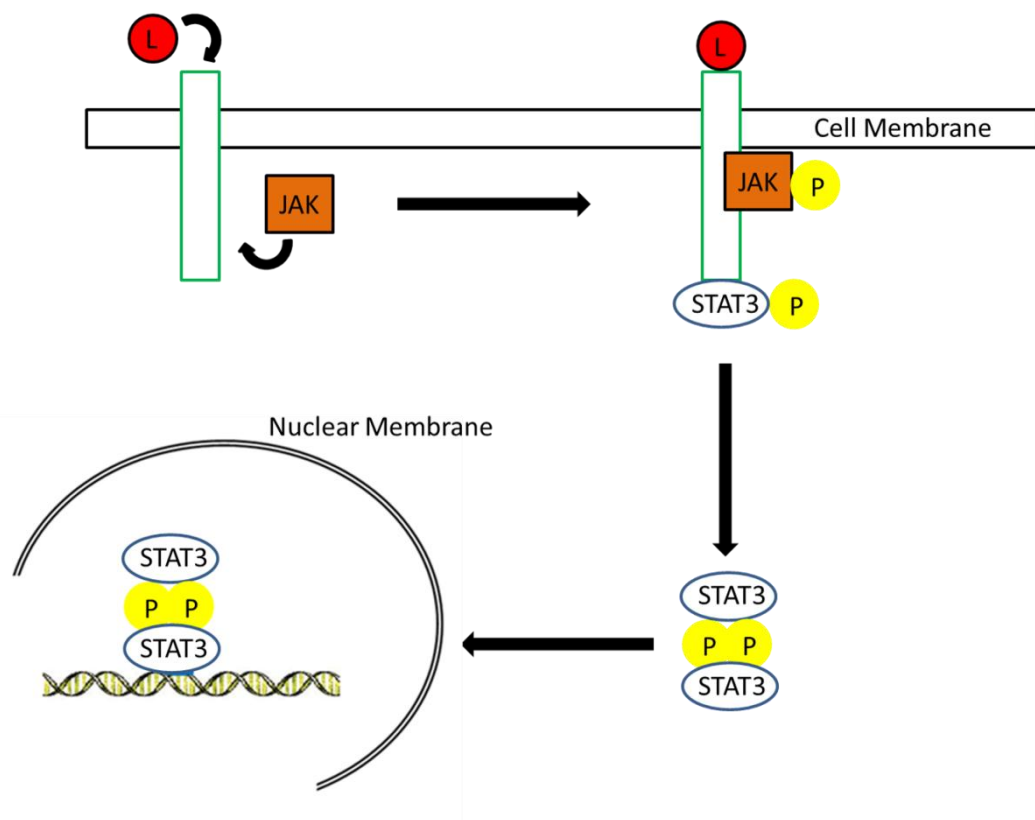
Figure 1.2. Representative schematic diagram of OB-Rb signaling through the JAK/STAT pathway. L - Leptin; P - phosphate group; JAK - Janus Kinase; STAT3 - signal transducer and acticator of transcription 3.

Leptin signaling is not solely mediated by the JAK/STAT pathway as its effects may also be mediated by the activation of extra-cellular signal-related kinase 1/2 (ERK1/2) (Kloek et al., 2002). In fact, the activation of ERK1/2 by leptin has previously been shown in the hypothalamus where it is known to mediate food intake, body weight and thermogenic sympathetic outflow (Rahmouni et al., 2009). Additionally, OB-Rb has been suggested to activate signaling through the phosphoinositide 3-kinase pathway (Fruhbeck et al., 2006).

As with any signaling molecule, the expression of the receptor is critical in determining the sensitivity of a cell or tissue to the peptide. Leptin and its receptors display a similar interaction as with many other ligand-receptor relationships in that the availability of the ligand can result in changes in receptor expression. In the *ob/ob* mouse, an animal model completely lacking the hormone, a prominent up-regulation of the OB-R is noticed, an effect which can be counteracted upon administration of exogenous leptin (Mercer et al., 1997). In a similar manner, conditions inducing lower levels of circulating leptin (eg. cold exposure, food restriction) result in up-regulation of OB-R within the hypothalamus (Lin & Huang, 1997; Mercer et al., 1997). Consistent with this interaction, hyperleptinaemia (such as that seen in the *db/db* mouse model) results in a decrease in OB-R expression (Malik & Young, 1996; Wilsey & Scarpace, 2004). This relationship would likely enhance leptin's effect in situations where there is low availability of the peptide and blunt leptin signaling when the ligand is present in excess.

1.3.3 Cardiovascular Effects of Leptin –Peripheral Administration

Leptin infusion in mice has been found to increase SNA to a variety of tissues including the kidney, brown adipose tissue, hindlimb and adrenal glands (Haynes et al., 1997). There is also evidence of leptin signaling through afferent nerves resulting in increases in SNA (Tanida et al., 2000). Although leptin-infusion data in humans are lacking, in non-human primates leptin has been shown to activate SNA as determined by plasma catecholamine levels (Tang-Christensen et al., 1999). These increases in SNA brought about by leptin, particularly on catecholamine levels and SNA to the kidney, naturally raise questions as to whether leptin then has an effect on blood pressure. The sympathetic nervous system plays an important role in renal function and if leptin-

mediated SNA were restricted to the kidney, one may expect arterial pressure to be increased due to vasoconstriction and tubular sodium reabsorption. In fact, sympathoexcitation by leptin is followed closely by an increase in arterial pressure (Dunbar et al., 1997). Intravenous infusion of leptin over 12 days resulting in decreased food intake resulted in increased arterial pressure and heart rate (Shek et al., 1998). Additionally, changes in the concentration of leptin within the circulation have been found to alter the discharge rate of NTS neurons that project to the sympathoexcitatory sites of the rostral ventrolateral medulla, neurons that were also found to respond to activation of the peripheral chemoreceptor reflex (Ciriello & Moreau, 2013). Not only do these leptin-sensitive neurons respond to peripheral chemoreceptor activation, the response was potentiated by leptin (Ciriello & Moreau, 2013).

1.3.4 Cardiovascular Effects of Leptin – Central Administration

Leptin can exert cardiovascular effects by acting centrally, as has been shown by studies introducing leptin directly into the CNS. Central administration serves to increase plasma catecholamines epinephrine and norepinephrine (Satoh et al., 1999). In other studies, leptin has been found to increase SNA, arterial pressure as well as heart rate and to inhibit the baroreflex (Arnold et al., 2009; Mark et al., 2009). Leptin may also have a role in modulating the peripheral chemoreflex at the level of the CNS as microinjection of leptin into caudal pressor areas of the NTS has been found to potentiate the sympathetic and blood pressure responses to chemoreflex activation (Ciriello & Moreau, 2012). These cardiovascular effects may not be debilitating under normal homeostatic conditions, however in conditions of increased circulating leptin, such as in OSA and obesity, the cardiovascular responses may be detrimental.

1.3.5 Leptin and Obstructive Sleep Apnea

The hypoxic condition is considered a pro-inflammatory state and thus may have an effect on a variety of circulating and local cytokines. Leptin is no exception to this and there is a body of evidence suggesting that circulating leptin is increased in response to IH. In fact, clinical studies have shown that OSA patients present with increased circulating levels of leptin compared to BMI-matched controls (Phillips et al., 2000; Ip et al., 2000). Additionally, OSA patients treated with CPAP show marked reductions in circulating leptin levels even with no change in BMI (Harsch et al., 2003; Ip et al., 2000). This would suggest increased peripheral effects of leptin during OSA due to increased levels in the circulation.

1.3.6 Leptin and Hypoxia

As would be predicted, animals treated with IH show increased circulating leptin levels compared to their normoxic counterparts (Polotsky et al., 2003), indicating IH may well play a role in leptin up-regulation and secretion. In fact, Grosfeld and others (2002) provide direct evidence that hypoxia can up-regulate leptin mRNA expression as well as increase leptin promoter activity and leptin secretion from cultured adipocytes. The evidence supporting leptin up-regulation by the hypoxic state, combined with the previously discussed cardiovascular implications of leptin may provide an avenue in which cardiovascular comorbidities are brought about in the OSA population.

1.3.7 Leptin and the Carotid Body

Considering the growing body of evidence displaying expression of leptin in a variety of tissues and the cardiorespiratory implications of leptin signaling, it comes as no surprise that leptin protein and mRNA are expressed in carotid body type-I cells of both

the rat and human (Porzionato et al., 2011). To the date of this writing, there have been no additional studies investigating the presence of leptin and its receptors in the carotid body and it is unknown if leptin activates traditional signaling cascades and serves to activate or inhibit these cells. Additionally, leptin and leptin receptor transcripts may be altered by hypoxia potentially resulting in changes of signaling strength and ultimately modulation on the chemoreceptor reflex at the level of the carotid body.

1.3.8 Leptin and Angiotensin II

The relationship between leptin and ANGII has been studied in detail in various tissues (Cassis et al., 2004; Danser et al., 1999; Haznedaroglu and Buyukasik, 1997; Haznedaroglu et al., 1996; Hilzendeger et al., 2012). However, data are lacking at the level of the carotid body. ANGII effects on leptin have been investigated in other tissues including rat ventricular myocytes where ANGII has been shown to stimulate leptin production and secretion. Additionally, the hypertrophic effects of ANGII on these cells were negated upon leptin receptor blockade (Rajapurohitam et al., 2006, 2012). In cultured rat adipocytes, incubation with ANGII resulted in increased leptin secretion and mRNA expression whereas inhibition of ANGII using the ACE inhibitor captopril resulted in decreased leptin secretion (Cassis et al., 2004). Similarly, in the brain, ANGII signaling through AT_1R is required for leptin's central sympathetic effects (Hilzendeger et al., 2012). These data may suggest a similar interaction between the carotid body RAS and leptin signaling in the carotid body.

1.4 Hypothesis and Specific Objectives

The hypothesis that was tested in these studies was that leptin signals directly in the carotid body and this signaling will be affected by IH exposure. Leptin may therefore alter the chemoreceptor reflex by acting directly within the carotid body. A schematic outlining the general hypothesis can be seen in Figure 1.3. Leptin may exert an effect via the circulation or its potential release from glomus cells to act in an autocrine/paracrine fashion or to signal the CSN afferents. Leptin up-regulation, as seen in OSA patients, may be involved in the hypertension observed in these patients by directly affecting the signaling of the chemoreceptor reflex.

Specific Objectives:

1. a. Completed in Chapter 2. To determine effect of intermittent hypoxia (IH) on systemic leptin release.

    b. Completed in Chapter 2. To determine whether systemic leptin can signal in carotid body glomus cells by identifying downstream regulators of leptin receptor signaling pSTAT3 and immediate early gene expression fos and fos-related antigen-1 (Fra-1).

2. a. Completed in Chapter 3. To determine the effect of IH on leptin and Ob-Rb protein levels in glomus cells

    b. Completed in Chapter 3. To determine the effect of IH on down-stream regulators of leptin receptor signaling including pSTAT3 and SOCS3.

3. Completed in Chapter 4. To determine the effect of CIH (7, 95 days) on leptin and Ob-Rb expression and leptin receptor signaling indicators.

4. Completed in Chapter 5. To determine the effect of ACE inhibitor on carotid body leptin and leptin receptor expression in normoxic and IH conditions revealing a potential role of angiotensin II in carotid body leptin signaling.
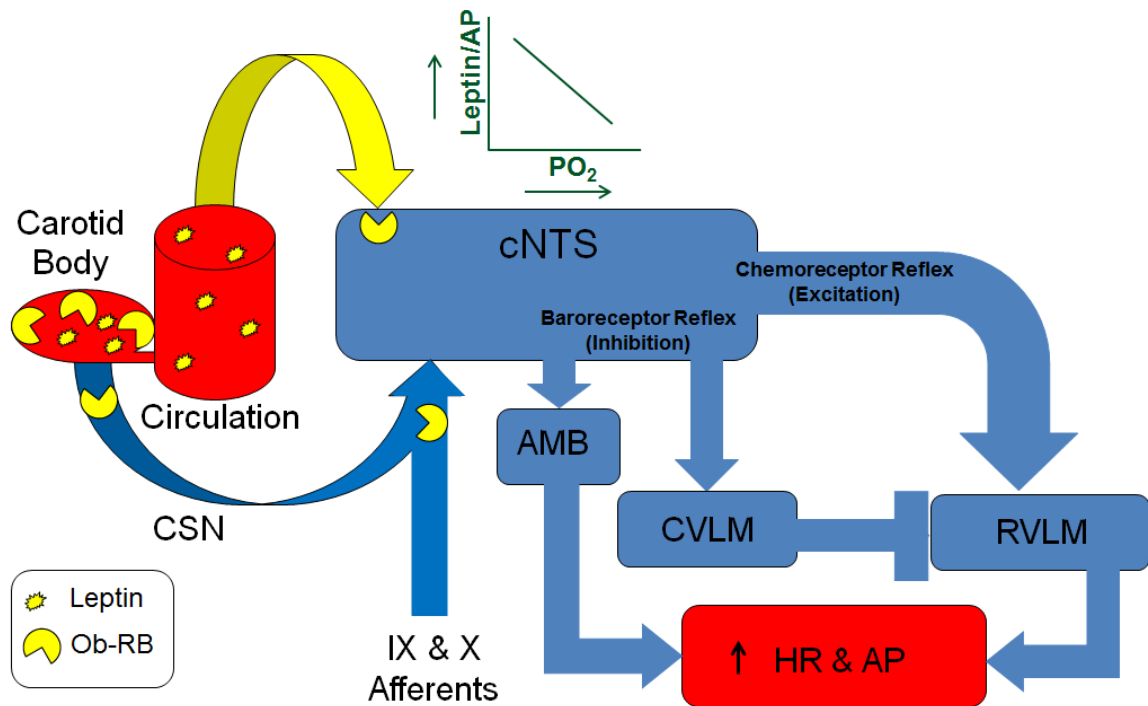
Figure 1.3. Schematic diagram of overall hypothesis. CSN-carotid sinus nerve; cNTS-caudal nucleus of the solitary tract; AMB-nucleus ambiguous; CVLM-caudal ventrolateral medulla; RVLM-rostral ventrolateral medulla; HR-heart rate; AP-arterial pressure; IX & X afferents-cranial nerve IX and X afferents.

Chapter 2

2   Intermittent Hypoxia and Systemic Leptin Administration Induces pSTAT3 and Fos/Fra-1 in the Carotid Body

2.1   Introduction

Intermittent hypoxia (IH), the repeated episodes of hypoxia followed by re-oxygenation, is an experimental model in animals (Fletcher et al., 1992b) used to mimic obstructive sleep apnea (OSA) (Dempsey et al., 2010). OSA is a common pathological condition, observed especially within overweight and obese patients (Dempsey et al., 2010; Young et al., 1993; Yu and Berger, 2011). OSA is characterized by a brief but repeated collapse of the upper pharyngeal airway throughout the sleep period which in turn induces a state of chronic IH (Dempsey et al., 2010; Young et al., 1993). Studies have previously shown that patients with OSA have an increased risk of heart disease, stroke, myocardial ischemia, and hypertension that may later contribute to increased mortality in these patients (Dempsey et al., 2010; He et al., 1988; Shamsuzzaman et al., 2003). Animals exposed to IH also develop an increased arterial pressure that is dependent on an intact peripheral chemoreceptor reflex (Fletcher et al., 1992a-b). Thus, it is has been suggested that the resultant hypoxia during OSA and IH activates peripheral chemoreceptors which in turn drive sympathetic nerve activity that leads to chronic hypertension (Dempsey et al., 2010; Fletcher et al., 1992b; Peled et al., 1998; Somers et al., 1995).

Peripheral chemoreceptors are located throughout the circulatory systems and within specific organs, although those that sense the partial pressure of oxygen (PO_2) within the blood are found predominantly within the carotid bodies located at the

bifurcation of the internal and external branches of the carotid artery (Heymans et al., 1930, West 1995). Type-1 glomus cells within the carotid bodies play an integral role in detecting changes in PO_2 by transducing this chemical signal to sensory afferent neurons within the petrosal (PG) and nodose (NG) ganglia that signal brainstem autonomic reflex pathways (Fung et al., 2001). Although the evidence is ambiguous with regard to the transmitter in type-1 glomus cells responsible for the signal transduction of the peripheral chemoreceptor reflex, dopamine (Gomez-Nino et al., 1990), norepinephrine (Gomez-Nino et al., 1990), acetylcholine (Eyzaguirre &Zapata, 1968), as well as adenosine triphosphate (Bock, 1980) have been suggested as signaling molecules. Recently, leptin, a 16 kDa adipokine protein product of the *Ob* gene, and 4 of its receptor isoforms (Lee et al., 1996; Wang et al., 1996) have been identified within only type-1 glomus cells of the carotid bodies (Porzionato et al., 2011).

Leptin is normally secreted by adipocytes in proportion to the amount of body adiposity (Lonnquist et al., 1995; Caro et al., 1996). Leptin's primary action is to elicit a satiety effect, as well as exert an effect on glucose homeostasis, energy expenditure, and weight loss through the activation of hypothalamic autonomic regulatory systems (Ahima et al., 1996; Bjørbaek & Kahn, 2004; Jacob et al., 1997; O'Donnell et al., 2000; Shirasaka et al., 2003). Additionally, leptin has been suggested to modulate respiratory activity (Tankersley et al., 1998). In *ob/ob* mice, those with a mutation in the *obese* gene encoding leptin, chronic leptin administration restores their rapid breathing pattern (Tankersley et al., 1998). However, it is worth noting that acute leptin replacement significantly increases baseline ventilation as well as chemosensitivity during sleep, a characteristic independent of weight gain (O'Donnell et al., 1999). Leptin microinjection

into the nucleus of the solitary tract (NTS), the primary site of termination of chemoreceptor afferent fibers (Ciriello et al., 1981, 1994) also produces respiratory effects, increasing pulmonary ventilation and respiratory volume as well as enhancing electrical activity to inspiratory muscles (Inyushkin et al., 2009). Furthermore, injections of leptin within NTS potentiate the cardiovascular responses during activation of the chemoreceptor reflex (Ciriello and Moreau, 2011), likely by altering the activity of the downstream regulator phosphorylated signal transducer and activator of transcription 3 (pSTAT3) (Hubschle et al., 2001). Peripheral administration of leptin has been shown to induce both pSTAT3 and fos-like immunoreactivity within the hypothalamus and within NTS (Elmquist et al., 1997; Huo et al., 2006, 2007; Wang et al., 1998).

In this study, experiments were done to determine whether carotid body glomus cells expressing the Ob-Rb can be functionally activated *in vivo*. The first series of experiments was done to determine whether carotid body glomus cells identified immunohistochemically by the presence of tyrosine hydoxylase (TH) (Bolme et al., 1977) and that expressed the Ob-Rb were activated by IH. This was done by examining immunohistochemically the immediate early gene products fos- and fos-related antigen-1 (Fra-1)-like proteins within these cells. The immediate early gene c-fos has been implicated as one of the third messengers in the intracellular signal transduction pathway in a variety of cells (Morgan et al., 1987; Morgan & Curran, 1990). The basal expression of the fos protein is relatively low, but it can be induced rapidly and transiently by growth factors, neurotransmitters, second messengers, or membrane depolarization (Morgan et al., 1987; Morgan and Curran, 1990; Sagar et al., 1988; Solano-Flores et al., 1997). On the other hand, Fra-1 is one of the transcription factor complex activator protein-1 that

plays a central role in regulating gene transcription in several biological processes. The activator protein-1 is made up of a variety of dimers composed of members of the fos and jun proteins, and the activating transcription factor families of proteins (Curran & Franza, 1988; Rauscher et al., 1988; Shaulian & Karin, 2002). The fos and Fra-1 immunohistochemical techniques have been used for the detection of changes in cellular activity during the application of a variety of specific physiological, chemical, and electrical stimuli (Hochstenbach et al., 1993; Morgan et al., 1987; Morgan and Curran, 1990; Sagar et al., 1988; Solano-Flores et al., 1997). However, while fos expression peaks within about 2 h after the stimulus and returns to baseline within 6-8 h, other immediate early gene proteins such as Fra-1 are also synthesized soon after their induction, but persist mainly in the cell nucleus for longer periods of time, which range from about 1-2 days to several weeks. Additionally, as it would be expected that activation of the carotid bodies would lead to increased activity within the sensory neurons, the PG and NG were processed for fos- and Fra-1-like immunoreactivity. The carotid bodies and associated ganglia of animals exposed to IH were further processed immunohistochemically for the expression of pSTAT3. The effects of leptin are mediated by receptors following the induction of a central signaling pathway that involves activation of signal transducer and activator-3 (Vaisse et al., 1996)

In the second series, to determine whether the pSTAT3 activity during IH was possibly related to changes in circulating levels of leptin, plasma levels of leptin were measured in both IH and normoxia exposed animals. As leptin levels were found to be elevated during IH, the effect of intravenous injections of leptin on fos-, Fra-1 and

pSTAT3-like immunoreactivity was determined in the carotid bodies and associated ganglia.

These studies were based on the hypothesis that cells within the carotid bodies expressing the Ob-Rb would express pSTAT3, fos- and Fra-1-like proteins during both IH and leptin administration.

2.2 Methods

General Animal Procedures: Experiments were done in male Sprague-Dawley rats (300–500g; Charles River Canada, St. Constant, Canada). All animals were housed under controlled conditions under a 12h light/dark cycle. Food and water was available *ad libitum*. All experimental procedures were done in accordance with the guidelines on the use and care of laboratory animals as set by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Western Ontario.

Induction of IH: IH (n=11) or normoxia (n=9) was induced in animals after placing them into custom made cylindrical plexiglass tubes inside an airtight plexiglass chamber. The chamber was attached to computer-controlled solenoid valves responsible for regulating the inflow of either pressurized room air (21% O_2) or 100% medical N_2. The solenoid valves were connected to a computerized timer system that allowed for the control of a total hypoxic (100% medical N_2) stimulus delivery of 80 s followed by a normoxic (21% O_2) time of 100 s, for a total continuous repetitive cycle period of 8 h. Additionally, the controller, attached to detectors for O_2 and CO_2 within the chamber, was set at a O_2 level of 6.5-7% and CO_2 level of 0.1% under isobaric conditions at all times during the delivery of the hypoxic stimulus and recovery phase. Animals were also run

simultaneously in a similar adjacent chamber through which only room air (21% O_2; normoxia) was used for the gas exchange in isobaric conditions for an 8 h period.

Leptin ELISA: Measurement of plasma levels of leptin were made immediately after the exposure of the animals to IH or the normoxic stimulus. Whole blood samples were taken through a cardiac puncture prior to perfusion of the animal for immunohistochemistry. Blood samples were drawn into centrifuge tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA; 7%; 10 µl EDTA/1 ml blood; Sigma, St. Louis, Missouri, USA) and kept on ice. Blood samples were then centrifuged at 10,000 g for 10 min at 4°C, and the plasma was immediately removed and stored at -80°C for later leptin analysis. Plasma was aliquoted and leptin was measured using a TiterZyme enzyme immunometric assay kit (Enzo Life Sciences (ADI-900-015A; Farmingdale, New York, USA)). Samples were diluted 1:4 and assayed in duplicate according to the manufacturer's instruction. The recovery is 99% at a known leptin concentration of 2700 pg/ml, 86% at a known leptin concentration of 900 pg/ml and 97% at a known leptin concentration of 300 pg/ml (Enzo Life Sciences, Farmingdale, New York, USA). The sensitivity of the assay is 67.2 pg/ml at the 95% confidence limits (Enzo Life Sciences, Farmingdale, New York, USA). The cross-reactivity of the recombinant leptin antiserum is 100% with rat leptin whereas it does not cross-react with mouse or human leptin (Enzo Life Sciences, Farmingdale, New York, USA). The intra-assay coefficient of variation was 2.5%, and the inter-assay coefficient of variation was 4.1%. The assay was read using a SpectraMax M5 plate reader (Molecular Devices; Sunnyvale, California, USA).

Intravenous Leptin Administration: On the day of the experiments, the animals were anesthetized with alpha-chloralose (40 mg/kg i.v. initially, and supplemented by

additional doses of 10 mg/kg every 30 min) after induction with equithesin (0.3 mL/100

g, i.p.). Body temperature was maintained at 36-37˚C by a heating pad (model K-20-C;

American Hospital Supply, Cincinnati, Ohio, USA). Prior to surgery, 0.2 mL of lidocaine

was injected into the neck region where the right jugular vein was to be isolated and

cannulated with a polyethylene catheter-50 (Clay Adams, Parsippany, New Jersey, USA).

This jugular vein cannula was used for administration of anesthetic, leptin and the saline

vehicle. Animals were allowed to recover for a period of 2 h in a dark and quiet room

immediately after the jugular vein cannulation. The animals then received three 0.1 mL

injections (at 0, 30 and 60 min) of either leptin (50 ng/0.1 mL, n=3; 200 ng/0.1 mL, n=3)

or physiological saline (0.9%, n=1) while remaining in the dark under silent conditions.

Immunohistochemistry: In the chronic studies, immediately after the application of the

8h IH or the normoxic (control animals) stimuli the animals were anesthetized with 0.3

ml/100g equithesin and perfused transcardially using 500 mL ice-cold phosphate buffered

saline (PBS; 0.01M, pH 7.4) followed by Zamboni's fixative containing 2%

paraformaldehyde in 0.1 M PBS and 15% saturated picric acid (Solano-Flores et al.,

1997). In the acute studies, 90 min after the initial intravenous injection of either leptin or

the saline vehicle the animals were similarly perfused transcardially with PBS followed

by the Zamboni fixative.

The carotid arteries on both sides at their bifurcations, along with the carotid

bodies, the PG and NG and adjacent ganglia (jugular and superior cervical ganglia) were

removed and stored overnight in Zamboni's fixative at 4 ℃. The following day the

tissues were gradually dehydrated through a series of alcohols, and placed in xylene

followed by paraffin wax. Serial transverse sections were cut at 6 µm on a RM 2155

microtome (Leica Microsystems Inc., Buffalo Grove, Illinois, USA) floated in a warm water bath and mounted on double-gelatinized glass microscope slides and placed on a slide warmer. Tissue sections were later de-paraffinized in xylene and rehydrated using graded alcohol solutions. For each animal, 1 in every 5 slides was stained with thionin to identify the region of the carotid body, ganglia and blood vessels. Adjacent de-paraffinized sections were rinsed in PBS and endogenous peroxidase activity was blocked using 1% hydrogen peroxide in PBS for 10 min at room temperature and processed for tyrosine hydroxylase (TH) -like immunoreactivity to identify the carotid body glomus cells. In brief, sections were rinsed twice in PBS before being placed in 5% normal goat serum (Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA) in PBS containing 0.3% Triton X-100 for 30 min. The sections were then rinsed in PBS and placed for overnight (12h) in primary polyclonal rabbit anti-TH (Cat.# ab112; Abcam, Cambridge, Massachusetts, USA) diluted 1:1000 in PBS/0.3% Triton X-100 at room temperature. Following two rinses in PBS, the sections were placed in goat biotinylated anti-rabbit IgG (Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA) diluted 1:200 in PBS/0.3% Triton X-100 for 1 h.

Tissue sections immediately adjacent to those immunostained for TH were processed to visualize Ob-Rb immunoreactivity. These tissue sections underwent an antigen-retrieval protocol using a citrate buffer (10 mM sodium citrate/0.05% Tween; pH 6.0) heated to 90-95ºC in a microwave for 15 min. Slides were then rinsed and endogenous peroxidase activity was inhibited by exposing the sections to a 1% hydrogen peroxide solution for 10 min. Sections were washed in PBS and allowed to incubate overnight at room temperature in affinity purified chicken anti-Ob-Rb (Cat. # CH14104,

LepRb/OBRb; Neuromics Inc., Edina, Minnesota, USA) antiserum (1:1000 in PBS/0.3% Triton-X 100 and 4% normal goat serum). Following two rinses in PBS, the sections were then placed in goat biotinylated anti-chicken IgY (Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA) diluted 1:200 in PBS/0.3% Triton X-100 for 1 h. Similarly, to visualize pSTAT3 immunolabeling, additional sections also underwent an antigen-retrieval protocol using a citrate buffer as described above. Slides were then rinsed and endogenous peroxidase blocking was done using a solution of 1% NaOH and 1% hydrogen peroxide. Sections were then exposed to 0.3% glycine in distilled water (10 min) and 0.6% SDS in distilled water (15 min) to ensure permeability of the tissue. Following a PBS rinse, sections were exposed to polyclonal rabbit pSTAT3 primary antibody (anti-pSTAT3; Cat. # 9145, phospho-stat3 (Tyr705) (D3A7) XP; Cell Signaling Technology Inc., Beverly, Massachusetts, USA).

Finally, additional tissue sections adjacent to those immunostained for TH and Ob-Rb were processed for fos- or Fra-1 like immunoreactivity as described above. However, the sections were placed overnight (12h) in primary polyclonal rabbit anti-fos (Cat. # sc52; Santa Cruz Biotechnology Inc.; Santa Cruz, California, USA) or primary polyclonal mouse anti-Fra-1 (Cat. # sc253; Santa Cruz Biotechnology Inc.; Santa Cruz, California, USA) diluted 1:2500 in PBS/0.3% Triton X-100 at room temperature.

Following exposure of the tissue to the secondary antibodies, sections for either TH, Ob-Rb, pSTAT3, fos, or Fra-1 were rinsed twice in PBS and acetate buffer (pH 5.5), and immersed for 40 min in a solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, Missouri, USA), or a solution of 0.05% of DAB with 0.01% nickel ammonium sulfate in acetate buffer, containing 0.2% β-D

glucose, 0.004% ammonium chloride and 0.001% glucose oxidase in PB for 10 min. The slides with sections were rinsed in acetate buffer, washed in PBS, air dried overnight, dehydrated in a graded series of alcohol, cleared in xylene and cover-glassed using DPX mounting medium.

Two types of controls for immunoreactivity were used: omission of the primary antibody and the preabsorption of the antisera with an excess of respective antigen. Under these conditions no immunoreactivity was demonstrated within the tissue sections. The antisera employed in these studies have been already been characterized for specificity (Chandrasekar and Dreyer, 2010; Tripathi and McTigue, 2008; Liu and Guthrie, 2011; Logullo et al., 2011).

Immunofluorescence: Additional de-paraffinized sections from IH-exposed animals and leptin-injected animals (200 ng/0.1ml) were rinsed in PBS and processed for double-immunofluorescence for Ob-Rb and Fra-1. Sections underwent antigen-retrieval as previously described previously and then rinsed in PBS before being placed in 5% normal goat serum (Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA) in PBS containing 0.3% Triton X-100 for 30 min. The sections were later rinsed in PBS and placed overnight (12 h) in primary polyclonal rabbit anti-Fra-1 diluted 1:1000 in PBS/0.3% Triton X-100 at room temperature. Sections were then rinsed in PBS and immersed in Streptavidin Texas Red (Code: RPN1233; GE Healthcare, Buckinghamshire, England) diluted 1:100 for 1 h. Following PBS rinses, sections were exposed to avidin solution for 15 min followed by biotin solution for 15 min (Avidin/Biotin blocking kit, Cat. # SP-2001; Vector Laboratories, Burlingame, California, USA) to block non-specific binding. Sections were rinsed in PBS and

incubated overnight at room temperature in affinity purified chicken anti-Ob-Rb antiserum (1:1000 in PBS/0.3% Triton-X 100 and 4% normal goat serum). Following PBS washes, the sections were placed in goat biotinylated anti-chicken IgY (Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA) diluted 1:200 in PBS/0.3% Triton X-100 for 1 h. Sections were rinsed in PBS and placed in Streptavidin Alexafluor-488 (Cat. # S11223; Invitrogen, Burlington, Ontario, Canada) diluted 1:100 for 1 h. Following PBS rinses, sections were placed in 100% ethanol containing 5% glacial acetic acid on dry ice for 10 min. Sections were immediately cover-glassed using Fluoromount mountant.

Histological Analysis: Cells containing TH-, Ob-Rb-, pSTAT3-, fos-, or Fra-1-like immunoreactivity were identified on sections throughout the region of the carotid body and ganglia using bright-field or fluorescence microscopy (Leica Diaplan, Leica Microsystems Inc., Concord, Ontario, Canada). Digital images of these cells were obtained with a Nikon DS-Fil camera and NIS Elements Basic Research 3.0 software (Nikon Canada, Mississauga, Ontario, Canada).

Statistical Analysis: Plasma leptin levels in the IH and normoxia exposed animals were compared using a one-tailed, unpaired Student t-test. A p-value of < 0.05 was taken as statistically significant.

2.3 Results

Effect of IH on plasma leptin: As shown in Figure 2.1, exposure of animals to IH for an 8 h period significantly ($p < 0.05$) raised the level of plasma leptin compared to animals

exposed to normoxic conditions. IH increased circulating leptin (8.78 ± 1.47 ng/mL) more than two-fold above that found in control animals (3.74 ± 0.17 ng/mL).
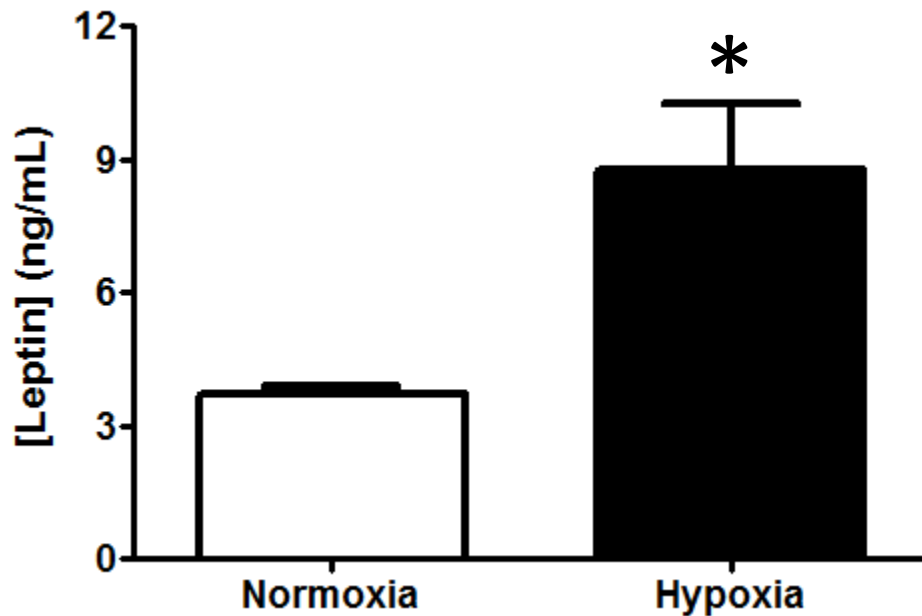
Figure 2.1. Bar chart showing the effect of IH (hypoxia) on plasma levels of leptin. Means $\pm$ S.E.; * $p < 0.05$. n=6.

Ob-Rb expression in rat carotid bodies: As previously described (Porzionato et al., 2011), Figure 2.2 shows that cells within the carotid body (Fig. 2.2a) express TH immunoreactivity (Fig. 2.2b). Additionally, carotid body cells express the Ob-Rb (Fig. 2.2c) in an overlapping distribution with cells that also expressed the enzyme TH, indicating that they were likely type-1 glomus cells (Bolme et al., 1977; Porzionato et al., 2011). Therefore, we have used these observations in the remaining experiments to determine whether carotid body glomus cells expressed pSTAT3, fos-, or Fra-1 -like immunoreactivity during CIH and intravenous injections of leptin.

pSTAT3-, fos-, and Fra-1 - like immunoreactivity in the carotid body after IH or normoxia: The finding of increased circulating levels of leptin during IH (Fig. 2.1) suggested that leptin may exert an effect on Ob-Rb expressing cells (Fig. 2.2) in the carotid body. Figure 2.3 shows the carotid body taken from a normoxic control animal. In these normoxic control conditions, cells within the carotid body express neither pSTAT3- (Fig. 2.3c), fos- nor Fra-1 (Fig. 2.3d) -like immunoreactivity. In contrast, Figures 2.4 and 2.5 show that after exposure of the animal to IH, carotid body cells express pSTAT3- (Fig. 2.4c-d), fos- and Fra-1 (Fig. 2.4d-e; Fig. 2.5a-c)- like immunoreactivity.
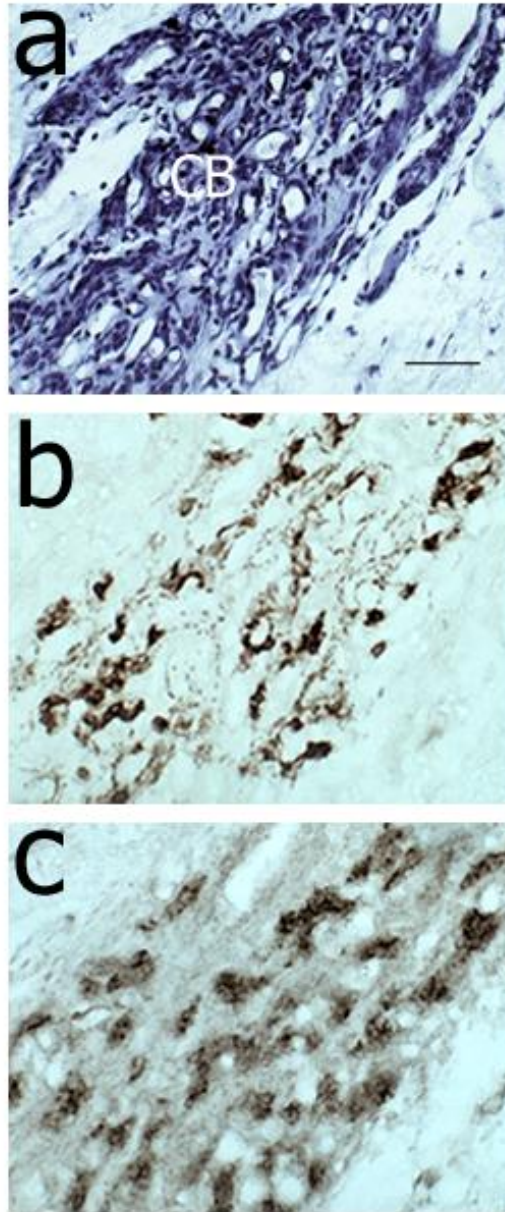
Figure 2.2. Bright-field photomicrographs through the region of the carotid body (CB) stained with thionin (a), or for TH- (b) and Ob-Rb (c) - like immunoreactivity. The distance between sections shown is 18 μm. Note that many cells within the carotid body stain for TH (b), which has been previously used for identification of glomus cells within the carotid body. Ob-Rb immunoreactivity appears to have an overlapping distribution. Calibrations mark in (a) represents 100 μm and applies to (a-c).
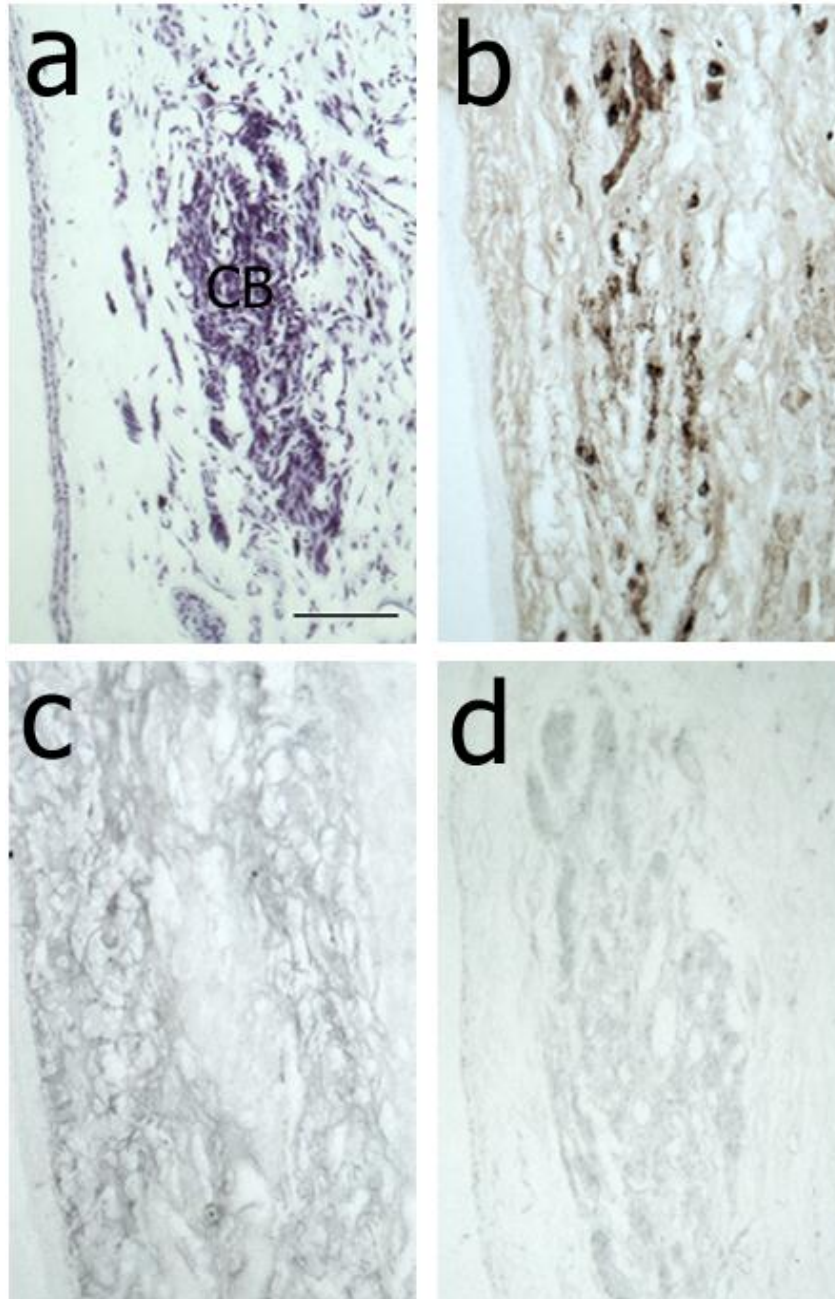
Figure 2.3. A series of bright-field photomicrographs through the region of the carotid body (CB) 18 µm apart stained with thionin (a), or for TH- (b), pSTAT3- (c), and Fra-1-(d) like immunoreactivity taken from an animal exposed to normoxia (control). Note the lack of both pSTAT3 and Fra-1 immunoreactivity in the CB. Calibrations mark in (a) represents 100 µm and applies to (a-d).
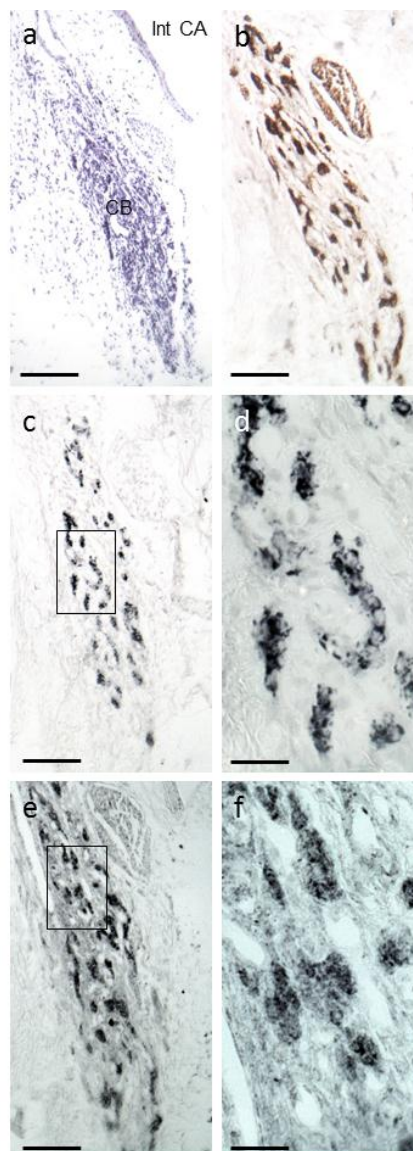
Figure 2.4. Bright-field photomicrographs taken through the region of the carotid body (CB) from an animal exposed to IH. The distance between each section shown is about 18 μm. (a), Nissl; stained section showing morphology of the CB and its proximity to the internal carotid artery (Int CA); (b), TH-immunoreactivity; (c-d), pSTAT3 immunoreactivity. Inset in (c) is shown at greater magnification in (d); (e-f), Fra-1 immunoreactivity. Inset in (e) is shown at greater magnification in (f). Note the overlapping distribution of TH, pSTAT3 and Fra-1 immunoreactivity within the CB. Calibration mark in (a) represents 100 μm and applies to (b, c and e), and represents 25 μm in (d and f).
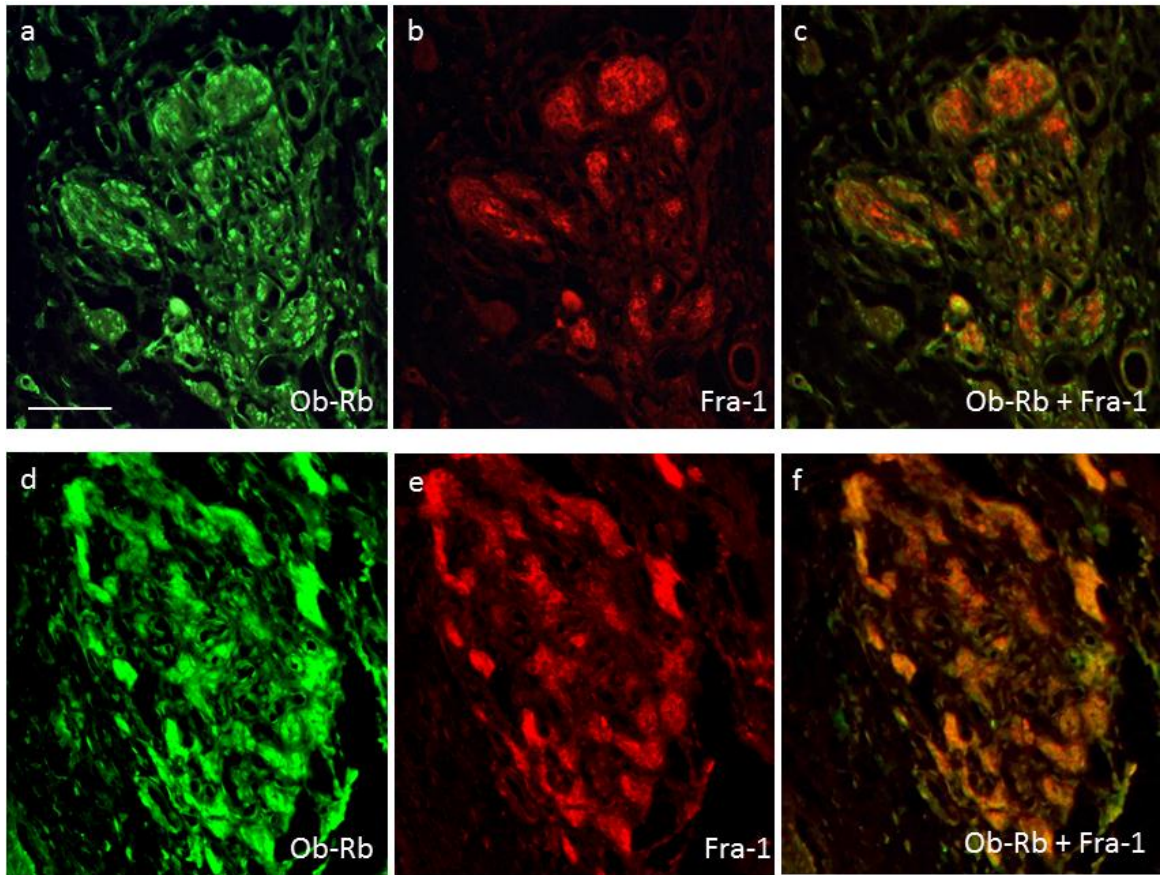
Figure 2.5. Fluorescent photomicrographs showing carotid body cells immunoreactive to Ob-Rb and Fra-1 following IH (a-c) and after injection of 200 ng/0.1 mL (iv) leptin (d-f). Calibration mark in (a) represents 100 μm and applies (a-f).

pSTAT3, fos-, and Fra-1 - like immunoreactivity in the carotid body after leptin injection: To determine whether circulating levels of leptin contributed to the changes in expression of pSTAT3, fos or Fra-1 in carotid body cells during IH, the effect of intravenously administering two different dosages of leptin on these proteins was examined within the carotid body. As shown in Figure 2.6, the low dosage of leptin did not induce either fos- or Fra-1 (Fig. 2.6d) -like immunoreactivity within carotid body cells (Fig. 2.6). However, a few cells express pSTAT3-like immunoreactivity (Fig. 2.6c). In contrast, as shown in Figures 2.5 and 2.7, injections of the higher dose of leptin induced pSTAT3- (Fig. 2.7c), fos-, and Fra-1 (Fig. 2.5 d-f; Fig. 2.7d) -like immunoreactivity within carotid body cells that had a similar distribution to CB cells that expressed the Ob-Rb (Fig. 2.7b). Figures 2.7e and f demonstrate that in the same animal, adjacent control stained sections in which the primary antibody was omitted show no immunoreactivity to either pSTAT3 or Fra-1.

Control intravenous injections of the vehicle saline, in the same volume as that used for injections of leptin, did not induce changes in either pSTAT3 (Fig. 2.8c), fos (Fig. 2.8d), or Fra-1.
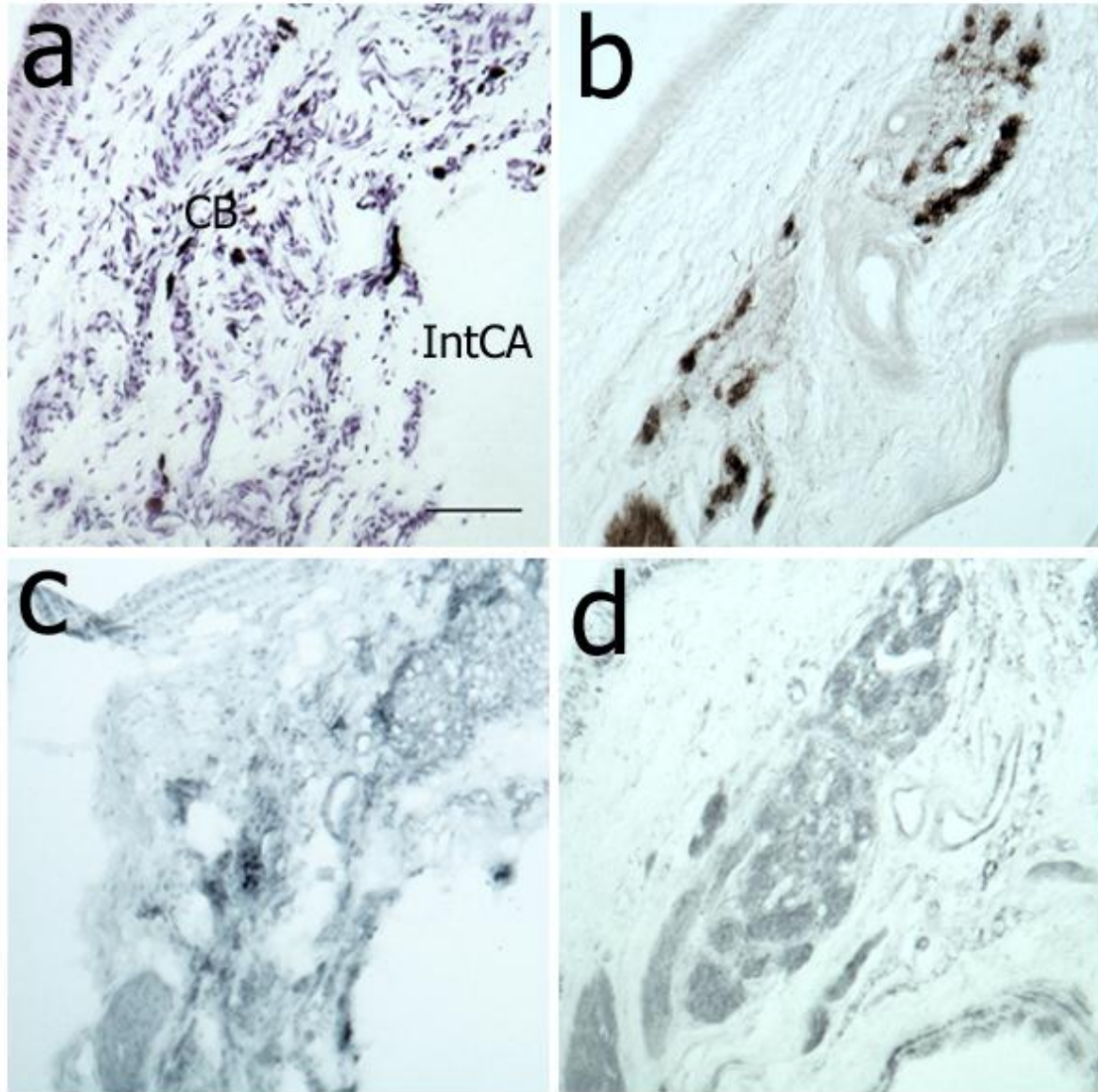
Figure 2.6. A series of bright-field photomicrographs of adjacent sections (18 μm apart) taken through the region of the carotid body (CB; a) showing the distribution of TH- (b), pSTAT3-(c), Fra-1- (d) like immunoreactivity, from an animal that received three 50 ng/0.1 mL injections of leptin every 30 min over a 90 min period. Note the CB lacks labelling for pSTAT3 and Fra-1. IntCA, lumen of internal carotid artery. Calibrations mark in (a) represents 100 μm and applies to (a -d).
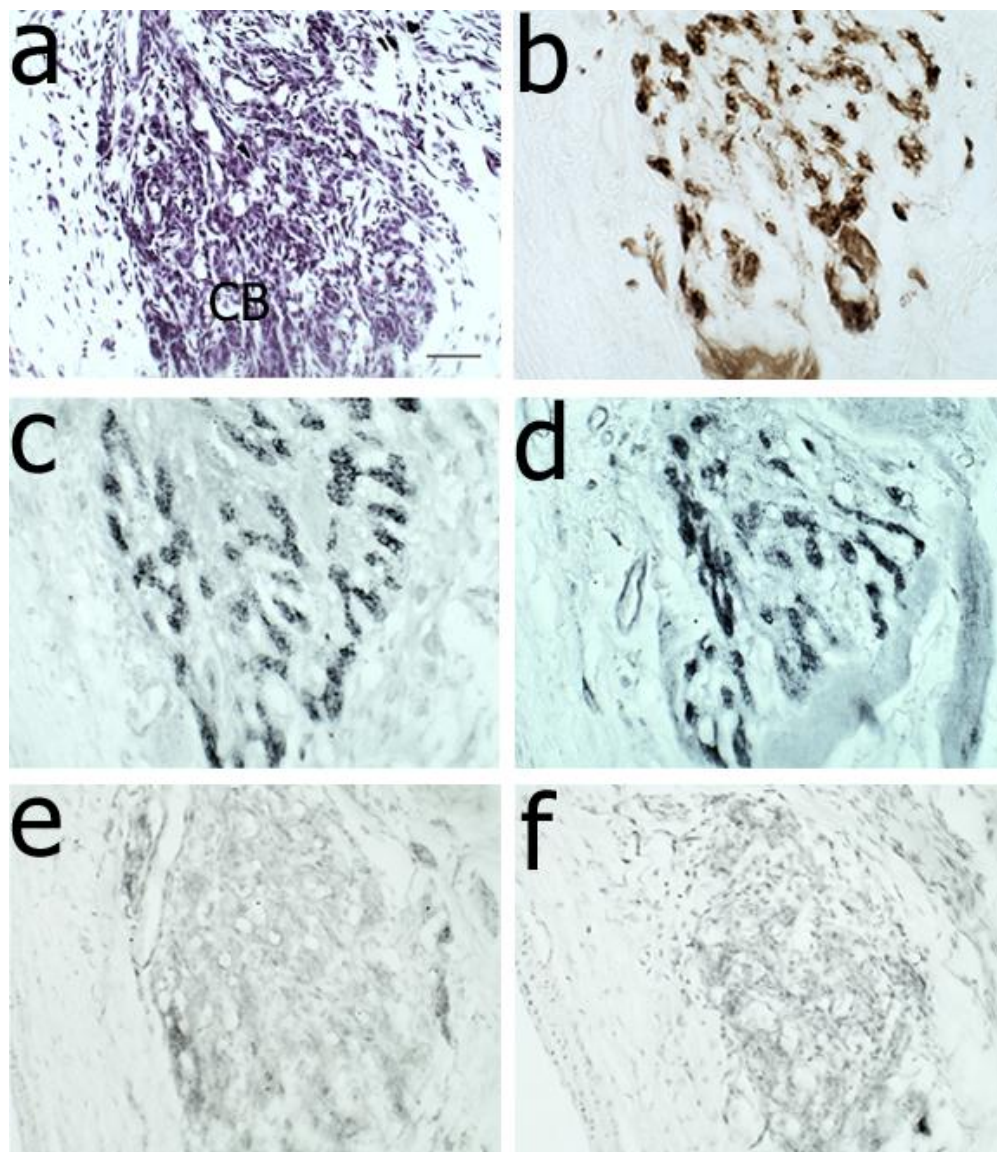
Figure 2.7. Bright-field photomicrographs of adjacent sections (18 µm apart) taken through the region of the carotid body (CB; a) showing the distribution of Ob-Rb- (b), pSTAT3-(c and e), Fra-1- (d and f), from an animal that received three 200 ng/0.1 mL injections of leptin every 30 min over a 90 min period. Panel (a) is a Nissl stained section of CB. Panels (e and f) are control sections processed immunohistochemically for pSTAT3 (e) and Fra-1 (f) following omission of the primary antibody. Note the lack of immunoreactivity in (e) or (f) compared to (c) and (d). Also note the overlapping distribution of Ob-Rb, pSTAT3 and Fra-1 in the CB. Calibrations mark in (a) represents 100 µm and applies to (a -f).
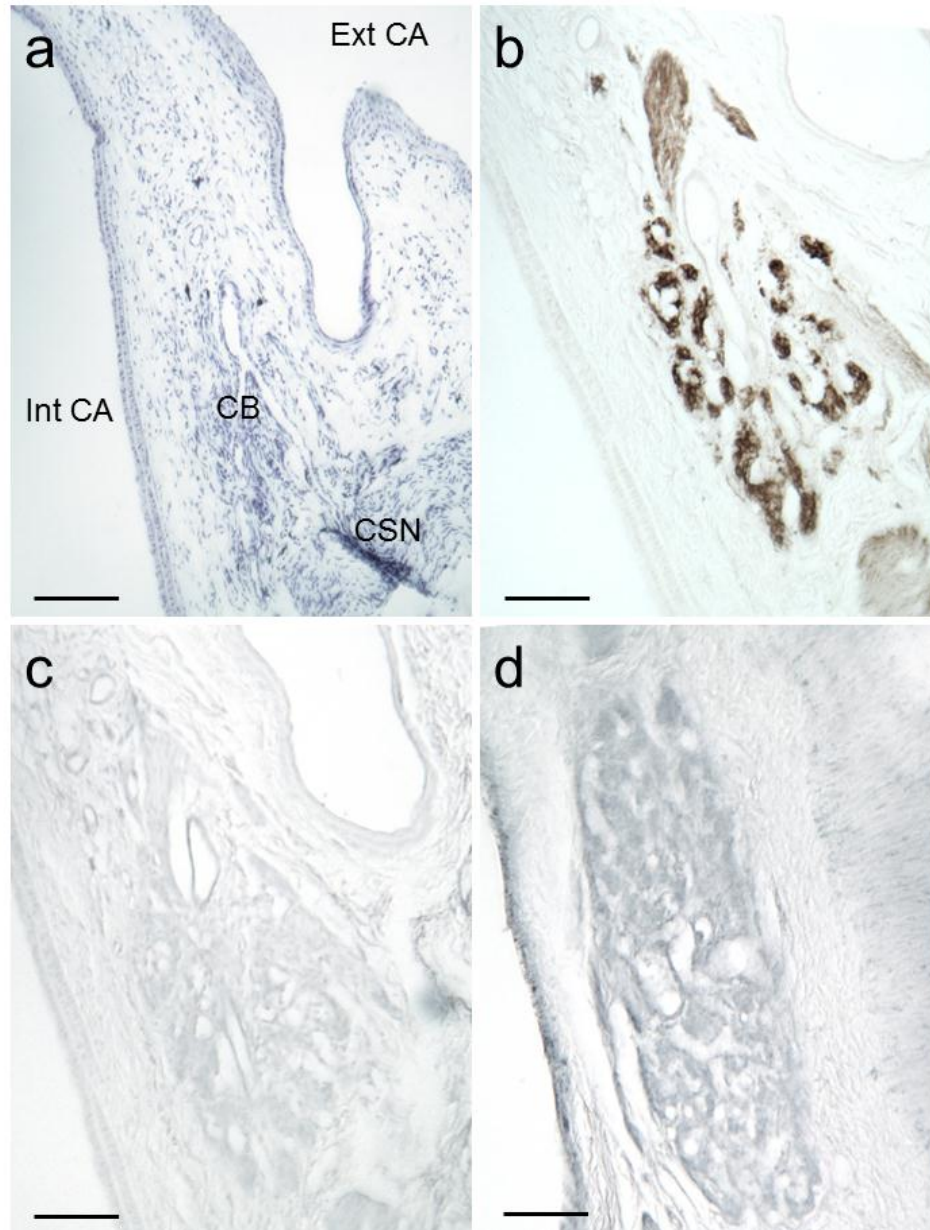
Figure 2.8. Bright-field photomicrographs of adjacent sections (18 μm apart) taken through the region of the carotid body (CB; a) showing the distribution of TH- (b), pSTAT3-(c), and fos (d) immunoreactivity in an animal that received three saline injections of equal volume to those for leptin (0.1 mL) over 30 min over a 90 min period. Note that saline injections do not induce either pSTAT3 (c), fos (d) or Fra-1 (not shown) immunoreactivity in the CB. IntCA, lumen of internal carotid artery; ExtCA, lumen of external carotid artery. Calibration mark in (a) represents 150 μm and represents 100 μm in (b-d).

fos-, Fra-1- and pSTAT3-like immunoreactivity in the nodose/petrosal complex after intravenous leptin injections:

The finding that intravenous injections of leptin induced pSTAT3, fos and Fra-1 immunoreactivity in the carotid body suggested that sensory neurons mediating the chemoreceptor information may also in turn be activated. To test this hypothesis, the PG, NG and other adjacent ganglia near the carotid body were examined for pSTAT3, fos or Fra-1 activity after leptin injections. Figure 2.9 shows a series of adjacent 6 μm sections stained for Nissl substance (Fig. 2.9a), and for TH- (Fig. 2.9b), Ob-Rb- (Fig. 2.9d and g), fos- (Fig. 2.9e and f), and Fra-1 (Fig. 2.9f and i) -like immunoreactivity within the PG. PG and NG afferent neurons contained Ob-Rb immunoreactivity (Fig. 2.9d and g), and fos and Fra-1 labeling (Fig. 2.9e and h and Fig. 2.9f and i, respectively). An unexpected finding was that in all animals studied, regardless if they were exposed to normoxia, IH, or injected with leptin or saline, the PG (Fig. 2.9b) and NG displayed very few TH labeled cells, while the superior cervical ganglion, as expected, contained many TH labeled neurons.

In addition to neurons within the PG and NG that contained Ob-Rb labeling, vagal nerve (Fig. 2.10b) and carotid sinus nerve fibers (Fig. 2.10c) were found to contain Ob-Rb immunoreactivity. Fibers immunoreactive to Ob-Rb were also observed coursing through the carotid body among Ob-Rb labeled cells (Fig. 2.10d), and through the PG and NG (Figs. 2.10e-f). Furthermore, a small number of neurons were found within the proximal pole of the jugular ganglion which also contained Fra-1- like immunoreactivity (Fig. 2.11).

A small number of fibers were also found that contained pSTAT3-like immunoreactivity (Fig. 2.12) within the carotid body of both the IH and the higher dose of leptin injected animals. These labeled fibers were seen to extend from labelled cells, and in some cases appeared to connect pSTAT3 labelled cells (Fig. 2.12c).
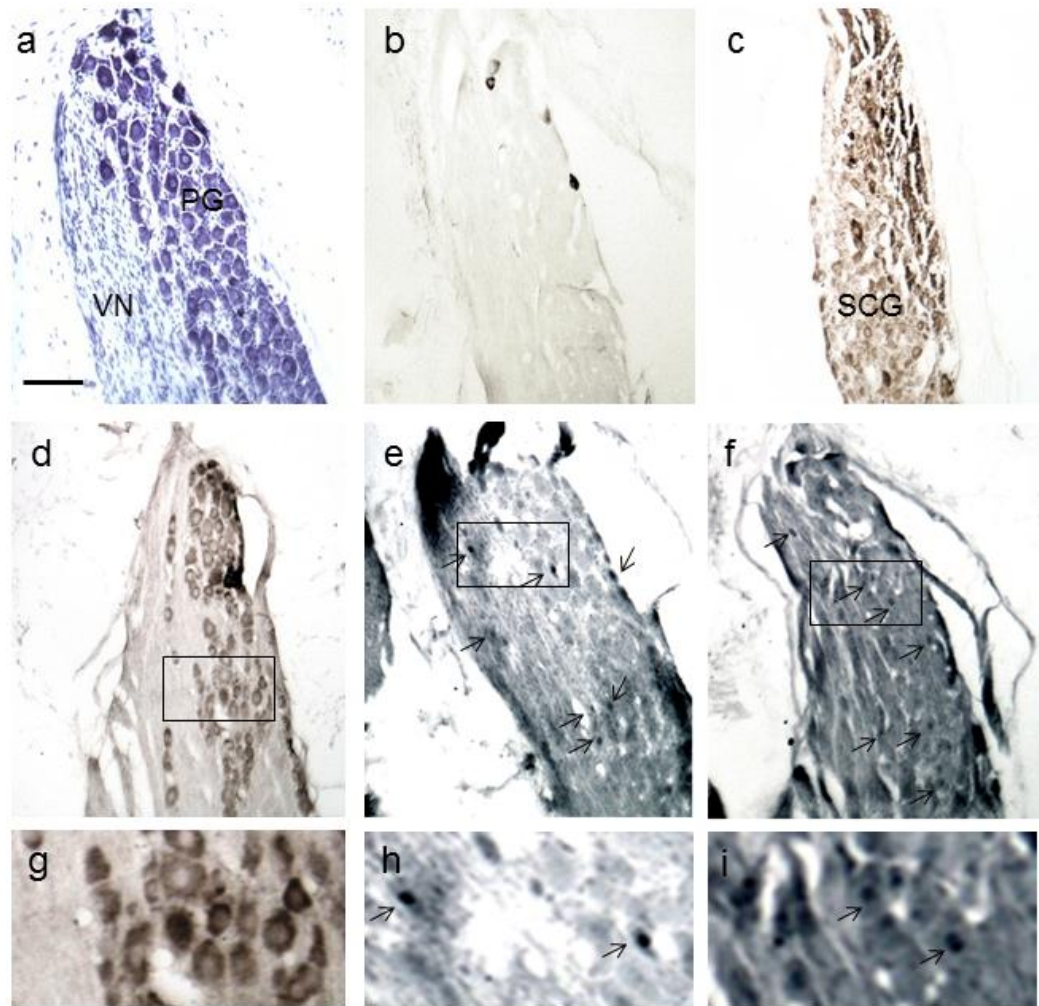
Figure 2.9. Bright-field photomicrographs taken through the petrosal ganglion (PG; a-b and d-i) showing neurons expressing TH (b), Ob-Rb (d and g), fos (e and h) and Fra-1 (f and i) immunoreactivity in an animal that received three 200 ng/0.1 mL injections of leptin every 30 min over a 90 min period. Note that PG neurons express Ob-Rb immunoreactivity (d and g) and leptin injections induce both fos (e and h), and Fra-1 (f and i) immunoreactivity within PG neurons (arrows). Insets in (d-f) correspond to photomicrographs (g-i, respectively) at higher magnification. Note also the small number of PG neurons that express TH (b) while all neurons within the superior cervical ganglion (SCG) express TH (c). VN, vagus nerve. Calibrations mark in (a) represents 100 μm and applies to (a -f) and represents 25 μm in (g-i).
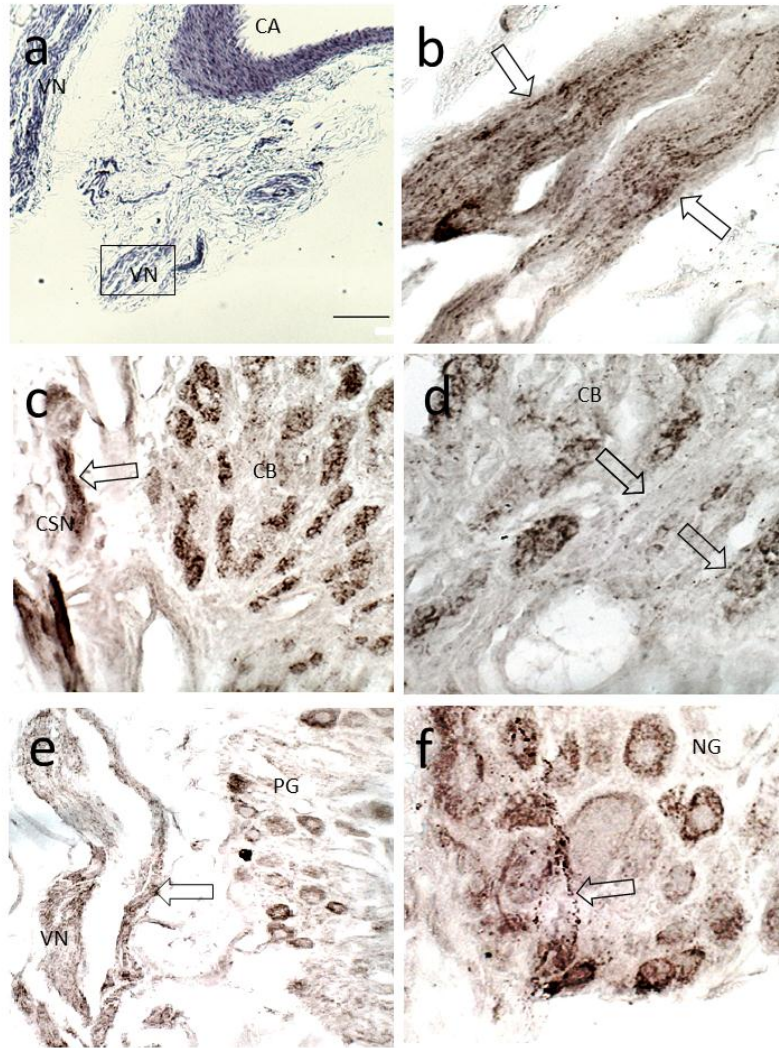
Figure 2.10. Bright-field photomicrographs taken through the region of the carotid body (CB) (a-d) and the petrosal (PG)/nodose (NG) complex showing the distribution of Ob-Rb immunoreactivity within nerve fibers. (a), thionin stained section showing cytoarchitecture of the CB region. VN, vagus nerve; CA, lumen of carotid artery. Inset in (a) is shown in (b) which is taken from an adjacent section 6 µm away; (b) Ob-Rb labeling within vagal nerve fibers (arrows); (c) shows Ob-Rb immunoreactivity within the CB and the carotid sinus nerve (CSN; arrow); and (d) Ob-Rb immunoreactivity in the CB. Note fibers expressing the Ob-Rb (arrows) coursing through the CB; (e) Ob-Rb immunoreactivity in PG neurons and the adjacent vagus nerve (VN) and glossopharyngeal nerve (arrow); (f) Ob-Rb expressing neurons and fibers (arrow) within the NG. Calibration mark in (a) represents 100 µm and represents 25 µm in (b-f).
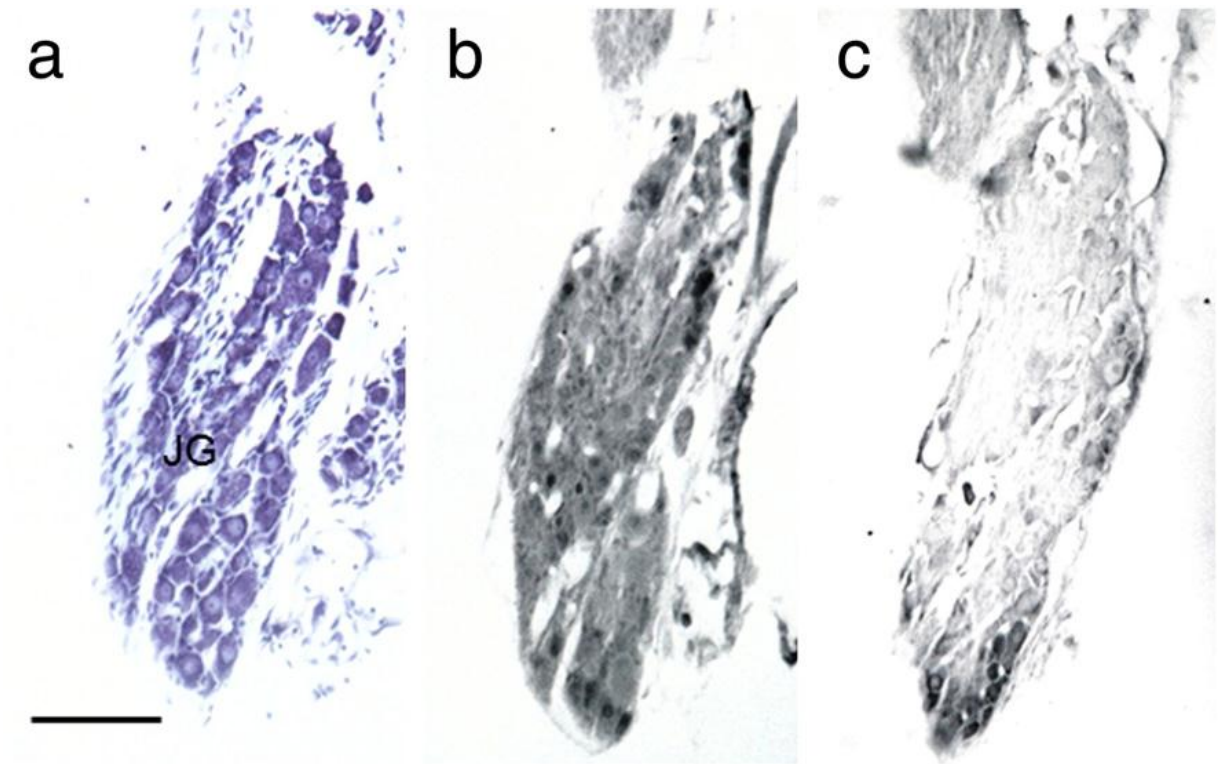
Figure 2.11. A series of adjacent bright-field photomicrographs (18 µm apart) through the jugular ganglion (JG) from an animal that received three 200 ng/mL injections of leptin over 30 min over a 90 min period. (a) thionin stained; (b) pSTAT3 immunoreactivity; (c) Fra-1 immunoreactivity. Note that leptin injections induce both pSTAT3 and Fra-1 express in the JG. Calibration mark in (a) represents 100 µm.
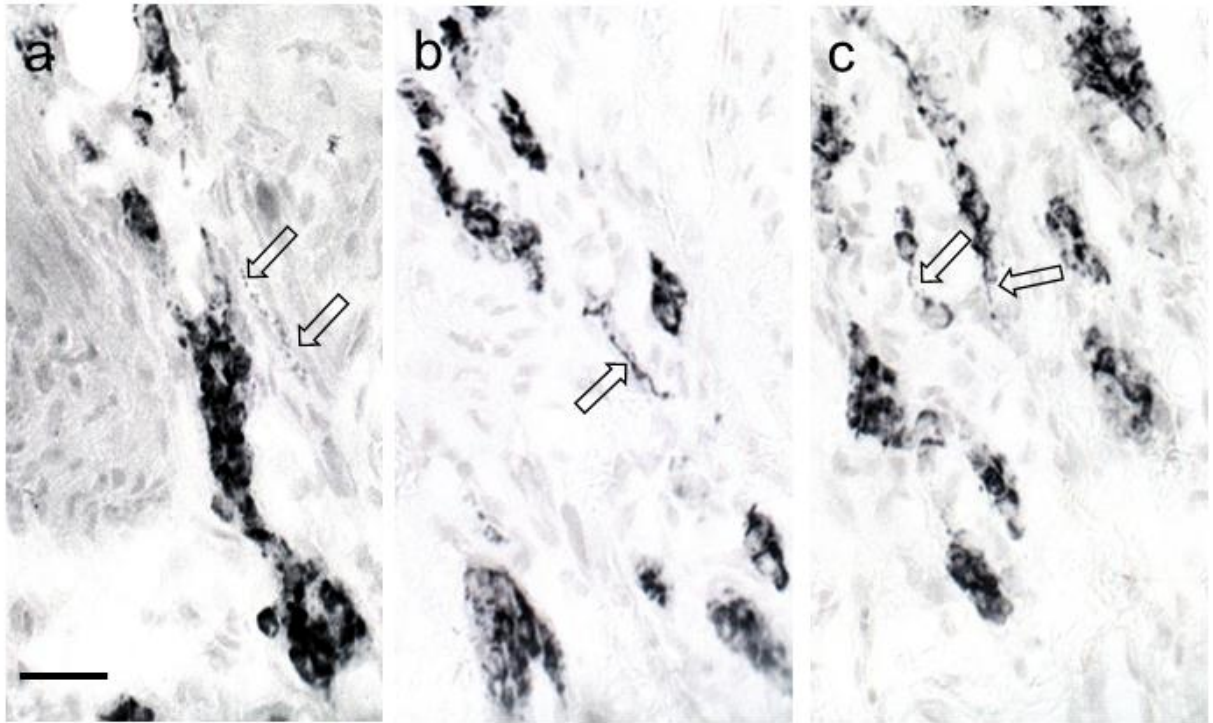
Figure 2.12. Bright-field photomicrographs taken through the region of the carotid body from an animal exposed to IH showing pSTAT3 immunoreactivity (a-c) within cells and fibers coursing through the carotid body (arrows). Calibrations mark in (a) represents 25 μm and applies to (a-c).

2.4 Discussion

This study has provided the first direct evidence showing that cells within the carotid body that express the Ob-Rb are functionally activated during IH and following intravenous administration of leptin. Both IH and leptin injections were found to induce the expression of pSTAT3, fos and Fra-1immunoreactivity. IH was also shown to result in a greater than two fold increase in circulating levels of leptin. Additionally, sensory neurons were found within the PG and NG that not only expressed the Ob-Rb, but were also activated in response to intravenous injections of leptin. Furthermore, afferent fibers within the vagus and carotid sinus nerves were observed to express the Ob-Rb. Taken together, these observations suggest that leptin may exert an effect on the peripheral chemoreceptors and their afferent pathway resulting in a modulation of the cardio-respiratory responses induced by IH both at the level of carotid body glomus cell and at the level of the nucleus of the solitary tract, the primary site of termination of chemoreceptor afferent fibers (Ciriello et al., 1981, 1994).

The finding that carotid body glomus cells express the Ob-Rb is in agreement with an earlier study (Porzionato et al., 2011). In addition, on the basis of their finding that carotid body cells also contained leptin (Porzionato et al., 2011), these authors suggested that leptin produced by the glomus cells may act in an autocrine/paracrine fashion to modulate their function and thus, the chemoreceptor reflex. This study has now shown that neurons within both the PG and NG also express the Ob-Rb. Although the function of these neurons is not known, the finding that a number of afferent axons in both the carotid sinus and vagus nerves that also express the Ob-Rb suggest that leptin may act presynaptically on both axons within the carotid body and the nucleus of the

solitary tract to influence transmission of chemoreceptor origin. Thus, a plausible explanation for the finding of PG and NG neurons expressing the Ob-Rb may be that these receptors are produced in the ganglion cells and transported to their terminal endings. This would not be unlike the production of angiotensin II receptors within NG cells that are transported to presynaptic sites in the nucleus of the solitary tract (Diz et al., 1986; Healy et al., 1989, Lewis et al., 1986). In support of this suggestion, neurons within the ganglia do not express any significant quantity of pSTAT3, either in animals exposed to IH or those injected with leptin. This finding indicates that these receptors are not activated by systemic leptin, even though pSTAT3 is induced in carotid body cells, or as previously reported within the nucleus of the solitary tract (Elmquist et al., 1997; Huo et al., 2006, 2007; Wang et al., 1998). However, the possibility cannot be eliminated that the specific blood brain barrier around ganglion neurons may not allow ready access by leptin to these Ob-Rb receptors. The finding that both afferent fibers and neurons in the PG and NG contain Ob-Rb is consistent with the observation that Ob-Rb labeled axons are found within the nucleus of the solitary tract (Barnes et al., 2010; Ellacott et al., 2006; Elmquist et al., 1997; Huo et al., 2006, 2007; Shioda et al., 1998).

It should also be noted that leptin has been suggested to play an important role in axonal growth (Bouret et al., 2004; Pinto et al., 2004; Valerio et al., 2006). Therefore, an alternate possibility may be that leptin acting through the Ob-Rb may help maintain the integrity of axons for transmission in a neuronal system that is chronically active in detecting changing levels of PO_2. This is not considered unreasonable as hypoxia has been shown to induce plasticity within neural circuits controlling respiration (Nurse et al., 1994; Ling et al., 2001).

This study has also provided the first evidence in a rat model of IH that during an 8 h period of exposure to IH circulating levels of leptin are elevated. This finding is consistent with observations in clinical studies showing that patients with OSA have higher circulating levels of leptin independent of body weight (Harsch et al., 2003; Phillips et al., 2000). The source of this leptin is unknown. However, as the plasma levels measured in this study are not as high as those expected in overweight or obese animals, it may be suggested that this increase in leptin is due to release from the carotid bodies themselves in response to the lower PO_2 during hypoxia. Carotid body cells have been shown to contain leptin (Porzionato et al., 2011). Regardless of the source of the leptin, it was found that IH induced pSTAT3, fos and Fra-1 in carotid body cells. The finding of pSTAT3 induction is suggestive of a leptin mechanism acting within the carotid body. STAT3 is phosphorylated upon activation of the JAK2/STAT3 pathway and is thought to be an important component in the Ob-Rb signal transduction in the nervous system (Bjørbaek et al., 1997; Hubschle et al., 2001). The suggestion of a leptin mechanism acting within the carotid body is further supported by the finding that intravenous injections of leptin induced pSTAT3, fos and Fra-1 within carotid body cells. The estimated amount of leptin injected in this study to induce pSTAT3 would be about 12.4 ng/mL plasma. This is well within the range of plasma leptin levels measured during IH. Thus, it was not surprising that no expression of these proteins was found in control normoxic animals, or in animal injected with saline or the low dose of leptin. It is calculated that the low dose of leptin used in this study would average in the circulation to be about 4.3 ng/mL plasma. This level is similar to that found within the plasma in the normoxic controls.

The carotid bodies taken from both IH and the higher dose of leptin injected animals expressed both fos and Fra-1 immunoreactivity. In the IH animals, a greater expression of Fra-1 compared to fos was observed in the carotid bodies. This was not unexpected as fos expression is known to peak within about 2 h after the stimulus and return to baseline within 6-8 h, whereas Fra-1 proteins are also synthesized soon after their induction, but persist from about 1-2 days to several weeks. On the other hand, fos and Fra-1 expression appeared not to be different within the animals that received the higher dose of leptin intravenously and were allowed to survive for only 90 min after the injection. The finding of activation of the immediate early gene products fos and Fra-1 in the carotid body cells, combined with the increased expression of pSTAT3 can be interpreted to indicate that these cells were activated in response to IH, likely through a leptin dependent mechanism. Activation of these cells would then be expected to affect the transduction of the chemoreceptor signal.

In addition to the carotid body, neurons within the PG/NG complex which are known to contain the perikarya of the carotid sinus nerve which relay chemoreceptor afferent inputs to the NTS, expressed fos and Fra-1 labelling in response to leptin injections. Additionally, a very small number of neurons expressed pSTAT3. The finding of fos/Fra-1 neurons is consistent with the interpretation that following leptin injections, the activation of carotid body glomus cells leads to activation of primary sensory afferents. However, the functional significance of the observation that a few neurons expressed pSTAT3 is not known. Similarly, the function of the finding that some axons within the carotid body expressed pSTAT3 is not known. However, it has been previously suggested that axonal pSTAT3 may function as retrograde signaling

transcription factor carrying information to the cell genome (Lee et al., 2004). STAT3 has been suggested to be involved in maintaining the neurochemical phenotype of the neuron (Lee et al., 2004; Nawa et al., 1990). STAT3 has also been associated with cellular protective mechanisms when found outside the central nervous system (Schwaiger et al., 2000; Suzuki et al., 2001; Wen et al., 2001).

Finally, an unexpected finding was the small number of neurons within the PG/NG complex that expressed TH. Although it could be argued that the sensitivity of the immunohistochemical procedure used in this study may have not been sufficient to detect the TH, this possibility is considered unlikely as the superior cervical ganglia contained extensive TH labeling, as did the carotid body. Although this finding is consistent with the small number of neurons identified in the distal end of the PG (Finley et al., 1992; Ichikawa et al., 1993), most studies indicate that a large number of afferent neurons from the carotid sinus and body express TH (Katz et al., 1983; Katz and Black, 1986), and that TH labeled neurons is often used as a marker for these afferent neurons within the ganglia.

In summary, the findings that carotid body glomus cells express the Ob-Rb, and are activated by circulating leptin suggest that leptin may function in the modulation of the chemoreceptor reflex. Although it remains to be determined whether the leptin that exerts its effects on the chemoreceptors is released by carotid body glomus cells or from adipocytes during IH, these findings suggest that leptin can now exert its effects on cardio-respiratory reflex pathways not only within the central nervous system at the level of the first synapse within the nucleus of the solitary tract, but also at the level of the peripheral receptors. Several recent studies have shown that leptin administration into

NTS not only alter cardio-respiratory activity (Arnold et al., 2009; Inyushkin et al., 2009, Mark et al., 2009), but potentiate the arterial pressure and sympathetic nerve responses to peripheral chemoreceptor activation (Ciriello and Moreau, 2011). On the basis of these earlier observations, combined with the findings in this study, it is not unreasonable to suggest that through leptin dependent mechanisms act at several different levels throughout the chemoreceptor reflex pathway, and that the autonomic effects associated with OSA may be exacerbated in obese patients with high circulating levels of leptin.

2.5 References

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Chapter 3

3 Effects of Intermittent Hypoxia on Leptin Signalling in the Carotid Body

3.1 Introduction

Obstructive sleep apnoea is a pathophysiological disorder commonly observed in overweight and obese patients that is characterized by brief repetitive collapse of the upper airway during the sleep cycle (Dempsey et al., 2010). If untreated, obstructive sleep apnoea increases the risk for the development of cardiovascular disease (He et al., 1988; Shamsuzzaman et al., 2003; Dempsey et al., 2012). These cardiovascular comorbidities (heart disease, stroke, myocardial ischaemia, atherosclerosis and hypertension) have been suggested to contribute later in life to increased mortality within this population. The cessation in respiration exposes the patient to intermittent hypoxia (IH), a recurring episodic decrease in blood oxygen saturation.

An animal model of IH has been developed to mimic obstructive sleep apnea (Fletcher et al., 1992b). In this animal model, IH has been shown to increase sympathetic nerve activity to different vascular beds, an effect dependent on an intact carotid chemoreceptor reflex (Fletcher et al., 1992a).

Specialized chemo-sensitive organs occur throughout the vascular system, though the organs predominantly involved in sensing changes in partial pressures of oxygen (PO_2) within the blood are located bilaterally at the bifurcation of the carotid artery (Heymans et al., 1930; Nurse, 2005). These carotid bodies contain specific chemosensory glomus cells responsible for the transducing the low PO2 signal, which in turn activate

the chemosensory nerve endings of the carotid sinus nerve (Heymans et al., 1930; Nurse 2005). These afferent signals arrive to the nucleus of the solitary tract (Ciriello et al., 1994) and activate homeostatic adaptations including the respiratory, cardiovascular and sympathetic nervous systems (Schultz and Li, 2007; Kumar, 2009).

Studies to determine the primary signalling molecule between glomus cells and carotid body afferent nerves have revealed a number of potential transmitter candidates (Spyer et al., 2003; Nurse, 2005, 2010; Shirahata et al., 2007) that include dopamine (Gomez-Nino et al., 1990), acetylcholine (Eyzaguirre and Zapata, 1968), and adenosine triphosphate (Bock, 1980). Recently, the finding of leptin receptor isoforms expressed within glomus cells (Porzionato et al., 2011; Messenger et al., 2012), combined with the observation that increased circulating leptin levels induce the expression of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and the immediate early gene Fra-1 within these glomus cells (Messenger et al., 2012) suggest that leptin may play an important role in modulating chemosensitivity.

Leptin, the 16 kDa highly conserved protein encoded by the *obese* gene, is produced predominantly, but not exclusively by adipocytes (Lonnquist et al., 1995; Caro et al., 1996). Leptin normally circulates in proportion to body adiposity and normally plays a critical role in body energy balance (Lonnquist et al., 1995; Caro et al., 1996). Leptin not only functions as a satiety hormone, but also functions to increase metabolism and thermogenesis, thus stimulating weight loss (Ahima et al., 1996; Jacob et al., 1997; O'Donnell et al., 2000; Bjorbaek and Kahn, 2004). In addition, central administration of leptin has been found to have an effect on the cardiovascular system. Central administration of leptin has been shown to increase sympathetic nerve activity, blood

pressure, heart rate and decrease baroreflex sensitivity (Arnold et al., 2009; Mark et al., 2009). Furthermore, microinjections of leptin into caudal pressor sites in the nucleus of the solitary tract have been shown to potentiate the arterial pressure and sympathetic nerve responses to activation of the chemoreceptor reflex (Ciriello and Moreau, 2012). Furthermore, leptin has also been shown to exert an effect on respiration (Tankersley et al., 1998). Leptin exerts these physiological effects through the activation of the obese receptors (Ob-R's), of which there are six known splice variants (Ob-Ra-f) (Lee at al., 1996; Wang et al., 1996). The long-form leptin receptor (Ob-Rb) operates via the JAK2/signal transducer and activator of transcription 3 (STAT3) pathway and has been implicated in transducing most of leptin's central signalling effects within the hypothalamus and the brainstem (Elmquist et al., 1997; Wang et al., 1998; Huo et al., 2006, 2007).

The finding that circulating levels of leptin are elevated during IH (Messenger et al., 2012), and that leptin injections into the nucleus of the solitary tract can alter the chemoreceptor reflex (Ciriello and Moreau, 2012), combined with the observation that leptin receptors exist within the carotid bodies (Porzionato et al., 2011; Messenger et al., 2012), suggest that leptin may also act through functional receptors on carotid body glomus cells to alter chemoreflex sensitivity. However, whether IH or leptin alters leptin signalling pathways within glomus cells, along with whether local leptin and leptin receptor (Porzionato et al., 2011; Messenger et al., 2012) expression are affected by changes in arterial PO_2 are not known. Therefore, the present study was done to determine: (1) whether glomus cells expressing the Ob-Rb also express leptin and whether these cells are activated during IH or during systemic leptin injections; (2)

whether IH alters leptin and OB-R expression; (3) whether IH alters the protein expression of STAT3 and pSTAT3, and suppressor of cytokine signalling 3 (SOCS3), which binds to JAK2, inhibiting leptin-induced signalling through the STAT3 mechanism (Bjorbaek et al., 2000); (4) whether IH has an effect on the expression of ERK1/2.

3.2 Methods

General: Experiments were done in male Sprague–Dawley rats (Charles River Canada, St. Constant, Canada) weighing 300-395 g. All animals were housed under controlled conditions with a 12 h light/dark cycle. Food and water were available *ad libitum*. All experimental procedures were done in accordance with the guidelines on the use and care of laboratory animals as set by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Western Ontario.

Induction of IH: IH (n = 16) or normoxia (n = 15) was induced in animals as previously described (Messenger et al., 2012). In brief, animals (one/tube) were placed into custom made cylindrical plexiglass tubes with a zero-pressure escape valve (11-cm diameter x 33-cm length; volume 3136 cm^3) inside an airtight plexiglass chamber which housed four tubes. The animal was allowed to freely move within each tube. The chamber was attached to computer-controlled solenoid valves responsible for regulating the inflow of either pressurized room air (21% O_2) or 100% medical N_2. The solenoid valves were connected to a computerized timer system that allowed for the control of a total hypoxic (100% medical N_2) stimulus delivery of 80 s followed by a normoxic (21% O_2) time of 100 s, for a total continuous repetitive cycle period of 8 h. Additionally, the controller, attached to detectors for O_2 and CO_2 within the chamber, was set at a O_2 level of 6.5 % and CO_2 level of 0.1% under isobaric conditions at all times during the delivery of the

hypoxic stimulus and recovery phase during which O_2 returned to 20.9%. A control (normoxic) set of animals was simultaneously run in a similar adjacent chamber through which only room air (21% O_2; normoxia) was used for the gas exchange in isobaric conditions for an 8-h period.

Plasma leptin levels: Measurement of plasma levels of leptin were made immediately after the exposure of the animals to IH or the normoxic stimuli (Messenger et al., 2012). Whole blood samples were taken through a cardiac puncture prior to perfusion of the animal for immunohistochemistry or collection of tissues for Western blot analysis. Blood samples were drawn into centrifuge tubes containing the anticoagulant EDTA (7%; 10 µL EDTA/1 mL blood; Sigma, St. Louis, Missouri, USA) and kept on ice. Blood samples were then centrifuged and the plasma was immediately removed and stored at −80 °C for later leptin analysis using a TiterZyme enzyme immunometric assay kit (Enzo Life Sciences (ADI-900-015A; Farmingdale, New York, USA) as previously described (Messenger et al., 2012). The assay was read using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, California, USA).

Intravenous leptin injections: On the day of the experiment, the animals were anaesthetized with alpha-chloralose (80 mg/kg i.v. initially, and supplemented by additional doses of 10 mg/kg every 30 min) after induction with equithesin (0.3 mL/100 g, i.p.). Body temperature was maintained at 36–37 °C by a heating pad (model K-20-C; American Hospital Supply, Cincinnati, Ohio, USA). Prior to surgery, 0.2 mL of lidocaine was injected into the neck region where the right jugular vein was to be isolated and cannulated with a polyethylene catheter-50 (Clay Adams, Parsippany,

New Jersey, USA). This jugular vein cannula was used for administration of anaesthetic, leptin and the saline vehicle. Animals were allowed to recover for a period of 2 h in a dark and quiet room immediately after the jugular vein cannulation. The animals then received three 0.1 mL injections (at 0, 30 and 60 min) of either leptin (50 ng/0.1 mL, n = 3; 200 ng/0.1 mL, n = 3) or physiological saline (0.9%, n = 3) while remaining in the dark under silent conditions.

Immunofluorescence: Immediately after the application of the 8-h IH or normoxic stimuli the animals were anaesthetized with 0.3 mL/100 g equithesin and perfused transcardially using 500 mL ice-cold phosphate-buffered saline (PBS; 0.01 M, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS. The carotid arteries at their bifurcations, along with the attached carotid bodies were removed bilaterally, and stored overnight in Zamboni's fixative at 4 ºC. The following day the tissues were gradually dehydrated through a series of alcohols, and placed in xylene followed by paraffin wax. Serial transverse sections were cut at 6 μm on a RM 2155 microtome (Leica Microsystems Inc., Buffalo Grove, Illinois, USA), floated in a warm water bath and mounted on double-gelatinized glass microscope slides and placed on a slide warmer. Tissue sections were later de-paraffinized in xylene and rehydrated using graded alcohol solutions. For each animal, one in every five slides was stained with Thionin to identify the region of the carotid body and blood vessels. Additionally, adjacent sections to those stained for thionin from the normoxic and IH-exposed animals were rinsed in PBS and processed for double-immunofluorescence for Ob-Rb and leptin. Sections underwent an antigen-retrieval protocol using a citrate buffer (10 mM sodium citrate/0.05% Tween; pH 6.0) heated to 90-95ºC in a microwave for 15 min and then were rinsed in PBS before

being placed in 5% normal goat serum (Vectastain Elite Kit, Cat. # PK6100; Vector Laboratories, Burlingame, California, USA) in PBS containing 0.3% Triton X-100 for 30 min (Messenger et al., 2012). The sections were later rinsed in PBS and placed overnight (12 h) into a primary polyclonal rabbit anti-leptin (Cat. # sc843; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA; 1:1000 in PBS/0.3% Triton X-100) at room temperature. Sections were then rinsed in PBS and incubated in goat biotinylated anti-rabbit IgG (Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA) diluted 1:500 in PBS/ 0.3% Triton X-100 for 1 h. Following PBS rinses, sections were placed in Streptavidin Alexafluor-488 (Cat. # S11223; Invitrogen, Burlington, Ontario, Canada) diluted 1:100 for 1 h. Following PBS rinses, sections were exposed to an avidin containing solution for 15 min followed by a biotin containing solution for an additional 15 min (Avidin/Biotin blocking kit, Cat. # SP-2001; Vector Laboratories, Burlingame, California, USA) to block non-specific binding sites. Sections were then rinsed in PBS and incubated overnight at room temperature in either affinity purified chicken anti-Ob-Rb antiserum (Cat. # CH14104, LepRb/OBRb; Neuromics Inc., Edina, Minnesota, USA) diluted 1:1000 in PBS/0.3% Triton-X 100 and 5% normal goat serum or primary polyclonal rabbit anti-Fra-1 (Cat. # sc253; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) diluted 1:1000 in PBS/0.3% Triton-X 100 and 5% normal goat serum. Following PBS washes, the sections were placed in goat biotinylated anti-rabbit IgG (Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA) diluted 1:500 in PBS/ 0.3% Triton X-100 or goat biotinylated anti-chicken IgY (Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA) diluted 1:200 in PBS/ 0.3% Triton X-100 for 1 h. Sections were rinsed in PBS and placed in Streptavidin Texas Red (Code:

RPN1233; GE Healthcare, Baie d'Urfe, Quebec, Canada) diluted 1:100 for 1 h. Following PBS rinses, sections were placed in 100% ethanol containing 5% glacial acetic acid on dry ice for 10 min. Sections were then immediately cover-glassed using Fluoromount mountant.

Immunohistochemistry: Tissues were processed as described above with the following exceptions. Following the antigen retrieval protocol, endogenous peroxidase activity was blocked by placing tissue sections into 1% hydrogen peroxide for 10 min. Sections were rinsed in PBS and then placed in 5% normal goat serum (Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA) in PBS containing 0.3% Triton X-100 for 30 min. The sections were later rinsed in PBS and placed overnight (12 h) at room temperature in primary monoclonal rabbit anti-pERK1/2 (1:100, Cat. # 4376S; Cell Signaling Technology, Danvers, Massachusetts, USA) in PBS/0.3% Triton X-100. After PBS rinses, sections were incubated in goat biotinylated anti-rabbit IgG (Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA) diluted 1:1000 in PBS/ 0.3% Triton X-100 for 1 h. Sections were rinsed in PBS and placed in an immunoperoxidase detection system (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, California, USA) for 75 min, followed by PBS rinses and immersion of the sections for 40 min into a solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, Missouri, USA). The slides with sections were rinsed in PBS, air dried overnight, dehydrated in a graded series of alcohol, cleared in xylene and cover-glassed using DPX mounting medium.

Histological analysis: Cells containing leptin-, Ob-Rb- and Fra-1 and ERK1/2-like immunofluorescence were identified in carotid body glomus cells using fluorescence

microscopy (Leica Diaplan). Digital images of these cells were obtained with a Nikon DS-Fil camera and NIS Elements Basic Research 3.0 software (Nikon Canada, Mississauga, ON, Canada).

Carotid body protein extracts and immunoblot analysis: Carotid body protein extracts were obtained following bilateral carotid body excision from the 8 h normoxia- or IH-exposed animals. Briefly, carotid bodies (bilaterally) from each animal were snap frozen and pooled and homogenized in 200 μL of RIPA buffer solution (150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 50 mM Tris-HCL at pH of 7.5) with a protease inhibitor cocktail (Roche Diagnostics, Laval, Quebec, Canada). The homogenate was centrifuged at 4 °C for 15 min at 2100 rpm. The supernatant was retained and then centrifuged a second time at 4 °C for 20 min at 13,200 rpm. The resultant supernatant was retained as the protein preparation. Equal concentrations of extracted proteins normalized by colorimetric BCA Protein Assay (Pierce Corp., Madison, Wisconsin, USA) were fractionated in 7.5% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. Blots were probed using polyclonal rabbit anti-leptin (1:1000, Cat. # sc843; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), polyclonal rabbit anti-OB-R (1:1000, Cat. # OBR12-A; Alpha Diagnostics International Inc., San Antonio, Texas, USA), affinity purified anti-OB-Rb (1:1000, Cat. # CH14104; Neuromics, Edina, Minnesota, USA), polyclonal rabbit anti-SOCS3 (1:1000, Cat. # ab16030; Abcam Inc., Cambridge, Massachusetts, USA), monoclonal rabbit anti-STAT3 (1:2000, Cat. # 4904S; Cell Signaling Technology, Danvers, Massachusetts, USA), polyclonal rabbit anti-pSTAT3 (1:1000, Cat. # 9131S; Cell Signaling Technology, Danvers, Massachusetts, USA), and monoclonal horseradish peroxidase-conjugated β-actin (1:50000, catalogue

no. A3854, Sigma-Aldrich, St. Louis, Missouri, USA) diluted in 5% milk-1X Tris-buffered saline-Tween 20 buffer and with horseradish peroxidase conjugated donkey anti-rabbit IgG or donkey anti-chicken IgG (1:10000, catalogue # 711-035-152 or 703-035-155, respectively; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) diluted in 5% milk-1X Tris-buffered saline-Tween 20 buffer as the secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Millipore Canada Ltd, Toronto, Ontario, Canada).

Statistical analysis: Statistical comparisons between plasma leptin levels and Western blots of the IH and normoxia exposed animals were made using a one-tailed, unpaired Student t-test. In all comparisons, a minimum *p*-value of <0.05 was taken to indicate statistical significance (GraphPad Prism; GraphPad Software, San Diego, California, USA).

3.3   Results

Plasma leptin levels during IH: As previously reported (Messenger et al., 2012), IH was found to significantly increase ($p < 0.002$) plasma leptin levels approximately 5.9-fold above those found in the normoxic control animals (Fig. 3.1).
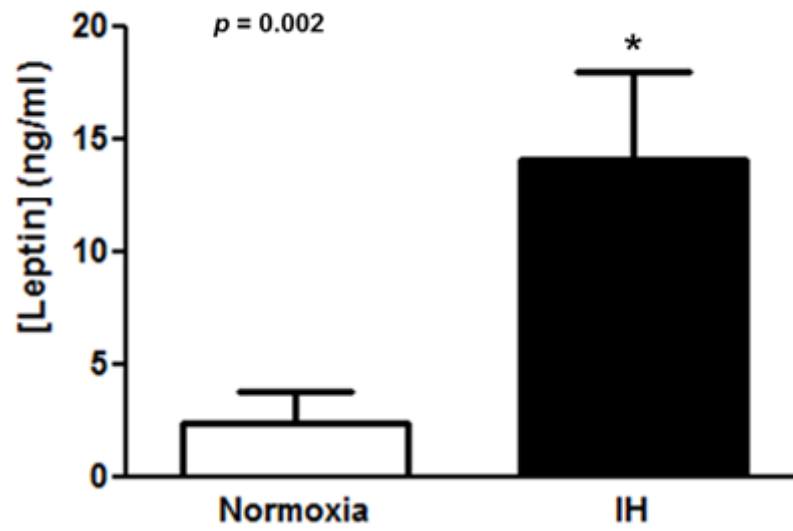
Figure 3.1. Effects of intermittent hypoxia (IH) upon plasma levels of leptin compared to levels observed in normoxic controls. Values are means ± S.E.; *p < 0.002. n=6 for each bar.

Leptin and Fra-1 co-expression in carotid body: Figure 3.2 shows the effect of IH and systemic injections of leptin on leptin expression and the expression of the immediate early gene Fra-1 protein within glomus cells of the carotid body. These cells within the carotid body express little leptin and no Fra-1 immunofluorescence under normoxic conditions (Figs. 3.2e, f and 3.3). However, following exposure to IH, carotid body glomus cells exhibited an increased expression of both leptin (Figs. 3.2 a, c, d and 3.3) and Fra-1 (Fig. 3.2b). Carotid body expression of leptin was increased (~ 60%) ($p <$ 0.001) after the IH (Fig. 3.3).

Intravenous injections of leptin induced an increased expression of leptin within the carotid body (Fig. 3.4a). These leptin immunoreactive cells also expressed Fra-1 immunoreactivity (Fig. 3.4b, c). The number of cells and the intensity of the leptin immunofluorescence appeared greater in those animals receiving the 200-ng injections of leptin compared to those receiving the 50-ng intravenous injections of leptin, although no statistical evaluation was used. In contrast, intravenous injections of the same volume of the vehicle (saline) did not alter leptin or Fra-1 labelling within the carotid body (Fig. 3.4d-f). Additionally, no immunoreactivity for leptin or Fra-1 was found with the carotid body in the immunohistochemical controls for leptin and fra-1 (Fig. 3.4g, h).

Leptin and OB-Rb co-expression in carotid body: As shown in Figure 3.2(g-i), carotid body cells expressing leptin also co-expressed the Ob-Rb. In addition, it was found that IH reduced (~ 10%) ($p < 0.002$) the protein expression of Ob-Rb within the carotid body (Fig 3.5a). On the other hand, the expression of the short-form leptin receptor (Ob-R$_{100}$) protein (Fig. 3.5b) was significantly increased (~ 35%) ($p < 0.03$).
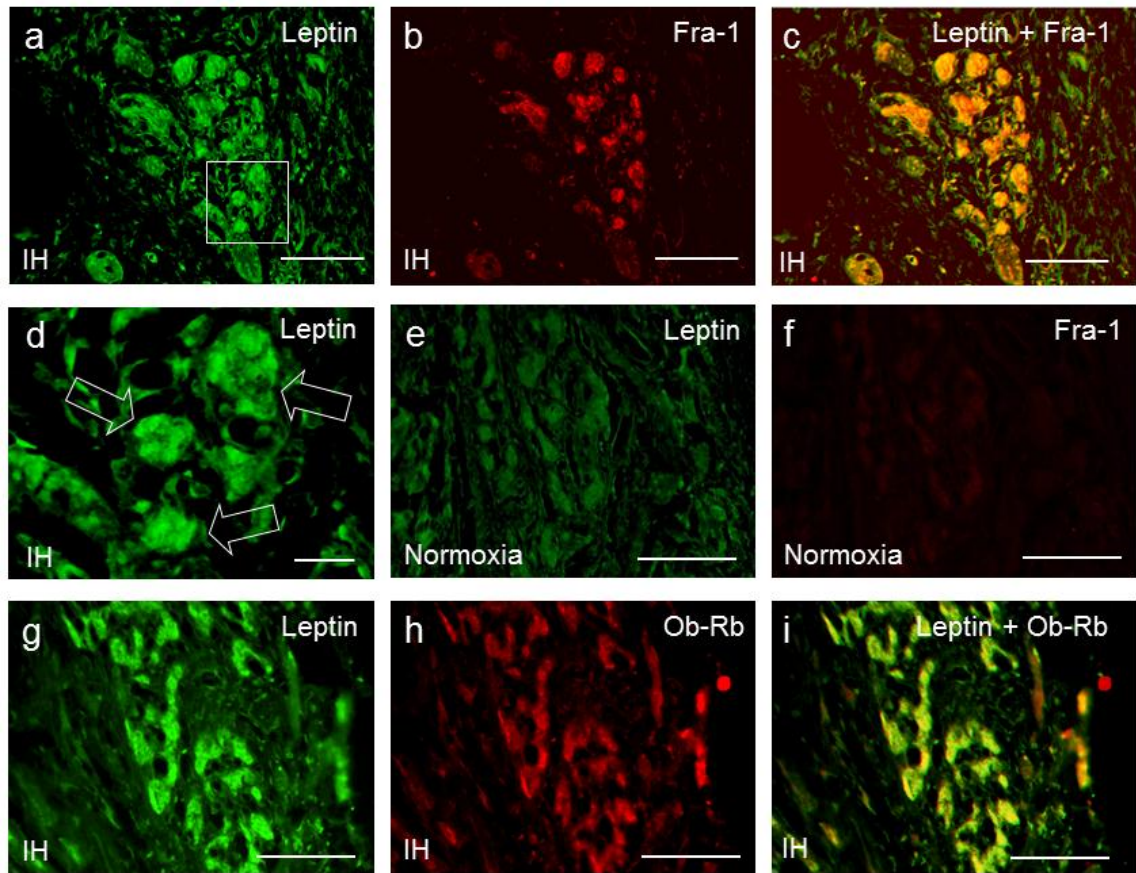
Figure 3.2. Fluorescent photomicrographs showing carotid body cells immunoreactive to leptin (a, c-e, g, i), Fra-1 (b, f) and Ob-Rb (h-i) following exposure to IH (a–d, g-i) or normoxia (e-f). Inset in (a) corresponds to (d) at higher magnification showing leptin immunoreactive glomus cells in the carotid body. Calibration marks indicate 100 μm and applies to (a-c, e-i) and 15 μm in (d).
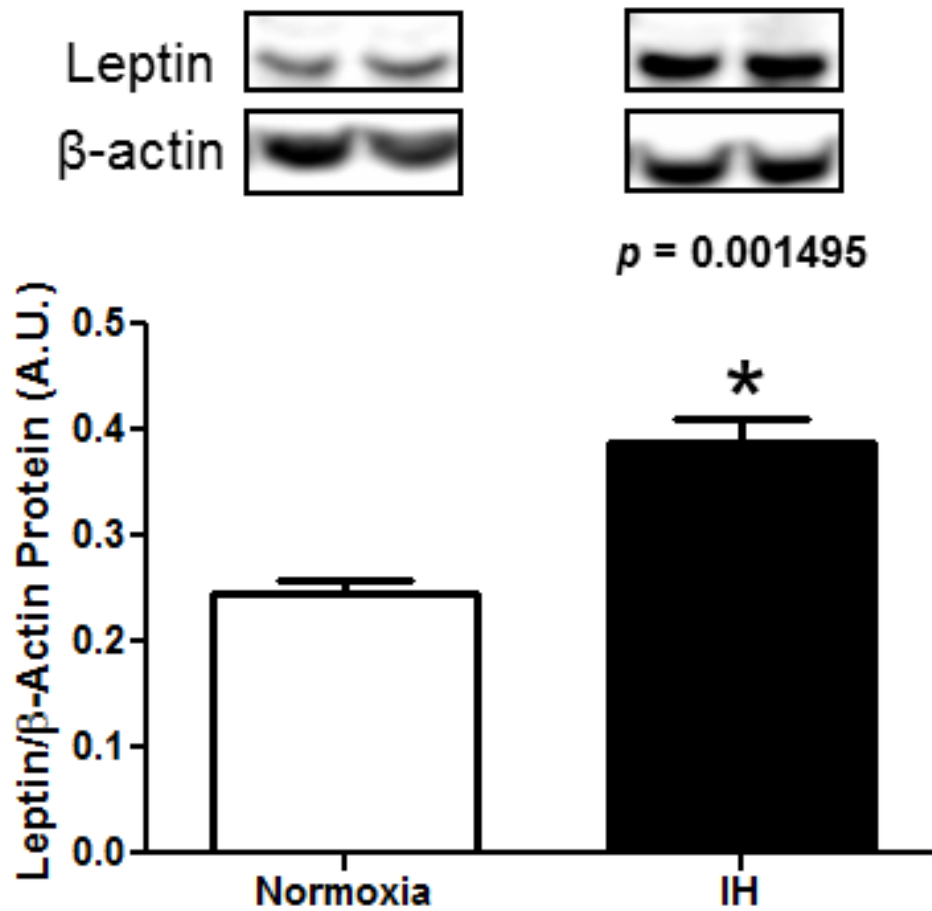
Figure 3.3. Western blots showing the presence of leptin protein within the carotid body.

Note the 1.6-fold increase in leptin protein expression following IH in the carotid body.

*, significantly different from normoxia control animals. *p* value is indicated. n=5-6.
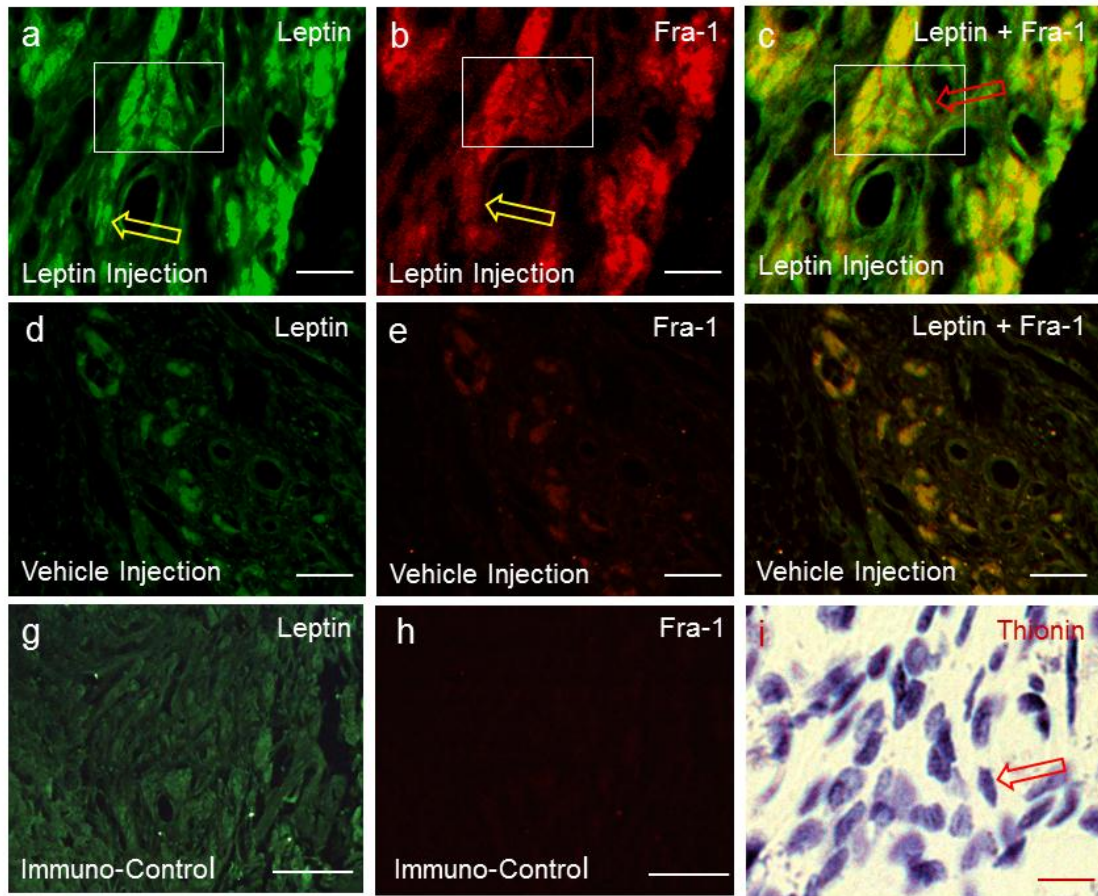
Figure 3.4. Fluorescent (a-h) and bright-field (i) photomicrographs of the carotid bodies. Effect of intravenous leptin injections (200 ng/0.1 mL) on leptin and Fra-1 expression in the glomus cells. Insets in (a-c) correspond to (i) stained with thionin at higher magnification. The red arrows in (c) and (i) point to the same glomus cell within the cluster. The arrows in (a) and (b) point to cell expressing leptin, but not Fra-1 immunoreactivity following injections of leptin intravenously. (d-f) show that intravenous injections of the same volume of vehicle did not induce leptin or Fra-1 expression the carotid body. (g-h) show the lack of immunoreactivity following omission of the primary antibody during the immunohistochemical processing of the tissue. Calibration mark in (a-h) represents 100 μm and calibration mark in (i) represents 15 μm.
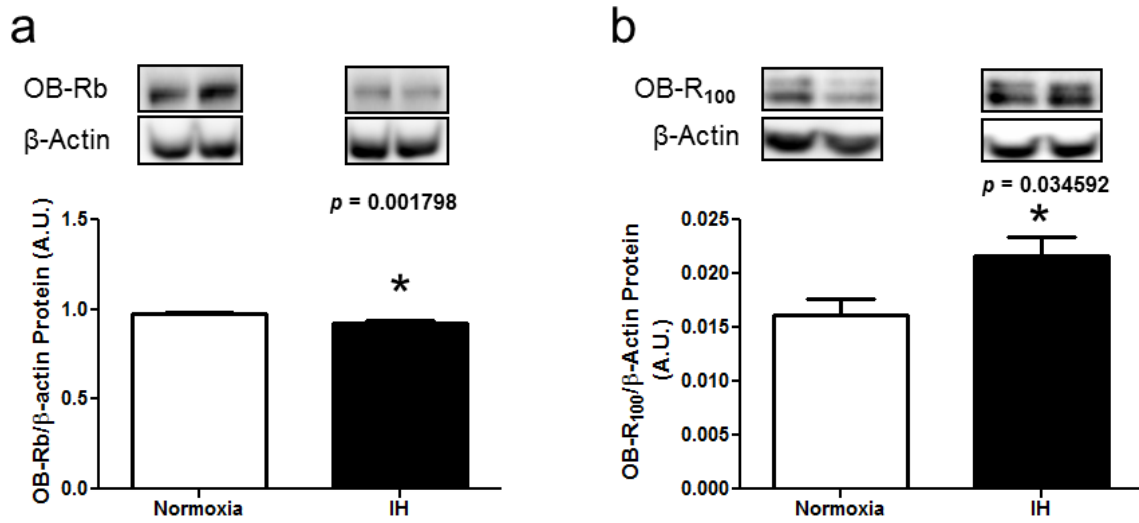
Figure 3.5. Western blots showing the presence of Ob-Rb (a) and Ob-R$_{100}$ (b) in carotid bodies after IH. Note that IH significantly (*) decreases the protein level of Ob-Rb (a), while significantly (*) increasing the protein level of Ob-R$_{100}$ (b) compared to normoxic controls (b). *p* values are indicated. n=5-6.

Effects of IH and leptin injections on ERK1/2 expression in carotid body: Carotid body glomus cells under normoxic conditions were found to express no ERK1/2 (Fig. 3.6). However, when exposed to IH, the expression of ERK1/2 immunoreactivity within the carotid body was increased (Fig. 3.6c). Similarly, intravenous injections of leptin (200 ng) induced ERK1/2 expression within the carotid body (Fig. 3.6d). The increased ERK1/2 in IH exposed animals was co-expressed within carotid body glomus cells that expressed the Ob-Rb (Fig. 3.7).

Effects of IH on STAT3, pSTAT3 and SOCS3 in carotid body: As shown in Figure 3.8, exposure of the animal to IH did not alter STAT3 protein expression within the carotid body (Fig. 3.8a). However, there was a small but significant ($p < 0.002$) increase in the expression of pSTAT3 following IH (Fig. 3.8b). Similarly, IH significantly (~ 10%) ($p < 0.001$) increased the expression of SOCS3 within the carotid body (Fig. 3.8c).
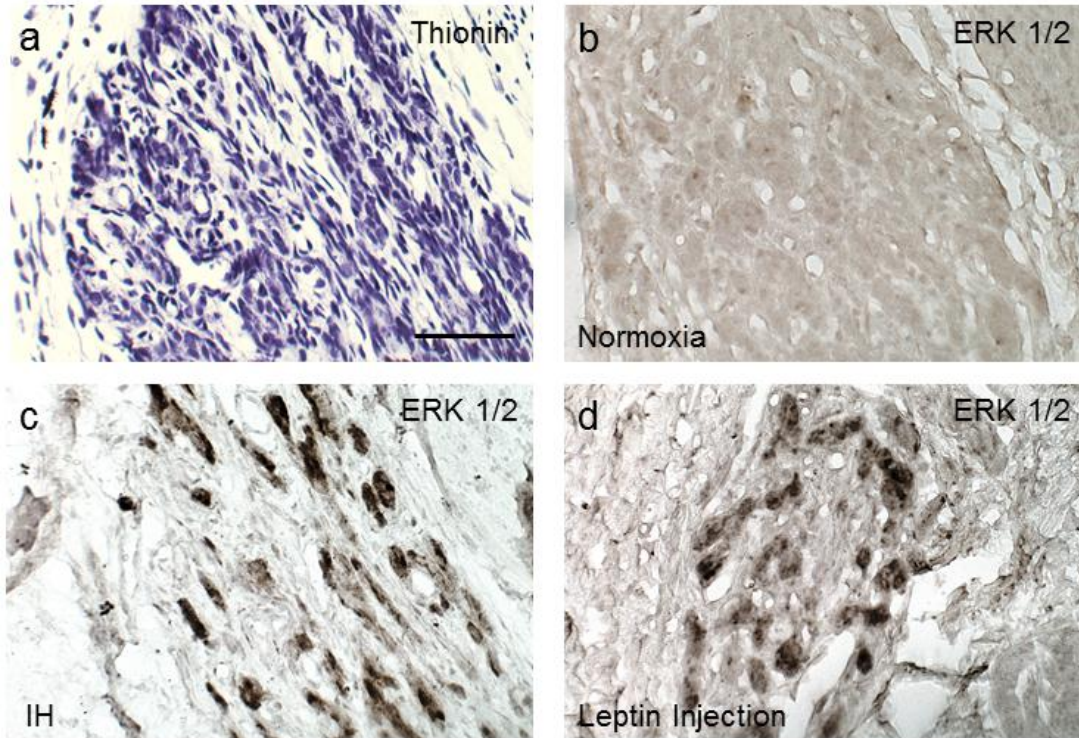
Figure 3.6. Bright-field photomicrographs of carotid bodies stained with thionin (a), or exhibiting ERK1/2 immunoreactivity after exposure to normoxia (b), acute IH (c), and after intravenous injections of leptin (200 ng/0.1 mL; d). Note the lack of ERK 1/2 immunoreactivity in control animals and its increased expression after IH and leptin injections. Calibration marks in panel (a) indicates 100 μm and applies to (a-d).
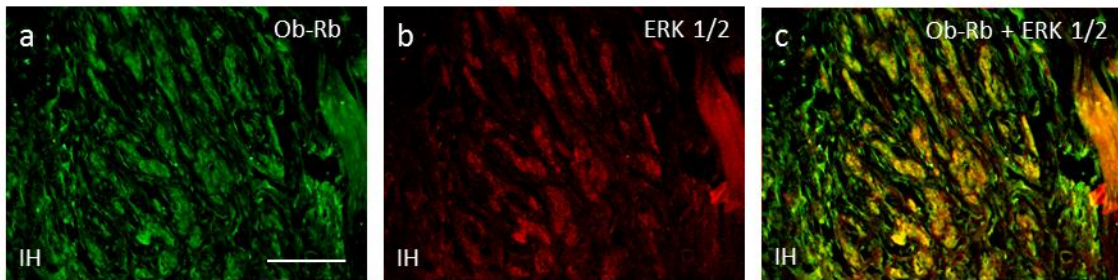
Figure 3.7. Fluorescent (a-c) photomicrographs showing the effect of IH on Ob-Rb (a) and ERK 1/2 (b) expression in carotid body glomus cells. Note that Ob-Rb and ERK 1/2 are co-expressed in the same cells (c). Calibration mark in (a) represents 100 μm and applies to (a-c).
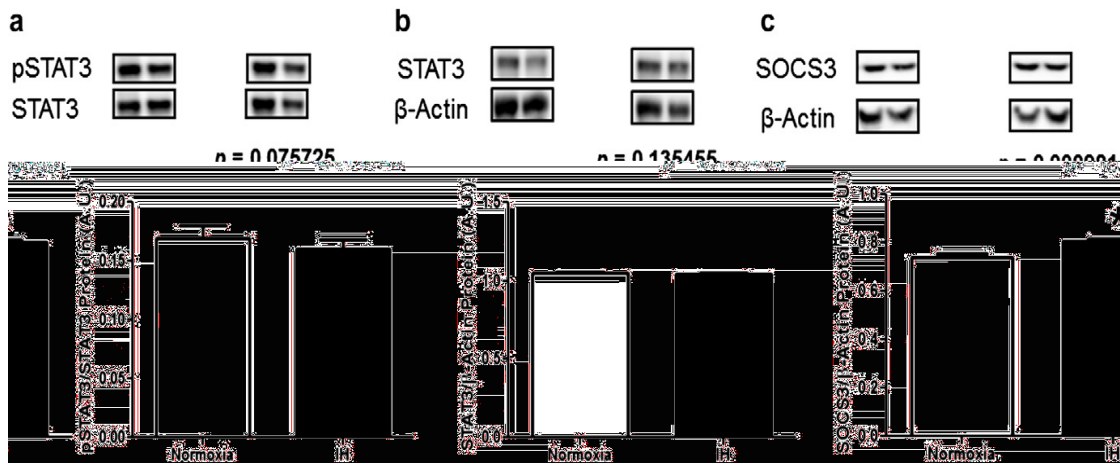
Figure 3.8. Western blots showing the presence of pSTAT3 (a), STAT3 (b), and SOCS3 (c) in carotid bodies after IH. Note that IH does not alter the level of pSTAT3 (a) or STAT3 (b), although there was a clear trend towards a decrease expression of the pSTAT3 protein above that seen in normoxic controls (a). Also note that IH significantly increases (*) the expression of the SOCS3 protein above that seen in normoxic controls. *p* values are indicated. n=5-6.

3.4 Discussion

These data provide the first direct evidence that the activity of cells within the carotid body which express both leptin and the Ob-R's are altered during IH during the time plasma levels of leptin are elevated by IH. These data can be interpreted to suggest that leptin, either through an autocrine/paracrine mechanism or from other sources that contribute to circulatory levels, may alter the function of carotid body cells and possibly modulate chemosensitivity during hypoxic conditions. This suggestion is supported by the finding in this study that intravenous injections of leptin not only induced Fra-1 activity within carotid body cells that expressed leptin and the Ob-Rb, but also increased leptin expression within these cells. Additionally, it was found that IH induced changes in a number of downstream signalling molecules associated with the JAK2/STAT3 signalling pathway following leptin receptor stimulation. It is known that activation of Ob-Rb by leptin results in the phosphorylation of STAT3 which leads to an increase in the expression of the SOCS3 protein. SOCS3 can then act as a negative feedback of the Ob-Rb signalling mechanism (Bjorbaek et al., 1998a, 1999). In this study, SOCS3 levels were found to be increased in carotid bodies following IH exposure, further supporting the suggestion that activation of Ob-Rb occurred as a result of elevated leptin levels. Finally, it was observed that leptin injections and IH increased the expression of ERK1/2 also suggesting that activation of Ob-Rb may also use an alternate signalling pathway within these cells to alter transcription. However, the source of the leptin exerting an effect on the carotid body cells remains unknown.

The finding that leptin is expressed within the carotid body is supported by an earlier study showing that the glomus cells of the carotid body expressed leptin

(Porzionato et al., 2011). However, this study has also shown that IH increases the expression of leptin within these cells, a finding also observed following systemic injections of leptin. Furthermore, these leptin producing carotid body cells expressed the immediate early gene product Fra-1 in responses to IH and the intravenous injection of leptin. Taken together, these findings can be interpreted to suggest that carotid body glomus cells, when activated by leptin during IH, further increase their production of leptin so that leptin can either exert an effect on the cell itself or on neighbouring cells to alter their ability to transduce changes in arterial PO_2. The co-expression of Ob-Rb within these carotid body leptin producing cell types further supports a potential autocrine/paracrine mechanism of action that is well documented in various tissues including pituitary, adipose tissue and the gastrointestinal tract (Siegrist-Kaiser et al., 1997; Fruhbeck et al., 1997; Jin et al., 2000; Bado et al., 1998; Mix et al., 2000; Sobhani et al., 2000). The finding that carotid body glomus cells express the Ob-Rb is consistent with previous studies (Messenger et al., 2012; Porzionato et al., 2011). Thus, these combined observations suggest the possibility that the release of leptin by carotid body cells during IH may contribute to the elevated plasma leptin levels observed after IH, although it is unlikely to account for the total rise in the plasma leptin levels, and that leptin may then exert an effect back onto the carotid body cells.

Interestingly, it was observed that IH induced change in the expression of both the long form (Ob-Rb) and the short form (Ob-R$_{100}$) of the leptin receptor within the carotid body. No other isoforms of Ob-R's were detected in these studies. As expected with an elevated plasma level of leptin, the Ob-Rb was found to be down-regulated (Mercer et al., 1997; Bennett et al., 1998; Uotani et al., 1999; Mitchel et al., 2009). The Ob-Rb is

generally accepted as the main signalling form of the Ob-R isoforms and a decrease in the levels of this receptor within glomus cells would then be expected to reduce the sensitivity of the cells to leptin. The exact mechanism by which the decrease in carotid body Ob-Rb expression occurs is unknown. However, one possible explanation may be that the increased exposure to leptin, both local and circulating, stimulates ligand-induced endocytosis (Bennett et al., 1998; Uotani et al., 1999). In animals lacking circulating leptin, such as the *ob/ob* mouse, or lacking Ob-Rb signalling due to mutations in the Ob-Rb gene, such as in the Zucker obese rat, Ob-Rb expression is significantly up-regulated (Mercer et al., 1997; Bennett et al., 1998) most likely due to lack of Ob-Rb signalling. Additionally, during fasting, where leptin levels drop, Ob-Rb gene expression is up-regulated (Baskin et al., 1998; Bennett et al., 1998). However in the case of increased leptin exposure, as seen in the IH model used (Messenger et al., 2012), previous studies have supported a down-regulation of Ob-Rb (Bennett et al., 1998; Uotani et al., 1999).

It was also found in this study that the short form of the leptin receptor (Ob-R$_{100}$), which likely represents the Ob-Ra isoform of the receptor, was up-regulated after the IH stimulus. Although the exact functions of the Ob-Ra isoform are not clear, it is generally believed that the OB-Ra may function in leptin's transport across the blood-brain barrier (Golden et al., 1997; Bjorbaek et al., 1998). In addition, it has been suggested that OB-Ra may function in leptin's degradation (Iida et al., 1996; Merabet et al., 1997; Sharma et al., 1997). Thus increased Ob-Ra levels may aid in mediating leptin transport from the circulation into the carotid body, as well as the degradation of the over-expressed cytokine within the carotid body cells after exposure to IH. Finally, it has been documented that leptin signalling is not exclusively mediated via the long-form receptor

but may also occur via the MAPK pathway through activation of both OB-Rb and OB-Ra isoforms (Bjorbaek et al., 1997) both of which are present in glomus cells.

Transcription within cells following leptin activation of the Ob-Rb is not mediated by only the JAK/STAT signalling pathway (Kloek et al., 2002). Activation of JAK2 also leads to activation of ERK1/2 (Kloek et al., 2002). The activation of ERK1/2 by leptin has previously been shown in the hypothalamus where it is known to mediate food intake, body weight and thermogenic sympathetic outflow (Rahmouni et al., 2009). Although we have shown that leptin activates the JAK2/STAT3 pathway in carotid body cells, a finding consistent with an earlier study (Messenger et al., 2012), this study has also shown the potential for an additional signalling pathway within carotid bodies by identifying a downstream regulator of MAPK signalling, pERK1/2 in animals exposed to IH. As increased pERK1/2 was also observed in carotid body cells following systemic leptin injections suggests multiple signalling pathways for leptin signalling in the carotid body.

It was found that IH did not alter STAT3 or pSTAT3 protein levels within the carotid body. This suggests that STAT3 activation in the carotid bodies of animals exposed to IH was the resulted of the activation of the Ob-Rb. The subsequent reduction in levels of the Ob-Rb after IH was likely due the increased exposure of the receptor to leptin which is known to induce a down-regulation of Ob-Rb (Uotani et al., 1999; Bennett et al., 1998). The trend towards decreased levels of pSTAT3 also suggest that SOCS3 would eventually rise and thus inhibit further phosphorylation of STAT3 by exerting an effect on JAK2 (Bjorbaek et al., 2000), which then may account for the relatively small change in pSTAT3 expression observed after IH. It was found in this

study that SOCS3 was elevated in the IH exposed animals. Thus activation of SOCS3 would essentially shut down Ob-Rb signalling through the activation of JAK2/STAT3 pathway. In *ob/ob* mice, peripheral administration of leptin induces SOCS3 mRNA (Bjorbaek et al., 1998a). The same result has been shown in *Ay/α* mice, a rodent model of leptin-resistant obesity (Bjorbaek et al., 1998a). In conditions of chronically increased leptin signalling, such as obesity (Ahima and Flier, 2000; Friedman and Halaas, 1998), high SOCS3 levels are found within various regions of the nervous system, including the nodose ganglion and the hypothalamus (Reed et al., 2010; de Lartigue et al., 2011).

In summary, this study has provided evidence for the co-expression of leptin, Ob-Rb and Fra-1 within carotid body cells, and that during IH these proteins are altered, as is the plasma levels of leptin. Additionally, IH activates a series of down-stream signalling pathways following the activation of the Ob-Rb. Taken together, these data suggest leptin produced locally by the glomus cells exerts an excitatory effect in an autocrine/paracrine fashion on glomus cells and possibly directly on carotid body afferent nerve fibers which have been also shown to express the Ob-Rb (Messenger et al., 2012). Leptin has been implicated in respiratory control (Tankersley et al., 1998; O'Donnell et al., 2000; Inyushkin et al., 2009) and cardiovascular control (Arnold, et al., 2009; Mark et al., 2009; Ciriello and Moreau, 2012) involving carotid chemoreceptors. In *ob/ob* mice, leptin replacement restored their rapid breathing pattern (Tankersley et al., 1998). Additionally, leptin microinjection into the NTS, the site of primary chemoreflex afferent termination, results in increased pulmonary ventilation, respiratory volume and electrical activity to inspiratory muscles (Inyushkin et al., 2009), increases in sympathetic activity and a potentiation of the chemoreceptor reflex (Ciriello and Moreau, 2012). Thus, these

data suggest the possibility that leptin exerts modulatory effects at multiple levels throughout the chemoreceptor reflex pathway.

3.5 References

This article was published in Neuroscience, 232, Messenger, SA, Ciriello, J, Effects of intermittent hypoxia on leptin signalling in the carotid body, 216-225, Copyright Elsevier (2013).

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Chapter 4

4 Effect of Chronic Intermittent Hypoxia on Leptin and Leptin Receptor Protein Expression in the Carotid Body

4.1 Introduction

Obstructive sleep apnea (OSA), characterized by brief, repeated upper airway collapse during the patients sleep cycle (Dempsey et al., 2010; Gonzalez et al., 2012), is a pathophysiological disorder observed in both normal weight (Pamidi et al., 2012) and obese individuals (Lubrano et al., 2012). The repetitive cessation of night time breathing exposes these individuals to chronic intermittent hypoxia (CIH), a repeated depression of arterial partial pressure of oxygen (PO_2) (Dempsey et al., 2010; Gonzalez et al., 2012). Furthermore, if left untreated, OSA in these individuals increases the risk of developing cardiovascular and metabolic disorders (Dempsey et al., 2010; Marin et al., 2005; Shamsuzzaman et al., 2003). An animal model has been developed to study the effects of CIH resulting from OSA (Fletcher et al., 1992), in which both arterial pressure and sympathetic nerve activity have been shown to be increased during exposure to CIH, and these autonomic effects are dependent on the integrity of the carotid body chemoreceptor reflex (Fletcher, 2000).

The carotid bodies, located bilaterally at the bifurcation of the carotid arteries, have been shown to contain oxygen-sensing type-I (glomus) cells (McDonald, 1981). The glomus cells within the carotid body transduce the PO_2 within arterial blood and transmit this information via the carotid sinus nerve to the central nervous system (Heymans et al., 1930; Nurse, 2005), which in turn activates multiple homeostatic mechanisms including those involving the respiratory and cardiovascular systems (Kumar, 2009; Schultz and Li,

2007). Although the mechanisms by which PO_2 in the glomus cells signals carotid body afferent neurons remain equivocal, a number of signaling molecules have been implicated including acetylcholine, dopamine, norepinephrine, histamine, adenosine and adenosine triphosphate (Bock, 1980; Conde and Monteiro, 2004; Eyzaguirre and Zapata, 1968; Gomez-Nino et al., 1990; McQueen and Ribeiro, 1981; Nurse, 2005; Nurse, 2010; Shirahata et al., 2007; Spyer et al., 2004).

Recently, leptin and isoforms of its receptor have been identified within carotid body glomus cells (Messenger and Ciriello, 2013; Messenger et al., 2012; Porzionato et al., 2011). Additionally, 8 hours of intermittent hypoxia exposure activates glomus cells that expressed the long form of the leptin receptor (OB-Rb) (Messenger et al., 2012), and increases the levels of leptin, the short form of the leptin receptor (OB-R_{100}), and downstream mediators of leptin signaling including, extracellular signal-regulated kinase 1/2 (ERK 1/2) and suppressor of cytokine signaling 3 (SOCS3) proteins, while decreasing phosphorylated signal transducer and activator of transcription 3 (pSTAT3), and OB-Rb protein expression within these cells (Messenger and Ciriello, 2013). Leptin, the 16 kDa hormonal product of the *obese* gene, produced primarily by adipocytes in proportion to adiposity (Caro et al., 1996; Lonnquist et al., 1995), normally plays an important role in metabolism and energy balance (Bjørbaek and Kahn, 2004; O'Donnell et al., 2000). This weight-reducing class-I cytokine is thought to signal primarily through the OB-Rb, one of the six isoforms identified for the leptin receptor (OB-Ra-f) (Lee et al., 1996; Wang et al., 1996). In addition, leptin signaling has been suggested to play an important role in cardiovascular regulation. Central administration of leptin increases sympathetic nerve activity, arterial pressure, and heart rate (Dubinion et al., 2011;

Rahmouni et al., 2003; Shirasaka et al., 2003). Furthermore, central injections of leptin potentiate the chemoreceptor reflex (Ciriello and Moreau, 2012) while decreasing the sensitivity of the vagal component of the baroreceptor reflex (Arnold et al., 2009).

Although 8 hours of intermittent hypoxia, as well as systemic administration of leptin, alters leptin and downstream leptin signaling proteins within the carotid body (Messenger et al., 2012; Messenger and Ciriello, 2013), the long term effects of CIH on leptin and its receptors and downstream signaling molecules within the carotid body are not known. This study was done to determine whether leptin and leptin signaling proteins in the carotid bodies were altered in response to short term (7 days) or long term (95 days) exposure to CIH by examining whether: 1) CIH alters leptin and OB-R expression; 2) CIH alters the protein expression of signal transducer and activator of transcription 3 (STAT3) and pSTAT3; 3) CIH alters the expression of suppressor of cytokine signaling 3 (SOCS3), which binds to JAK2 and thus inhibits leptin-induced signaling through the STAT3 mechanism (Bjørbaek et al., 2000; Fruhbeck, 2006); 4) CIH alters the expression of ERK1/2 and pERK1/2. OB-Rb, through the activation of the JAK2/STAT3 pathway, is implicated in transducing most of leptin's signaling effects within the central nervous system (Elmquist et al., 1997; Huo et al., 2006; Huo et al., 2007).

4.2   Methods

General Animal Procedures: Experiments were done in male Sprague–Dawley rats (Charles River Canada, St. Constant, Canada) weighing 211-400 g. All animals were housed under controlled conditions with a 12 h light/dark cycle. Food and water were available *ad libitum*. All experimental procedures were done in accordance with the

guidelines on the use and care of laboratory animals as set by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Western Ontario.

Induction of CIH: CIH (n=7 for 7 days; n=4 for 95 days) or normoxia (n=5 for 7 days; n=4 for 95 days) was induced in animals as previously described (Messenger et al., 2012; Messenger and Ciriello, 2012). In brief, animals (one/tube) were placed into custom made cylindrical plexiglass tubes with a zero-pressure escape valve (11 cm diameter x 33 cm length) inside an airtight plexiglass chamber. The animal was allowed to freely move within each tube. The chamber was attached to computer-controlled solenoid valves responsible for regulating the inflow of either pressurized room air (21% O_2) or 100% medical N_2. The solenoid valves were connected to a computerized timer system that allowed for the control of a total hypoxic (100% medical N_2) stimulus delivery of 80 s followed by a normoxic (21% O_2) time of 100 s, for a total continuous repetitive cycle period of 8 h. Additionally, the controller, attached to detectors for O_2 and CO_2 within the chamber, was set at a O_2 level of 6.5 % and CO_2 level of 0.1% under isobaric conditions at all times during the delivery of the hypoxic stimulus and recovery phase during which O_2 returned to 20.9%. A control (normoxic) set of animals was simultaneously run in a similar adjacent chamber through which only room air (21% O_2; normoxia) was used for the gas exchange in isobaric conditions for an 8 h period. Animals were exposed to the 8 h IH stimuli each day of the experimental period during daylight hours.

Carotid Body Protein Extracts and Immunoblot Analysis: Carotid body protein extracts were obtained following bilateral carotid body excision from the normoxia- or CIH-exposed animals as described previously (Messenger and Ciriello, 2012). In brief, the

carotid bodies (bilaterally) from each animal were snap frozen and pooled and homogenized in 200µL of RIPA buffer solution (150 mM NaCl, 1 mM NaF, 1 mM NaVO$_4$, 0.5 mM β-glycerophosphate, 1mM EDTA, 1% Triton-X 100, 50mM Tris-HCL at pH of 7.5) with a protease inhibitor cocktail (Roche Diagnostics, Laval, Quebec, Canada). The homogenate was centrifuged at 4°C for 15 min at 2100 RPM. The supernatant was retained and then centrifuged a second time at 4°C for 20 min at 13200 RPM. The resultant supernatant was retained as the protein preparation. Equal concentrations of extracted proteins normalized by colorimetric BCA Protein Assay (Pierce Corp., Madison, Wisconsin, USA) were fractionated in 10% polyacrylamide gel (Novex, Carlsbad, California, USA) and transferred onto a polyvinylidene fluoride membrane. Blots were probed using polyclonal rabbit anti-leptin (1:1000, Cat. # sc843; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), polyclonal rabbit anti-OB-R (1:1000, Cat. # OBR12-A; Alpha Diagnostics International Inc., San Antonio, Texas, USA), affinity purified anti-OB-Rb (1:1000, Cat. # CH14104; Neuromics, Edina, Minnesota, USA), polyclonal rabbit anti-SOCS3 (1:1000, Cat. # ab16030; Abcam Inc., Cambridge, Massachusetts, USA), monoclonal rabbit anti-STAT3 (1:2000, Cat. # 4904S; Cell Signaling Technology, Danvers, Massachusetts, USA), polyclonal rabbit anti-pSTAT3 (1:1000, Cat. #9131S; Cell Signaling Technology, Danvers, Massachusetts, USA), monoclonal rabbit anti-ERK1/2 (1:1000, Cat. # 4695S; Cell Signaling Technology, Danvers, Massachusetts, USA), monoclonal rabbit anti-pERK1/2 (1:1000, Cat. # 4376S; Cell Signaling Technology; Danvers, Massachusetts, USA), and monoclonal horseradish peroxidase-conjugated β-actin (1:50000, catalog no. A3854, Sigma-Aldrich, St. Louis, Missouri, USA) diluted in 5% milk-1X Tris-buffered saline-

Tween 20 buffer and with horseradish peroxidase conjugated donkey anti-rabbit IgG or donkey anti-chicken IgG (1:10000, catalog # 711- 035-152 or 703-035-155, respectively; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) diluted in 5% milk-1X Tris-buffered saline-Tween 20 buffer as the secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Millipore Canada Ltd, Toronto, Ontario, Canada).

Statistical Analysis: Statistical comparisons between Western blots of the normoxia and CIH exposed animals at each time point, and those between 7 and the 95 day time point were made using a two-tailed, unpaired Student t-test. In all comparisons, a minimum *p*-value of < 0.05 was taken to indicate statistical significance (GraphPad Prism; GraphPad Software, San Diego, California, USA).

4.3   Results

Effects of 7 and 95 day CIH on Leptin, OB-Rb and OB-R$_{100}$ Protein Expression within the Carotid Body: Figure 4.1a shows that the protein expression of leptin within the carotid body of animals exposed to CIH for 7 days was significantly ($p = 0.040$) lower by approximately 20%, compared to normoxic controls. Additionally, the protein expression of OB-Rb was significantly ($p = 0.042$) increased by about 92% (Fig. 4.2a), whereas that for OB-R$_{100}$ was not changed (Fig. 4.3a) compared to normoxic controls. After 95 day exposure to CIH, carotid body leptin protein levels were significantly ($p = 0.027$) lower by about 45% compared to normoxic controls (Fig. 4.1b). Furthermore, the expression of OB-Rb was significantly ($p=0.040$) increased by about 124% (Fig. 4.2b), whereas that for OB-R$_{100}$ was not changed (Fig. 4.3b) compared to normoxic controls at the 95 day period.

Figure 4.1. Western blots showing protein expression of leptin in carotid bodies after 7 day (a) and 95 (b) days of exposure to normoxia or CIH (IH). Note that CIH significantly (*) decreases the level of leptin below that seen in normoxic controls at both time points. *p* values are indicated. n=4-7.

Figure 4.2. Western blots showing protein expression of OB-Rb in the carotid bodies after 7 day (a) and 95 (b) days of exposure to normoxia or CIH (IH). Note that CIH significantly (*) increases the level of OB-Rb protein above that seen in normoxic controls at both time points. *p* values are indicated. n=4-7.

Figure 4.3. Western blots showing protein expression of OB-R$_{100}$ in the carotid bodies after 7 day (a) and 95 (b) days of exposure to normoxia or CIH (IH). Note that CIH does not significantly alter the expression of the Ob-R$_{100}$ protein above that seen in normoxic controls. *p* values are indicated. n=4-7.

Figure 4.4. Western blots showing protein expression of STAT3 in the carotid bodies after 7 day (a) and 95 (b) days of exposure to normoxia or CIH (IH). Note that CIH does not alter the expression of the STAT3 protein above that seen in normoxic controls at 7 days, but significantly increases it at 95 days compared to normoxic controls . p values are indicated. n=4-7.

Effects of 7 and 95 day CIH on STAT3, pSTAT3, SOCS3, ERK1/2, and pERK 1/2

Protein Expression within the Carotid Body: As shown in Figure 4.4, exposure of the animal to 7 (Fig. 4.4a) days of CIH did not significantly alter STAT3 protein levels within the carotid body. However, after 95 days of CIH exposure, STAT3 protein levels were significantly ($p = 0.050$) higher ($\sim 40\%$) compared to normoxic controls (Fig. 4.4b). On the other hand, only within the 7 day group was there a significant ($p = 0.024$) decrease by about 66% in pSTAT3 (Fig. 4.5a) compared to normoxic animals, while there were no changes in pSTAT3 observed within the 95 day group between normoxic and CIH groups (Fig. 4.5b).

Protein expression levels of SOCS3 were significantly ($p = 0.034$) higher by 111% in the 7 day group compared to normoxic controls (Fig. 4.6a). On the other hand, there were no changes in SOCS3 protein after exposure to 95 days of CIH compared to normoxic animals (Fig. 4.6b).

Figure 7 summarizes the effects of CIH on ERK 1/2 protein expression within the carotid body. After 7 days of CIH exposure, ERK 1/2 protein levels were decreased ($p=0.033$; by about 43% of normoxic controls) (Fig. 4.7a), whereas at the 95 day period, the protein levels of ERK 1/2 were significantly increased ($p=0.010$) by about 114% compared to normoxic controls (Fig. 4.7b). pERK 1/2 levels followed a similar pattern to that observed for ERK 1/2, but were only significant ($p=0.008$) at the 95 day period (Fig. 4.8b) where protein levels of pERK 1/2 increased by about 252%.
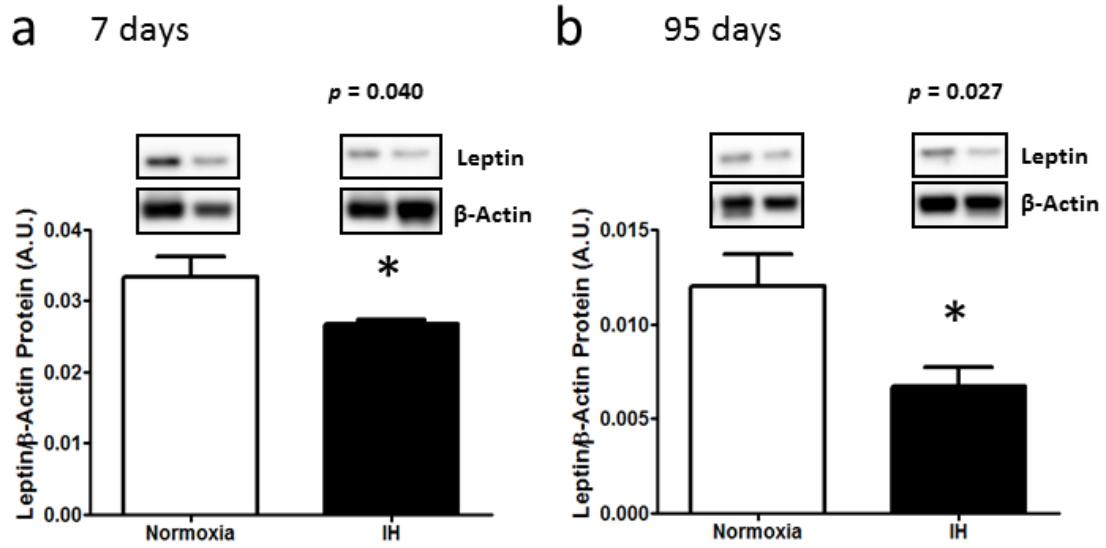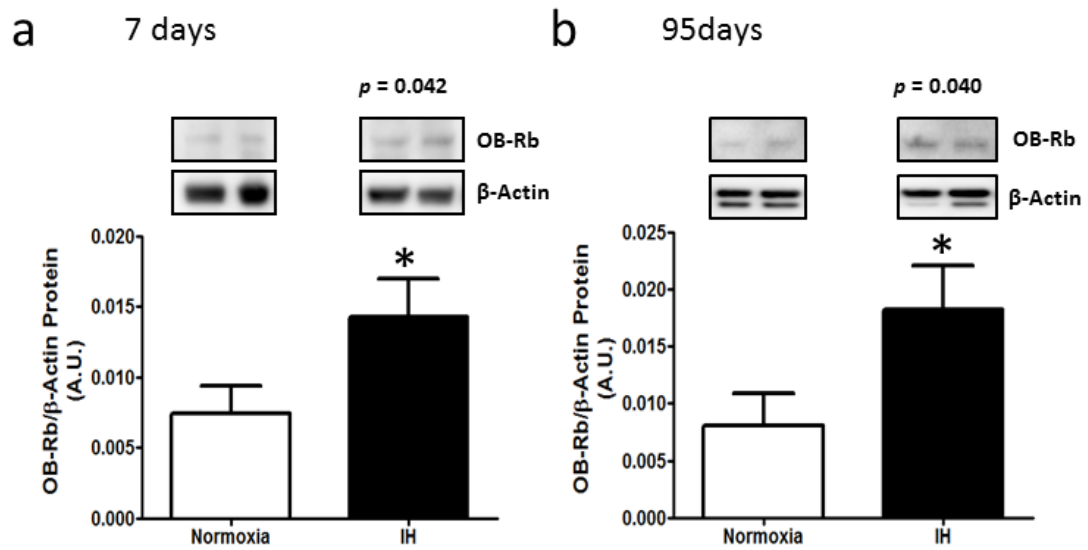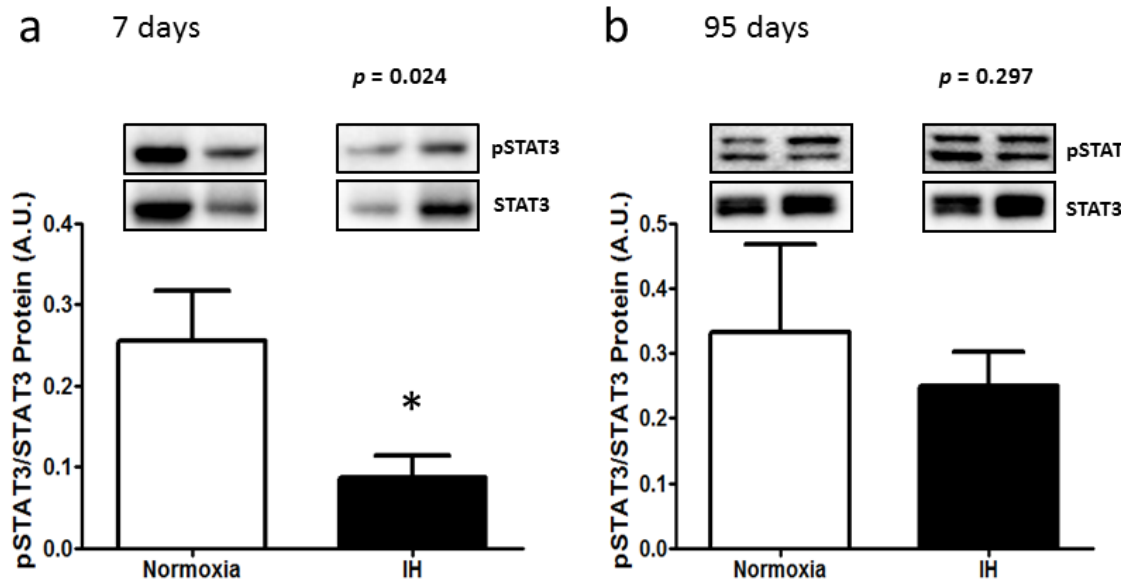
Figure 4.5. Western blots showing protein expression of pSTAT3 in the carotid bodies after 7 day (a) and 95 (b) days of exposure to normoxia or CIH (IH). Note that CIH decreases the expression of the STAT3 protein below that seen in normoxic controls at 7 days, but does not alter it after 95 days compared to normoxic controls. *p* values are indicated. n=4-7.
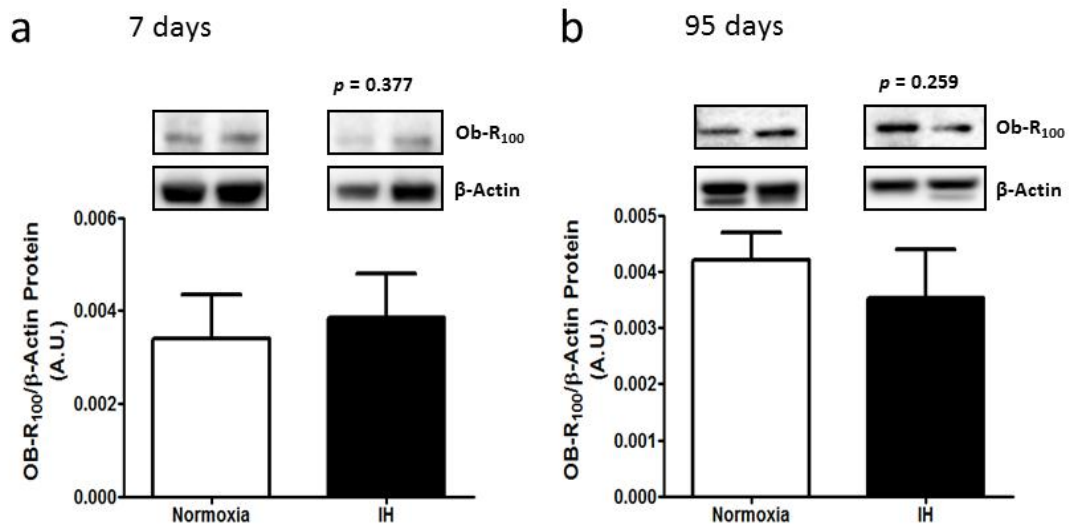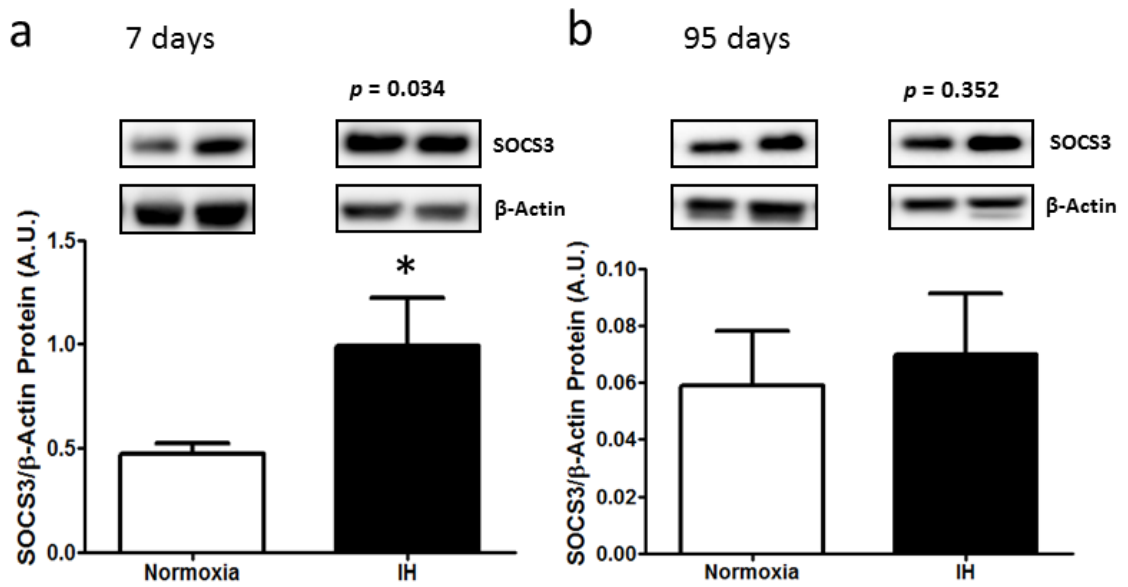
Figure 4.6. Western blots showing protein expression of SOCS3 in the carotid bodies after 7 day (a) and 95 (b) days of exposure to normoxia or CIH (IH). Note that CIH increases the expression of the SOCS3 protein above that seen in normoxic controls at 7 days, but does not alter it after 95 days compared to normoxic controls. *p* values are indicated. n=4-7.
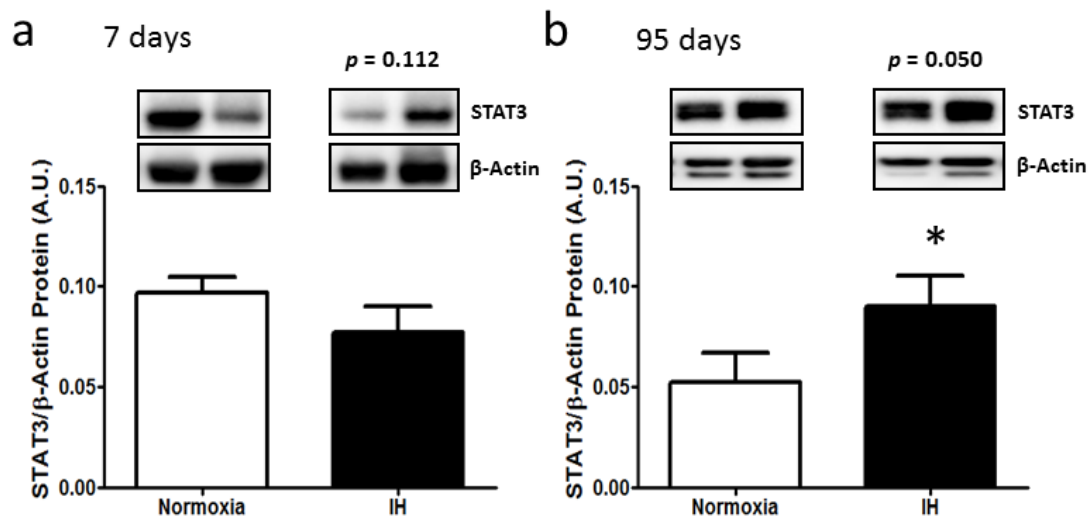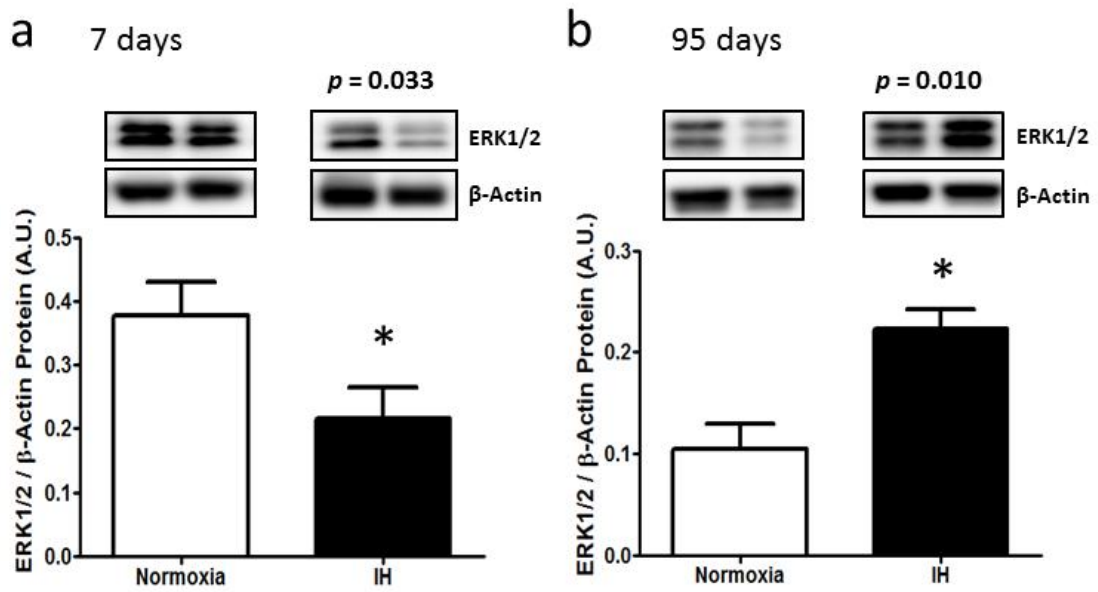
Figure 4.7. Western blots showing protein expression of ERK1/2 in the carotid bodies after 7 day (a) and 95 (b) days of exposure to normoxia or CIH (IH). Note that CIH decreases the expression of the ERK1/2 protein below that seen in normoxic controls at 7 days, while increasing it above that seen in normoxic controls at 95 days. p values are indicated. n=4-7.
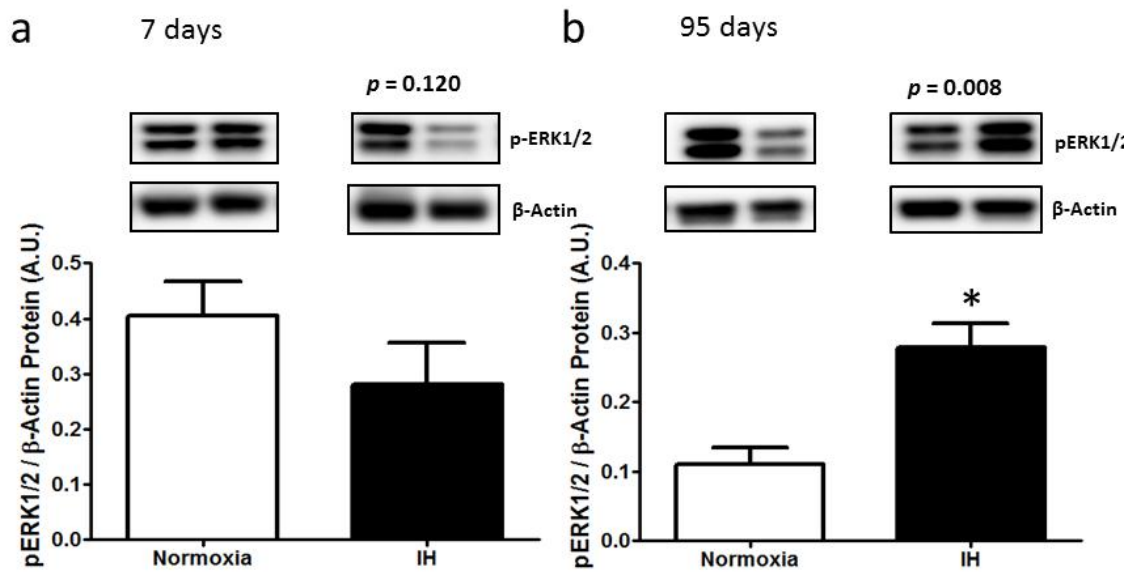
Figure 4.8. Western blots showing protein expression of pERK1/2 in the carotid bodies after 7 day (a) and 95 (b) days of exposure to normoxia or CIH (IH). Note that CIH does not alter pERK1/2 after 7 days compared to normoxic controls, but increases the expression of the pERK1/2 protein above that seen in normoxic controls at 95 days. *p* values are indicated. n=4-7.

Effects of Normoxic and CIH Exposure Time on Carotid Body Leptin, OB-Rb, OB-R_{100}, pSTAT3/STAT3 ratio, STAT3, ERK1/2, pERK 1/2 and SOCS3 Protein Levels:
Table 1 summarizes comparisons between the effects of normoxia or CIH on protein expression within the carotid body between the 7 and 95 day exposure groups. Leptin protein levels were found to be significantly decreased in both the normoxic controls and the CIH group at the 95 day period compared to the 7 day period. On the other hand, no changes were found between the two time periods in either the normoxic or CIH group for OB-Rb or OB-R_{100}. Similarly, no changes were observed in either STAT3 or pSTAT3 protein levels in the normoxic group between the two different time points. However, in the CIH group, STAT3 levels were lower by about 51% in the 95 day group compared to the 7 day group, while pSTAT3 levels were increased by about 58% in the 95 day group compared to the 7 day group. SOCS3 levels were not different between to the two points in the normoxic animals, but in animals exposed to CIH SOCS3 levels were significantly reduced by about 66% in the 95 day group compared to the 7 day group. Finally, only in the normoxic controls were the levels of ERK 1/2 reduced (by about 73%) at the 95 day time period compared to the 7 day time period.

Table 1.

Effects of normoxia and CIH exposure time on the protein expression within the carotid body of leptin, OB-Rb, OB-R$_{100}$, pSTAT3/STAT3 ratio, STAT3, ERK1/2, pERK 1/2 and SOCS3 protein levels.

Stimulus	Normoxia	CIH
	p value	*p* value
Protein		
Leptin	↓ 0.0006*	↓ 0.050*
OB-Rb	0.496	0.163
OB-R$_{100}$	0.355	0.111
STAT3	0.385	↓ 0.016*
pSTAT3	0.289	↑ 0.0004*
SOCS3	0.442	↓ 0.050*
ERK1/2	↓ 0.004*	0.455
pERK1/2	0.242	0.266

p values are from statistical comparisons between 7 day and 95 day (↑, increase; ↓, decrease) exposure times for the normoxic control group or the CIH group. n=4-7. *, statistical significance.

4.4 Discussion

This study has provided evidence indicating that CIH exerts an effect on leptin and leptin receptors, and on downstream signaling proteins associated with leptin receptor activation within the carotid body. This is based on the finding that carotid body cells not only express leptin and the leptin receptors OB-Rb and OB-R_{100}, but also that these receptors and leptin protein levels were altered after exposing them to short term (7 days) and long term (95 days) CIH. Additionally, changes were induced by CIH in a number of downstream signaling molecules associated with the leptin JAK2/STAT3 signaling pathway following activation of the OB-Rb (Fruhbeck, 2006). It is well documented that OB-Rb activation by leptin results in the phosphorylation and activation of STAT3 and ERK 1/2. pSTAT3 leads to an increase in the expression of the SOCS3 protein, which can then act as a negative feedback regulator of the OB-Rb signaling mechanism (Bjørbaek et al., 1999; Bjørbaek et al., 1998a; Fruhbeck, 2006).The increased phosphorylation of ERK 1/2 suggests that activation of OB-Rb may also use an alternate signaling pathway within these cells to alter cellular responses in addition to pSTAT3 (Fruhbeck, 2006). Taken together, these data can be interpreted to suggest that leptin, either through an autocrine/paracrine mechanism or from other sources that contribute to circulatory levels, may alter the function of carotid body cells and possibly modulate chemosensitivity during CIH conditions.

This study has shown that leptin is expressed within the carotid body and that it is decreased after exposure to CIH for either 7 days or 95 days compared to normoxic controls. This finding is supported by an earlier study showing that the glomus cells of the carotid body not only expressed leptin (Messenger and Ciriello, 2013; Porzionato et

al., 2011), but also that the carotid bodies contain mRNA for leptin (Porzionato et al., 2011), suggesting that leptin is likely produced within these cells. The decreased leptin protein expression observed after CIH is in contrast to the increase observed within the carotid bodies following an acute exposure of 8 hours of intermittent hypoxia exposure (Messenger and Ciriello, 2013). The increase observed following 8 hours of intermittent hypoxia was suggested to be due to the increased production of leptin with the glomus cells or transport of leptin from plasma into these carotid body cells (Messenger and Ciriello, 2013), as plasma levels of leptin were elevated (Messenger and Ciriello, 2013; Messenger et al., 2012) and as corresponding changes in the expression of the OB-R$_{100}$ (likely OB-Ra) were observed in that study (Messenger and Ciriello, 2013). OB-Ra is thought to function as a carrier protein for leptin's transport across cell membranes (Bjørbaek et al., 1998b; Golden et al., 1997). The decreases observed in leptin after CIH may suggest that a number of different factors may account for these changes, including alterations in production, uptake, secretion and degradation of the protein within the carotid body. Although these data could also be interpreted to suggest that carotid body leptin is no longer involved in leptin signaling within glomus cells, this may not be the case as plasma levels of leptin remain elevated at both 7 and 95 days after CIH (Moreau and Ciriello, 2013, unpublished observations), and there is an up-regulation of the expression of the OB-Rb protein within the carotid body at these time points.

As the promoter of the gene encoding the leptin protein has been shown to be activated by hypoxia-inducible factor 1-alpha in humans (Grosfeld et al., 2002), and CIH has been found to increase hypoxia-inducible factor 1-alpha (Peng et al., 2006), a possible explanation for the decrease in leptin during long term exposure to CIH may be

that there is a change in secretion of leptin by the glomus cells. This in turn may function to modulate glomus cell activity or to signal the carotid body afferent fibers. Consistent with this suggestion, a previous study has identified OB-Rb-like immunoreactivity on fibers within the carotid sinus nerve and on those within the carotid body (Messenger et al., 2012). This study also demonstrated intermittent hypoxia and systemic leptin injection induces pSTAT3 in these fibers coursing through the carotid body. In addition, acute intermittent hypoxia and systemic injections of leptin have been shown to increase Fra 1/2 protein expression within both glomus cells and petrosal ganglion neurons, suggesting a change in the cellular activity of the carotid chemoreflex (Messenger et al., 2012). Furthermore, the co-expression of OB-Rb within these carotid body leptin producing glomus cells (Messenger et al., 2012) supports the suggestion of a potential autocrine/paracrine mechanism, similar to that demonstrated in other tissues (Bado et al., 1998; Fruhbeck et al., 1997; Jin et al., 2000; Mix et al., 2000; Siegrist-Kaiser et al., 1997; Sobhani et al., 2000). However, changes in secretion of leptin from the carotid body is unlikely to account for the decreased leptin levels within the carotid body as the protein expression levels of the $OB-R_{100}$ were not changed.

Exposure to CIH resulted in changes to OB-Rb, but not $OB-R_{100}$. Following both 7 days and 95 days of CIH, OB-Rb protein expression levels were increased whereas those for $OB-R_{100}$ were unchanged compared to normoxic controls. $OB-R_{100}$ denotes a 100 kDa band potentially representing OB-Ra, OB-Re or both. OB-Ra is a well-known transport receptor, playing a role in leptin passage into and out of cells (Bjørbaek et al., 1998b, Golden et al., 1997). Additionally, the OB-Ra may play a role in leptin degradation (Merabet et al., 1997; Sharma et al., 1997). The OB-Re is considered a

soluble receptor which binds leptin in the circulation and is involved in maintenance of circulating leptin levels by binding or releasing leptin within the blood for physiological functions (Huang et al., 2001; Li et al., 1998).

The change observed in OB-Rb, the primary receptor transducing leptin's physiological effects (Fruhbeck, 2006), suggests a possible change in sensitivity or signaling strength. It is well-known that leptin and its receptor may undergo ligand-induced endocytosis as well as increase receptor expression in situations of low ligand availability (Bennett et al., 1998; Mercer et al., 1997; Mitchell et al., 2009; Uotani et al., 1999). Thus, as observed in this study, the decreased local leptin levels may account for an increase in OB-Rb expression. However, if it is accepted that leptin levels have decreased within the glomus cells in response to increased release, the increased OB-Rb expression may suggest a mechanism by which leptin plays a role in the facilitation of the carotid chemoreceptor reflex (Ciriello and Moreau, 2012).

To investigate whether leptin receptor activity was altered during CIH the ratio of pSTAT3:STAT3 protein was examined in the carotid body. Decreased pSTAT3:STAT3 was observed following 7 days of CIH suggesting OB-Rb activation was decreased. As it is well-known that SOCS3 represents a negative feedback on the STAT3 signaling cascade (Bjørbaek et al., 1999; Bjørbaek et al., 2000; Fruhbeck, 2006), it was not unexpected to find that CIH concomitantly increased SOCS3 protein levels within the carotid body. This increase in SOCS3 would be expected to eventually reduce STAT3 phosphorylation (Bjørbaek et al., 1999; Bjørbaek et al., 2000; Fruhbeck, 2006), although the time course for this to occur, as well as the strength of inhibition of this system in the carotid body, is not known. Interestingly, no changes in pSTAT3 or SOCS3 were

observed after 95 day exposure to CIH compared to the normoxic groups; however, when compared to animals exposed to 7 days of CIH, pSTAT3 was elevated while SOCS3 was decreased after 95 days of CIH exposure. These findings can be interpreted to suggest that an increased sensitivity may have occurred to any incoming signal that there is an elevated sensitization of the system to incoming signals mediated by the JAK2/STAT3 pathway (Howard et al., 2004; Kievit et al., 2006; Mori et al., 2004). This would be the result of reduced SOCS3, which would be expected to increase the phosphorylation of STAT3 by JAK2, and thus allow the transduction of the incoming signal (Bjørbaek et al., 1999; Bjørbaek et al., 2000). In normoxic controls no changes in pSTAT3 or SOCS3 were observed between the animals exposed for 7 and 95 days.

Transcription within cells after leptin activation of the OB-Rb may also be mediated through the JAK2 pathway activating ERK1/2 (Fruhbeck, 2006; Kloek et al., 2002). Consistent with this suggestion, carotid body exposure to CIH for 95 days resulted in an increase in pERK1/2 protein expression. The functional significance of these findings is not clear. However, the observation of a downstream regulator of MAPK signaling within carotid bodies suggests an additional signaling pathway by which leptin may exert an effect on glomus cell chemoreceptor transduction. ERK 1/2 has also been implicated in the developmental regulation of the carotid body as ERK 1/2 protein levels have been shown to be lower in human fetuses compared to infants or young adults (Porzionato et al., 2010). Interestingly, in these studies ERK 1/2 protein levels within the carotid body were found to be decreased between 7 and 95 day normoxia exposure groups suggesting that these changes may be related to aging. However, the lack of change of ERK 1/2 between the two CIH groups could be interpreted that ERK1/2 was

activated as a result of CIH, consistent with previous observations (Messenger and Ciriello, 2013).

The data obtained in this study have provided evidence for a potential leptin signaling pathway within the carotid body, which is activated during CIH. These findings taken together with the finding that local leptin levels are altered after CIH suggest that leptin may exert an effect through an autocrine/paracrine mechanism to either activate neighboring glomus cells or carotid body afferent fibers that co-express OB-Rb (Messenger et al., 2012; Messenger and Ciriello, 2013). Although the functional role of the activation of JAK2/STAT3 pathway within the carotid body has not yet been delineated, it appears to be activated in response to elevations in circulating leptin levels (Messenger and Ciriello, 2013), which also occur following exposure to intermittent hypoxia (Messenger et al., 2012; Messenger and Ciriello, 2013).

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Chapter 5

5   Angiotensin Converting Enzyme Inhibition Affects Leptin and Downstream Indicators of Leptin Signaling in the Carotid Body

5.1   Introduction

The carotid body is a highly vascularized organ located bilaterally at the bifurcations of the common carotid arteries (Heymans et al., 1930; Nurse, 2005). The carotid body, composed of specialized oxygen-sensing type-I glomus cells, signals carotid sinus nerve endings that transmit the afferent information to the nucleus of the solitary tract (Ciriello et al., 1994), activating homeostatic mechanisms involving respiratory, cardiovascular and hormonal systems (Schultz and Li, 2007; Kumar, 2009).

There are now considerable data suggesting that substances within the circulation gain access to carotid body glomus cells and alter their excitability (Allen 1998; Chen et al., 2005; Leung et al., 2000, 2003; Nurse & Piskuric, 2012; Peng et al., 2006). One vasoactive compound that has been shown to alter the discharge of carotid body afferent fibers independent of its circulatory effects is angiotensin II (ANG II) (Allen 1998; Leung et al., 2000). The carotid body has been shown not only to contain ANG II binding sites (Allen, 1998), but to also possess its own intrinsic renin-angiotensin system (RAS) (Lam & Leung, 2002), suggesting that ANG II within the carotid bodies may act in an autocrine or paracrine manner. Key elements of the RAS, including protein and mRNA of angiotensinogen as well as mRNA of angiotensin I converting enzyme (ACE) have been localized to the type-I glomus cells (Lam & Leung, 2002). A role for ANG II in chemosensitivity is supported by the finding that gene expression for the ANG II type 1

receptor (AT_1R) is up-regulated in the carotid bodies during chronic hypoxia (Leung et al., 2000; Fung et al., 2002), and this increased expression of the AT_1R is associated with the increased sensitivity of carotid chemoreceptors (Leung et al., 2000; Fung et al., 2001, 2002). In addition, administration of the ANG II type 1 receptor (AT_1R) antagonist losartan abolishes these effects on chemoreceptors (Allen, 1998; Leung et al., 2000), including the increased intracellular calcium levels in type-I cells induced by ANG II (Fung et al., 2001). Consistent with these results, a recent study has demonstrated that signaling through the AT_1R is critical for carotid chemoreceptor sensitivity and signal transduction of the carotid chemoreceptor reflex during hypoxia (Marcus et al., 2010).

The increases in intracellular calcium induced by ANG II suggest that through activation of the AT_1R, ANG II may impact vesicle secretion from type-I glomus cells and therefore promote the release of other potential modulators involved in chemoreceptor function and for the signal transduction of glomus cells to the carotid sinus nerve (Gomez-nino et al., 1990; Eyzaguirre and Zapata, 1968; Bock, 1980). One possibility may be that ANG II may signal changes in leptin signaling within the carotid body as ANG II signaling is known to affect leptin production and secretion other tissues such as ventricular myocytes, bone marrow and adipocytes (Cassis et al., 2004; Danser et al., 1999; Haznedaroglu and Buyukasik, 1997; Haznedaroglu et al., 1996; Rajapurohitam et al., 2006, 2012). Leptin is a 16 kDa protein product of the *obese* gene and is produced in proportion to, and secreted primarily but not exclusively, by adipocytes (Lonnquist et al., 1995; Caro et al., 1996). Circulating leptin may be an indicator of body fat content and acts as a satiety hormone as well as increasing energy expenditure to balance body energy stores (Lonnquist et al., 1995; Caro et al., 1996). Leptin signals primarily through

the long-form leptin receptor (OB-Rb) although 6 splice variants of the OB-R exist (OB-Ra-OB-Rf) of which only a few have known functions (Lee et al., 1996; Wang et al., 1996). Leptin has been shown to possess a signaling capability within the carotid body (Messenger et al., 2012; Messenger and Ciriello, 2103), and both circulating and carotid body protein expression of leptin have been found to increase in response to intermittent hypoxia (IH) challenge (Messenger et al., 2012; Messenger and Ciriello, 2013), although its function within carotid body glomus cells remains unclear. The discovery of leptin as well as 4 of leptin's receptor isoforms within carotid body glomus cells (Messenger et al., 2012, 2013; Porzionato et al., 2011), as well as the finding that increased circulating leptin levels induce phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and immediate early gene Fra-1 expression within glomus cells (Messenger et al., 2012) is suggestive that the adipokine may play a role in chemosensitivity.

ANG II has been shown to be released and is required along with increased sympathetic nerve activity for the chronic hypertension in both human and the animal model of obstructive sleep apnea (OSA) (Fletcher et al., 1999, 1992a/b; Moller et al., 2003; Yuan et al., 2004). In addition, in the animal model of OSA, circulating and carotid body leptin is elevated (Messenger et al., 2012; Messenger and Ciriello, 2013), a finding consistent with clinical data (Phillips et al., 2000; Harsch et al., 2003). However, it is not known whether ANG II may affect leptin signaling in the carotid body which in turn may contribute to altered sensitivity within the chemoreceptor reflex pathway (Ciriello and Moreau, 2012, 2103). Therefore, this study was done to determine: (1) whether, selective chronic inhibition of ACE has an effect on circulating levels of leptin during IH; (2) whether glomus cells expressing the AT_1R co-express leptin and the OB-Rb, and whether

AT_1R levels in carotid bodies are altered following exposure to IH; (3) whether chronic inhibition of ACE alters leptin, OB-R or downstream mediators of long-form leptin receptor signaling within the carotid body.

5.2 Methods

General Animal Procedures: Experiments were done in male Sprague–Dawley rats (Charles River Canada, St. Constant, Canada) weighing 250-350 g. All animals were housed under controlled conditions with a 12 h light/dark cycle. Food and water were available *ad libitum*. All experimental procedures were done in accordance with the guidelines on the use and care of laboratory animals as set by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Western Ontario.

Angiotensin Converting Enzyme Inhibition: Inhibition of angiotensin converting enzyme was performed by administration of captopril (Sigma-Aldrich, St. Louis, Missouri, USA) in the drinking water (2 mg/ml) of each animal three days prior to IH (n=7) or normoxic conditions (n=7). Captopril was freshly dissolved daily in the drinking water which was monitored daily. It was calculated that the animals received 146 ± 6 mg/kg/day. At this dose, ACE inhibition has been shown to occur (Schiffrin & Genest, 1982; Ferrone & Antonaccio, 1979).

Induction of IH: IH or normoxia (8 h) was induced in the captopril or losartan treated animals and in a separate set of animals that were not treated with the drugs (IH, n=8; normoxia, n=8) as previously described (Messenger et al., 2012; Messenger and Ciriello, 2013). In brief, animals (one/tube) were placed into custom made cylindrical plexiglass

tubes with a zero-pressure escape valve (11 cm diameter x 33 cm length) inside an airtight plexiglass chamber. The animal was allowed to freely move within each tube. The chamber was attached to computer-controlled solenoid valves responsible for regulating the inflow of either pressurized room air (21% O_2) or 100% medical N_2. The solenoid valves were connected to a computerized timer system that allowed for the control of a total hypoxic (100% medical N_2) stimulus delivery of 80 s followed by a normoxic (21% O_2) time of 100 s, for a total continuous repetitive cycle period of 8 h. Additionally, the controller, attached to detectors for O_2 and CO_2 within the chamber, was set at a O_2 level of 6.5 % and CO_2 level of 0.1% under isobaric conditions at all times during the delivery of the hypoxic stimulus and recovery phase during which O_2 returned to 20.9%. A control (normoxic) set of animals was run simultaneously in a similar adjacent chamber through which only room air (21% O_2; normoxia) was used for the gas exchange in isobaric conditions for an 8 h period. Animals were exposed to the 8h IH stimuli during daylight hours.

Plasma Collection and Immunoassays: Measurement of plasma levels of leptin and ANG II were made immediately after the exposure of the animals to IH or the normoxic stimuli (Messenger et al., 2012; Messenger & Ciriello 2013). Blood samples were collected by cardiac puncture in 7% ethylenediaminetetraacetic acid at a volume of 10 μl/ml blood. The blood was immediately centrifuged at 10 000 RPM for 10 min at 4 °C to isolate the plasma which was removed and stored at -80 °C until analyzed for hormone content. Plasma samples were analyzed using enzyme immunoassays for rat leptin (inter-assay variability: 6.5% intra-assay variability: 7.1%; Enzo Life Sciences; Farmingdale, NY), and rat ANG II (inter-assay variability: <15%; intra-assay variability:

<10%; Phoenix Pharmaceuticals, Burlingame, California, USA) according to manufacturer instructions. Enzyme immunoassay plates were read on a SpectraMax M5 plate reader using SoftMax Pro v.5 microplate analysis software (Molecular Devices; Sunnyvale, California, USA).

Carotid Body Protein Extracts and Immunoblot Analysis: Carotid body protein extracts were obtained following bilateral carotid body excision from the normoxia- or CIH-exposed animals as described previously (Messenger et al., 2012; Messenger & Ciriello 2013). In brief, the carotid bodies (bilaterally) from each animal were snap frozen and pooled and homogenized in 200μL of RIPA buffer solution (150 mM NaCl, 1 mM NaF, 1 mM NaVO$_4$, 0.5 mM β-glycerophosphate, 1mM EDTA, 1% Triton-X 100, 50mM Tris-HCL at pH of 7.5) with a protease inhibitor cocktail (Roche Diagnostics, Laval, Quebec, Canada). The homogenate was centrifuged at 4°C for 15 min at 2100 RPM. The supernatant was retained and then centrifuged a second time at 4°C for 20 min at 13200 RPM. The resultant supernatant was retained as the protein preparation. Equal concentrations of extracted proteins normalized by colorimetric BCA Protein Assay (Pierce Corp., Madison, Wisconsin, USA) were fractionated in 10% polyacrylamide gel (Novex, Carlsbad, California, USA) and transferred onto a polyvinylidene fluoride membrane. Blots were probed using: polyclonal rabbit anti-AT$_1$R (Cat. # sc-1173; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), polyclonal rabbit anti-leptin (1:1000, Cat. # sc843; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), polyclonal rabbit anti-OB-R (1:1000, Cat. # OBR12-A; Alpha Diagnostics International Inc., San Antonio, Texas, USA), affinity purified anti-OB-Rb (1:1000, Cat. # CH14104; Neuromics, Edina, Minnesota, USA), polyclonal rabbit anti-SOCS3 (1:1000, Cat. #

ab16030; Abcam Inc., Cambridge, Massachusetts, USA), monoclonal rabbit anti-STAT3 (1:2000, Cat. # 4904S; Cell Signaling Technology, Danvers, Massachusetts, USA), polyclonal rabbit anti-pSTAT3 (1:1000, Cat. #9131S; Cell Signaling Technology, Danvers, Massachusetts, USA), monoclonal rabbit anti-ERK1/2 (1:1000, Cat. # 4695S; Cell Signaling Technology; Danvers, Massachusetts, USA), monoclonal rabbit anti-pERK1/2 (1:1000, Cat. # 4376S; Cell Signaling Technology; Danvers, Massachusetts, USA), and monoclonal horseradish peroxidase-conjugated β-actin (1:50000, catalog no. A3854, Sigma-Aldrich, St. Louis, Missouri, USA) diluted in 5% milk-1X Tris-buffered saline-Tween 20 buffer and with horseradish peroxidase conjugated donkey anti-rabbit IgG or donkey anti-chicken IgG (1:10000, catalog # 711- 035-152 or 703-035-155, respectively; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) diluted in 5% milk-1X Tris-buffered saline-Tween 20 buffer as the secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Millipore Canada Ltd, Toronto, Ontario, Canada).

Immunofluorescence: Immediately after the application of the 8h IH or normoxic stimuli the animals were anesthetized with 0.3 mL/100g equithesin and perfused transcardially using 500 mL ice-cold phosphate buffered saline (PBS; 0.01M, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (Messenger et al., 2012; Messenger & Ciriello 2013). The carotid arteries at their bifurcations, along with the attached carotid bodies were removed bilaterally, and stored overnight in Zamboni's fixative at 4 ºC. The following day the tissues were gradually dehydrated through a series of alcohols, and placed in xylene followed by paraffin wax. Serial transverse sections were cut at 6 μm on a RM 2155 microtome (Leica Microsystems Inc., Buffalo Grove, Illinois, USA.), floated in a

warm water bath and mounted on double-gelatinized glass microscope slides and placed on a slide warmer. Tissue sections were later de-paraffinized in xylene and rehydrated using graded alcohol solutions. For each animal, 1 in every 5 slides was stained with thionin to identify the region of the carotid body and blood vessels. Additionally, adjacent sections to those stained for thionin from the normoxic and IH-exposed animals were rinsed in PBS and processed for double-immunofluorescence for Ob-Rb and leptin. Sections underwent an antigen-retrieval protocol using a citrate buffer (10 mM sodium citrate/0.05% Tween; pH 6.0) heated to 90-95ºC in a microwave for 15 min and then were rinsed in PBS before being placed in 5% normal goat serum (Vectastain Elite ABC Kit, Cat. # PK6100; Vector Laboratories, Burlingame, California, USA) in PBS containing 0.3% Triton X-100 for 30 min (Messenger et al., 2012). The sections were later rinsed in PBS and placed overnight (12 h) into a primary polyclonal rabbit anti-leptin (Cat. # sc843; Santa Cruz Biotechnology Inc.; Santa Cruz, California, USA; 1:1000 in PBS/0.3% Triton X-100) or affinity purified anti-OB-Rb (1:1000, Cat. # CH14104; Neuromics, Edina, Minnesota, USA) at room temperature. Sections were then rinsed in PBS and incubated in goat biotinylated anti-rabbit IgG (Vectastain Elite ABC Kit, Burlingame, California, USA) diluted 1:500 in PBS/ 0.3% Triton X-100 for 1 h. Following PBS rinses, sections were placed in Streptavidin Alexafluor-488 (Cat. # S11223; Invitrogen, Burlington, Ontario, USA) diluted 1:100 for 1 h. Following PBS rinses, sections were exposed to an avidin containing solution for 15 min followed by a biotin containing solution for an additional 15 min (Avidin/Biotin blocking kit, Cat. # SP-2001; Vector Laboratories, Burlingame, California, USA) to block non-specific binding sites. Sections were then rinsed in PBS and incubated overnight at room temperature in

primary polyclonal rabbit anti-AT$_1$R (Cat. # sc-1173; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) diluted 1:1000 in PBS/0.3% Triton-X 100 and 5% normal goat serum. Following PBS washes, the sections were placed in goat biotinylated anti-rabbit IgG (Vectastain Elite ABC Kit, Burlingame, California, USA) diluted 1:500 in PBS/ 0.3% Triton X-100 or goat biotinylated anti-chicken IgY (Vectastain Elite ABC Kit, Burlingame, California, USA) diluted 1:200 in PBS/ 0.3% Triton X-100 for 1 h. Sections were rinsed in PBS and placed in Streptavidin Texas Red (Code: RPN1233; GE Healthcare, Baie d'Urfe, QC, Canada) diluted 1:100 for 1 h. Following PBS rinses, sections were placed in 100% ethanol containing 5% glacial acetic acid on dry ice for 10 min. Sections were then immediately cover-glassed using Fluoromount mountant.

Statistical Analysis: Statistical comparisons between normoxia and CIH exposed animals were made using a one-tailed, unpaired Student t-test. Statistical comparisons between plasma values of ANGII or leptin were made using two-way ANOVA followed by Bonferroni's post-hoc analysis. In all comparisons, a minimum *p*-value of < 0.05 was taken to indicate statistical significance (GraphPad Prism; GraphPad Software, San Diego, California, USA).

5.3 Results

Plasma ANG II and Leptin Levels Following Captopril Treatment: As previously reported (Messenger et al., 2012), IH was found to significantly increase plasma ANG II ($p < 0.01$) (Fig. 5.1a) about 3 fold above those found in the normoxic control animals and plasma leptin (Fig. 5.1b) levels approximately 5.9 fold ($p < 0.01$) above those found in the normoxic control animals. Following captopril treatment, no differences were found

between IH and normoxic controls. In addition, in animals treated with captopril, the increase in leptin following IH exposure was not only abolished, but basal circulating levels of leptin were found to be significantly (p<0.05) lower than then those observed in non-captopril treated normoxic controls (Fig. 5.1b).
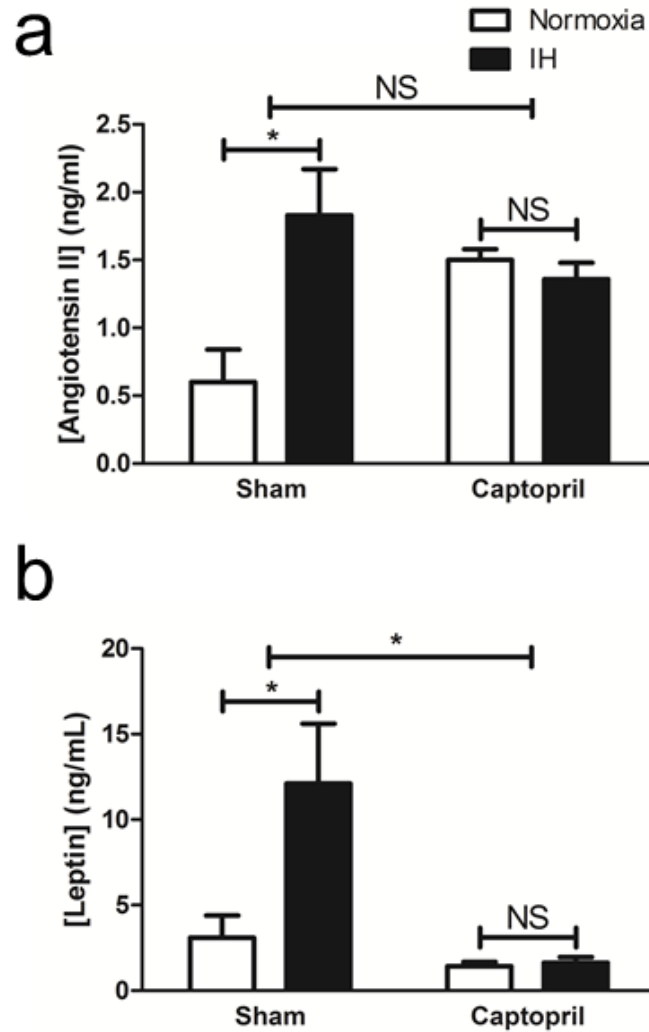
Figure 5.1. Bar charts showing the effect of IH on plasma levels of ANG II (a), and leptin (b) compared to levels observed in normoxic controls under sham (non-captopril treatment) and captopril treatment. Note that plasma ANG II (a) and leptin (b) are elevated following IH in the non-captopril treated (sham) group compared to normoxic group. Additionally, note that captopril treatment not only eliminated the rise in both ANG II and Leptin following IH, but also lowered the plasma levels of leptin below control sham levels (b). Values are shown as means ± S.E. *$p < 0.01$. n=7.

Leptin, OB-Rb and AT1R Co-expression in Carotid Body: Figure 5.2 shows the effect of IH on leptin, OB-Rb and AT_1R expression within glomus cells of the carotid body. These cells within the carotid body expressed little leptin (Fig. 5.2a), but some AT_1R (Fig. 5.2b) immunofluorescence under normoxic conditions. However, following exposure to IH, carotid body glomus cells exhibited an increased expression of leptin (Fig. 5.2c), OB-Rb (Fig. 5.3e), and AT_1R (Fig. 5.2d and 5.2f). As can be seen in Figure 5.2, both leptin and Ob-Rb are expressed within the same carotid body glomus cells that express the AT_1R.

Figure 5.2. Fluorescent photomicrographs of the carotid body showing glomus cells immunoreactive to leptin (a,c), Ob-Rb (e) and angiotensin type-1 receptor ($AT_1R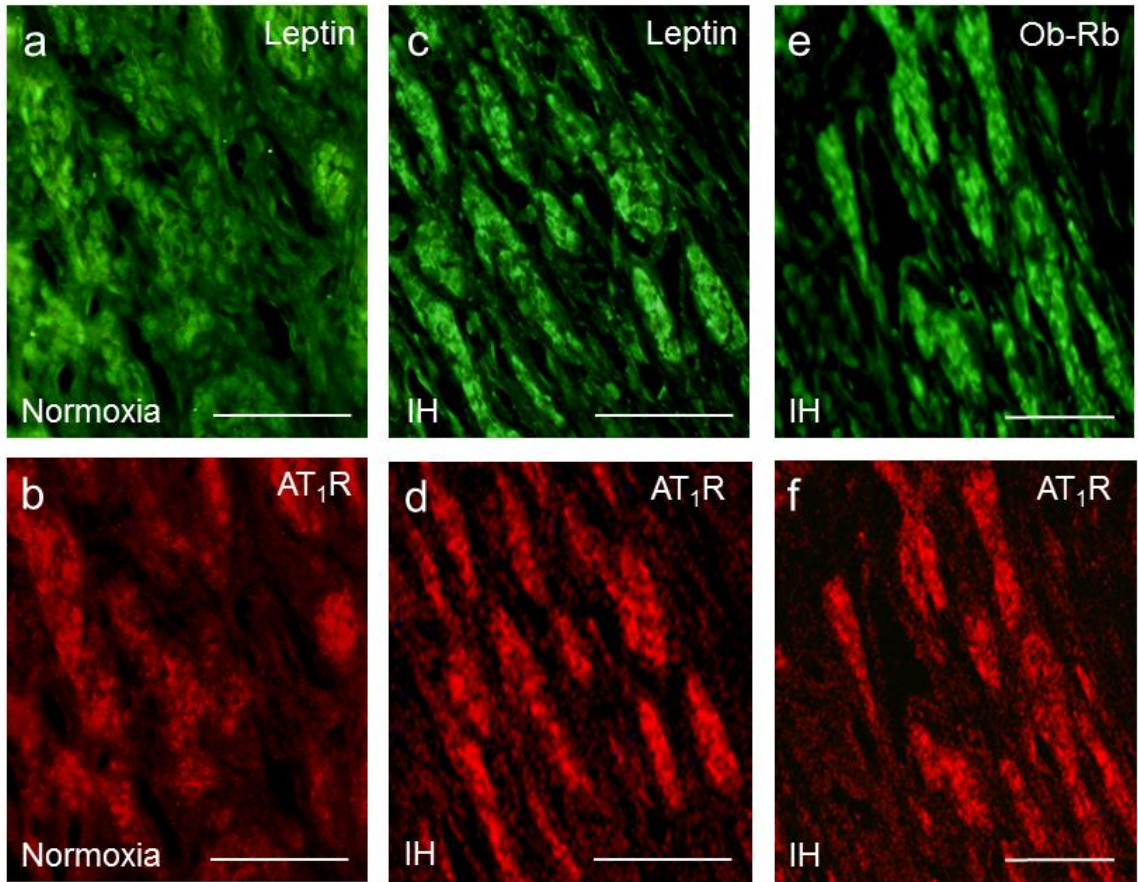$; b,d,f) following exposure to IH (c,d,e,f) or normoxia (a,b). Note that cells immunoreactive to leptin and Ob-Rb also contain immunoreactivity to AT_1R. Calibration marks indicate 25 μm.

Effect of IH on AT₁R and AT₂R in Carotid Body: Figure 5.3 shows the effect of IH on AT$_1$R and AT$_2$R protein expression in the carotid body. When exposed to IH, the expression of AT$_1$R was significantly increased by about 33% (Fig. 5.3a). However, the protein expression of AT$_2$R was not different following IH exposure (Fig. 5.3b).

Effects of Captopril on Leptin within Carotid Body: Figure 5.4 show the effect of captopril (Fig. 5.4a-c) treatment on carotid body leptin protein levels following IH exposure. In normoxic animals (Figs. 5.4a), captopril treatment lowered leptin levels to about 49% of non-treated animals however this effect was not statistically significant. In the IH animals, captopril (Fig. 5.4b) lowered leptin levels within the carotid body to about 58%, of levels found within the IH non-treated animals. In addition, when the effects of captopril (Fig. 5.4c) in the normoxic animals were compared to those in of the IH animals, it was found that captopril lowered the leptin levels of the IH animals to about 60% of the normoxic captopril animals (Fig. 5.4c) however this effect was not statistically significant.
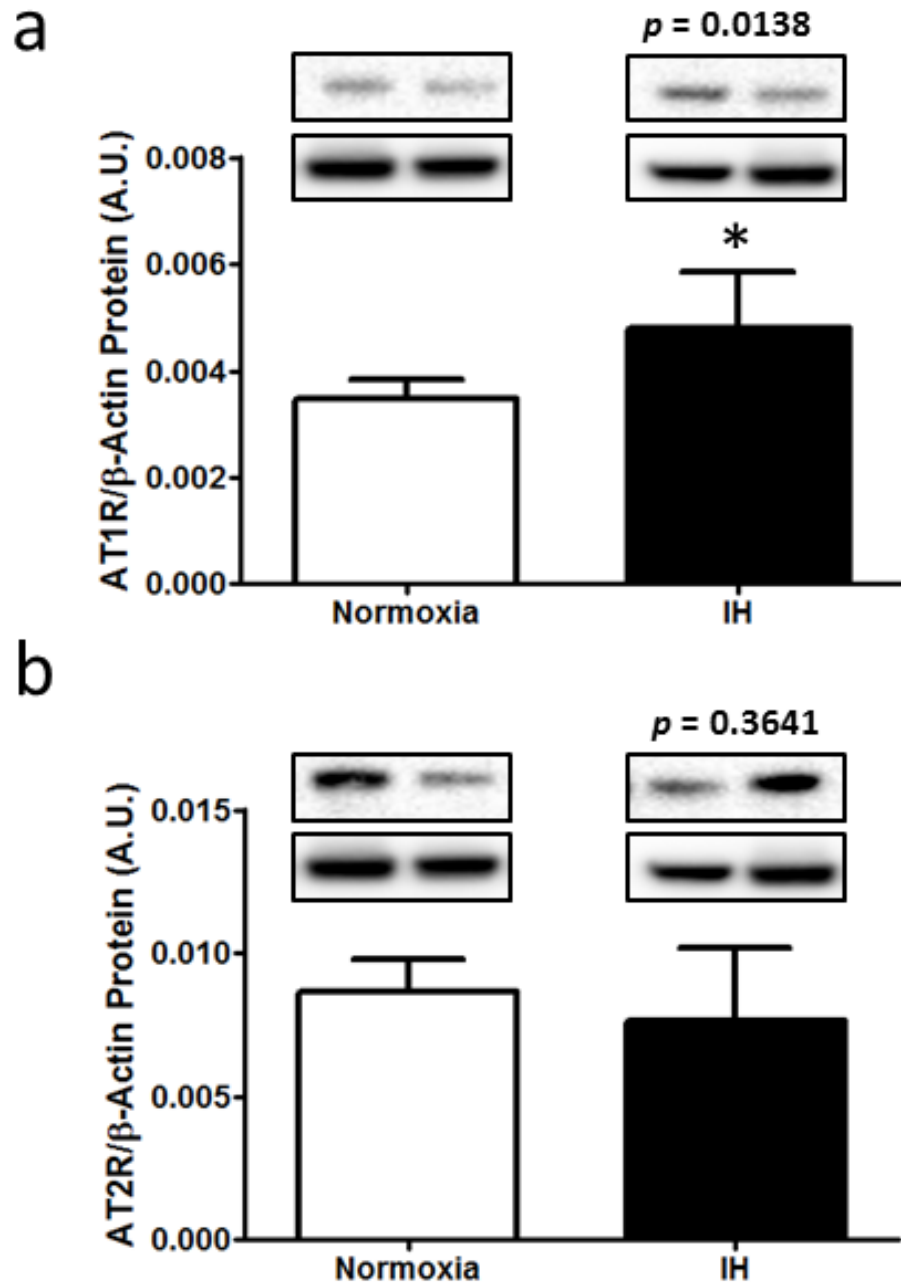
Figure 5.3. Western blots showing the effect of IH on AT_1R (a) and AT_2R (b) the protein expression in the carotid bodies. Note that AT_1R protein levels are significantly elevated following IH, whereas levels of the AT_2R are not altered in the carotid bodies. *, significantly different from normoxic control animals. n=7.
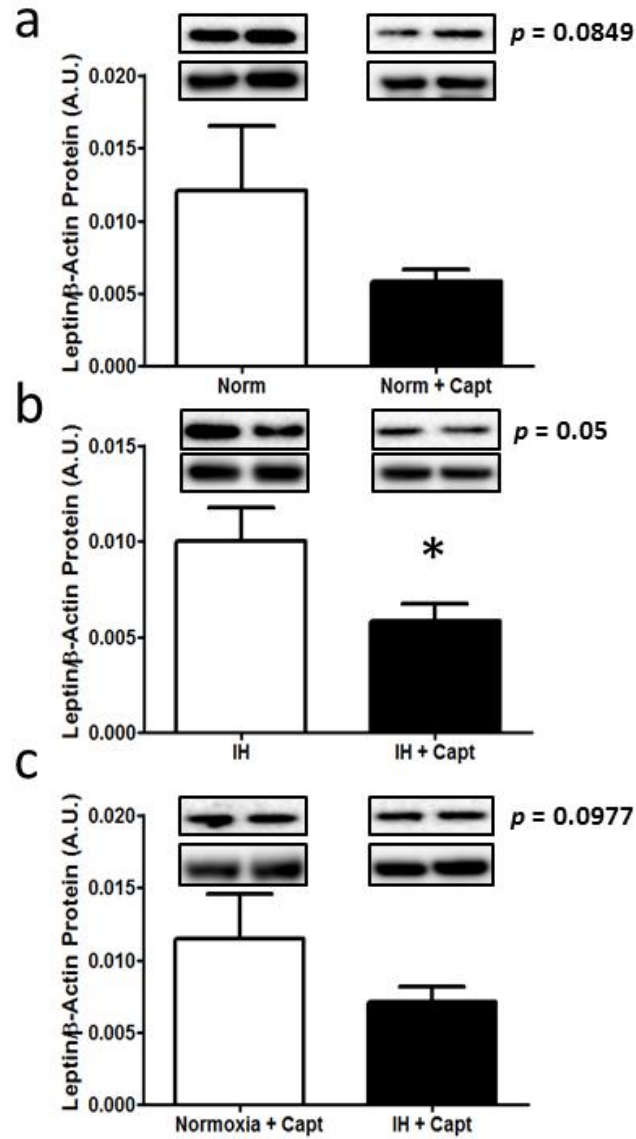
Figure 5.4. Western blots showing changes in leptin protein within the carotid body following IH with or without captopril (Capt) treatment. Note that captopril treatment lowered leptin levels within the carotid body in the IH (b) group. *, significantly different from non-captopril IH treated animals. n=7.

Effects of Captopril on OB-Rb and OB-R₁₀₀ within Carotid Body: Captopril treatment in either the normoxic controls (Fig. 5.5a) or the IH exposed (Fig. 5.5b) animals resulted in a lower carotid body OB-Rb (Fig. 5.5a-b) protein levels (31% and 44%, respectively), while the protein expression levels of OB-R$_{100}$ (Fig. 5.5c-d) were increased to about 100% and 140% of the levels within the non-treated normoxic (Fig. 5.5c) and IH (Fig. 5.5d) animals, respectively. Additionally, when the effects of captopril in the normoxic animals (Fig. 5.6a) were compared to those in of the IH animals (Fig. 5.6b), it was found that captopril lowered in the IH animals the expression levels of the OB-Rb protein to about 45% the normoxic controls (Fig. 5.6a). On the other hand, no differences were found with the expression of the OB-R$_{100}$ (Fig. 5.6b) between normoxic- and IH-captopril treated animals.

Figure 5.5. Western blots showing changes in OB-Rb (a-b) and OB-R$_{100}$ (c-d) protein within the carotid body following IH and captopril treatment. Note that captopril treatment lowered Ob-Rb protein levels within the carotid body in both normoxic (Norm) (a) and the IH (b) group. On the other hand, the protein levels of OB-R$_{100}$ were increased in both the normoxic (c) and the IH (d) groups. *, significantly different from non-captopril treated animals within each group. n=7.

Figure 5.6. Western blots showing changes in OB-Rb (a) and OB-R$_{100}$ (b) protein within the carotid body following captopril treatment in both the normoxic and IH groups. Note that the protein level of Ob-Rb was significantly (*) lower in the IH animals compared to the normoxic animals (a) treated with captopril. n=7.

Effects of Captopril on STAT3, pSTAT3, SOCS3 and pERK 1/2 in the Carotid Body:
Figures 5.7-5.11 summarize the effects of captopril treatment on carotid body protein expression of STAT3 (Figs. 5.7a-b and 5.8a), pSTAT3 (Figs. 5.7-c-d and 5.8b), SOCS3 (Fig. 5.9a-c), and pERK 1/2 (Figs. 5.10 and 5.11a-c) in normoxic and IH exposed animals. Captopril treatment in normoxic animals resulted in decreased STAT3 protein expression (Fig. 5.7a; to about 47% of non-treated group), whereas no differences were found between IH-exposed animals (Fig. 5.7b) or between normoxic and IH animals treated with captopril (Fig. 5.8a).

Captopril treatment in normoxic and IH exposed animals also resulted in decreased pSTAT3 (52% and 49% of non-captopril treated animals, respectively) (Fig. 5.7c-d). Furthermore, pSTAT3 protein levels in the IH-captopril treated animals were decreased to about 58% of normoxic-captopril treated animals (Fig. 5.8b).

Captopril treatment in normoxic and IH exposed animals also resulted in increased SOCS3 protein expression within the carotid body. In normoxic animals, captopril increased the expression of SOCS3 protein by almost 1036% (Fig. 5.9a), while in the IH exposed animals captopril increased the expression of SOCS3 protein by about 2490% (Fig. 5.9b). However, the protein expression level of SOCS3 in the IH captopril treated animals was reduced to about 70% of that found within the normoxic captopril treated animals (Fig. 5.9c).

ERK1/2 and pERK 1/2 protein levels were decreased (to about 48% and 42% of non-captopril treated animals, respectively) following captopril treatment (Fig 10a and c). On the other hand, neither ERK 1/2 or pERK 1/2 protein levels in the IH captopril treated

animals were different from those in the non-captopril treated IH exposed group (Fig. 5.10b and d) or from the normoxic captopril treated groups (Fig. 5.11).
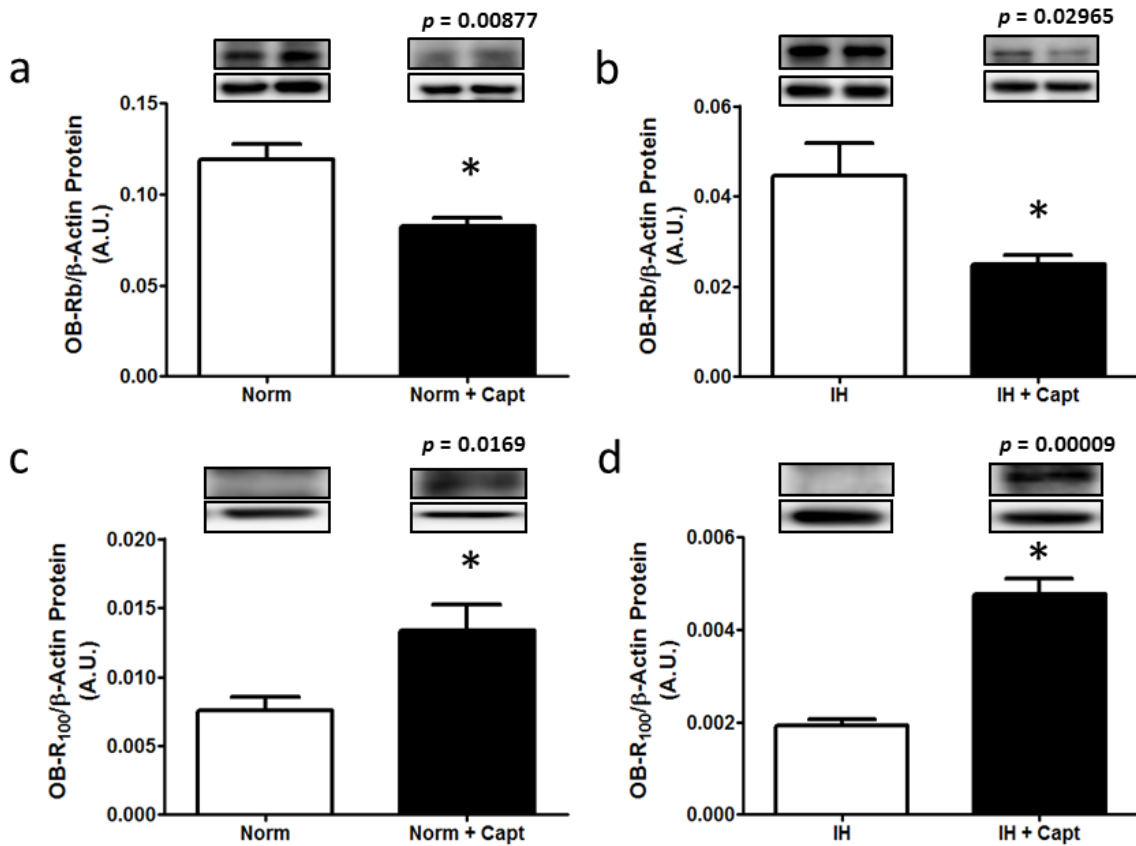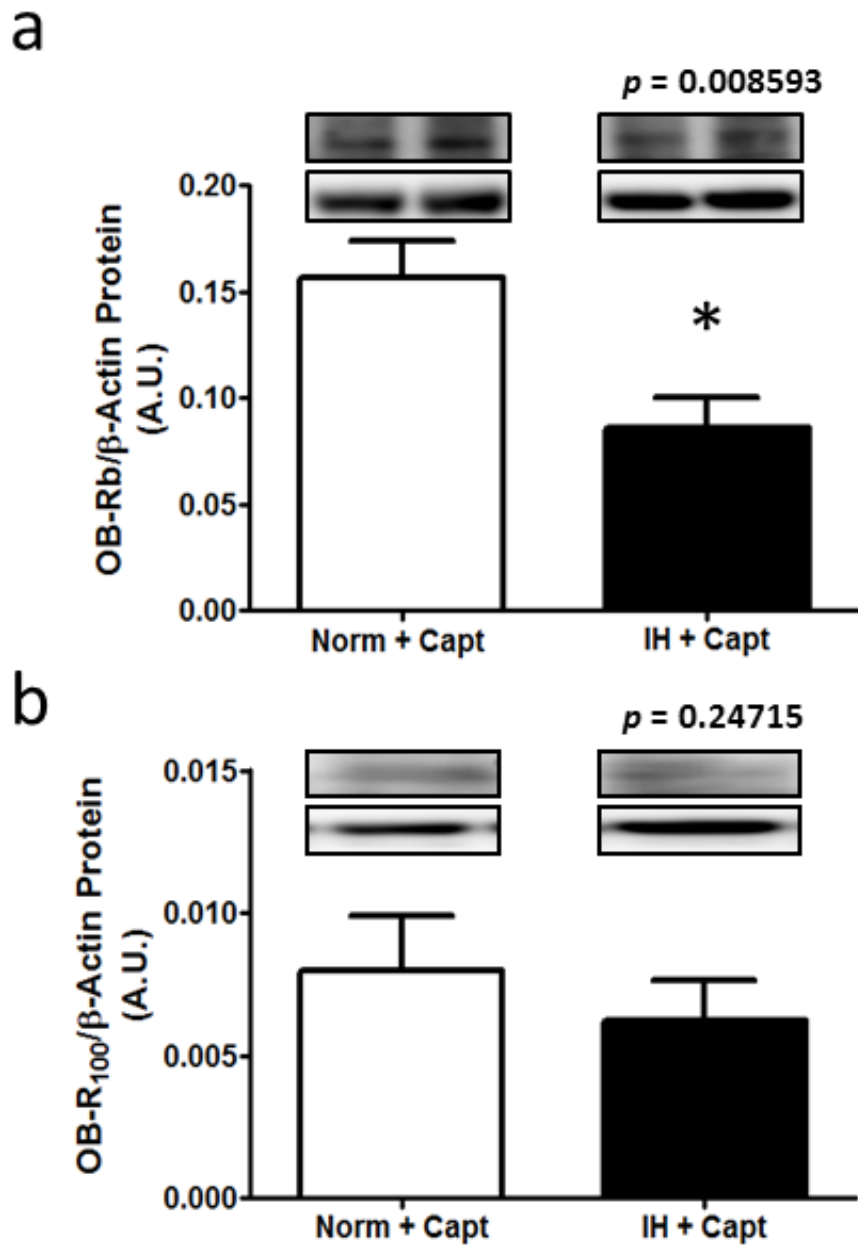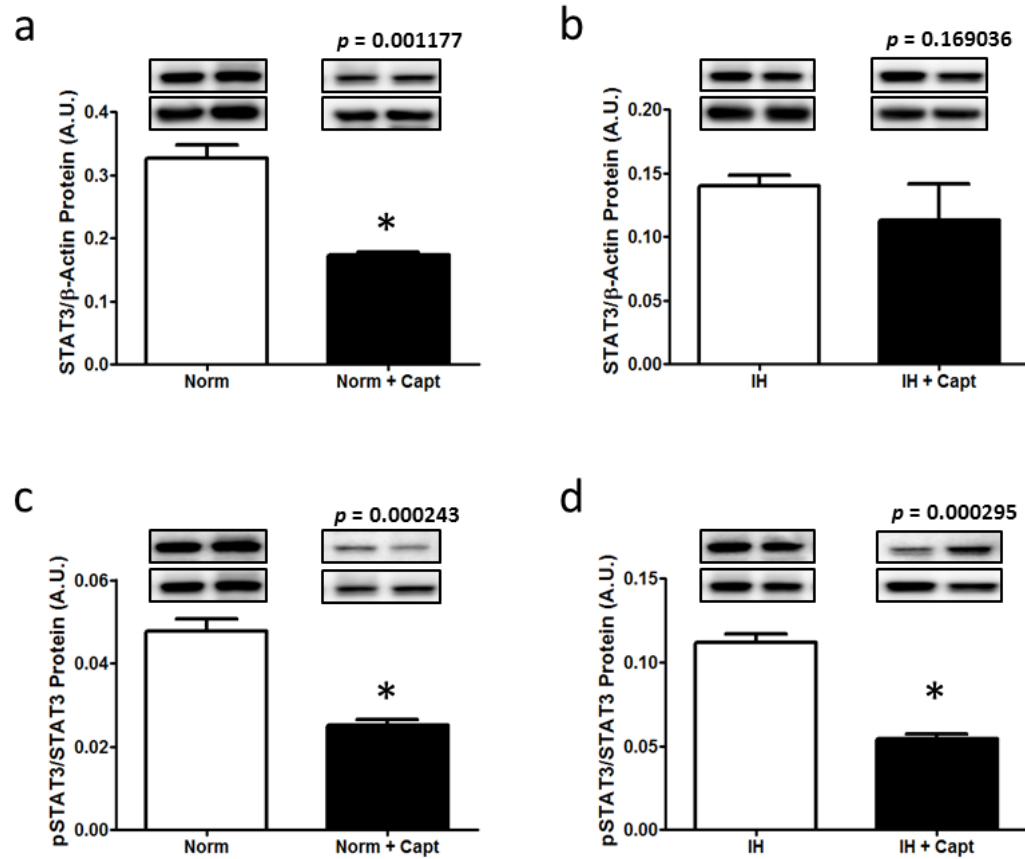
Figure 5.7. Western blots showing changes in STAT3 (a-b) and pSTAT3 (c-d) protein within the carotid body following IH and captopril treatment. Note that captopril treatment significantly (*) lowered STAT3 protein levels within the carotid body only in normoxic (Norm) group (a). However, pSTAT3 levels were significantly (*) lower in both the normoxic (c) and the IH (d) groups after captopril treatment. n=7.

Figure 5.8. Western blots showing changes in STAT3 (a) and pSTAT3 (b) protein within the carotid body following captopril treatment in both the normoxic and IH groups. Note that the protein level of pSTAT3 was significantly (*) lower in the IH animals compared to the normoxic animals (b) treated with captopril. n=7.
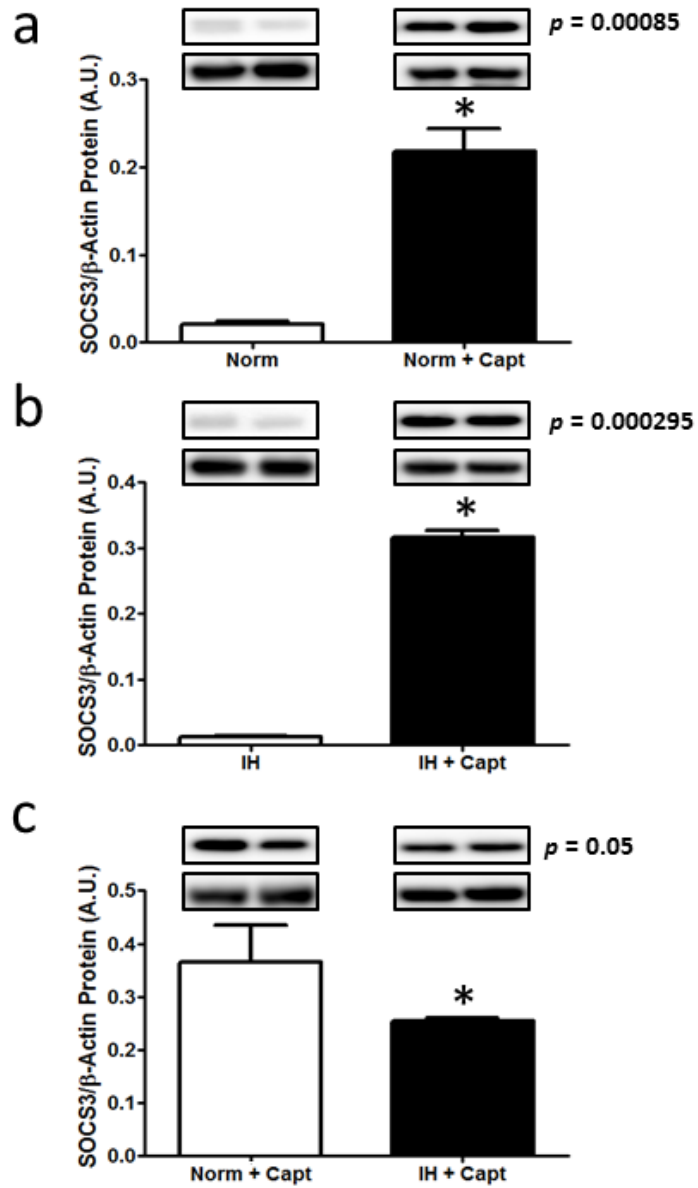
Figure 5.9. Western blots showing changes in SOCS3 protein expression within the carotid body following IH and captopril treatment. Note that captopril treatment significantly (*) elevated SOCS3 protein levels within the carotid body in the normoxic (Norm) (a) and IH group (b). However, SOCS3 levels were significantly (*) lower in the IH captopril treated group compared to the normoxia captopril treated group (c). n=7.

Figure 5.10. Western blots showing changes in ERK 1/2 (a-b) and pERK 1/2 (c-d) protein within the carotid body following IH and captopril treatment. Note that captopril treatment significantly (*) lowered ERK 1/2 and PERK 1/2 protein levels within the carotid body only in normoxic (Norm) group (a and c). n=7.
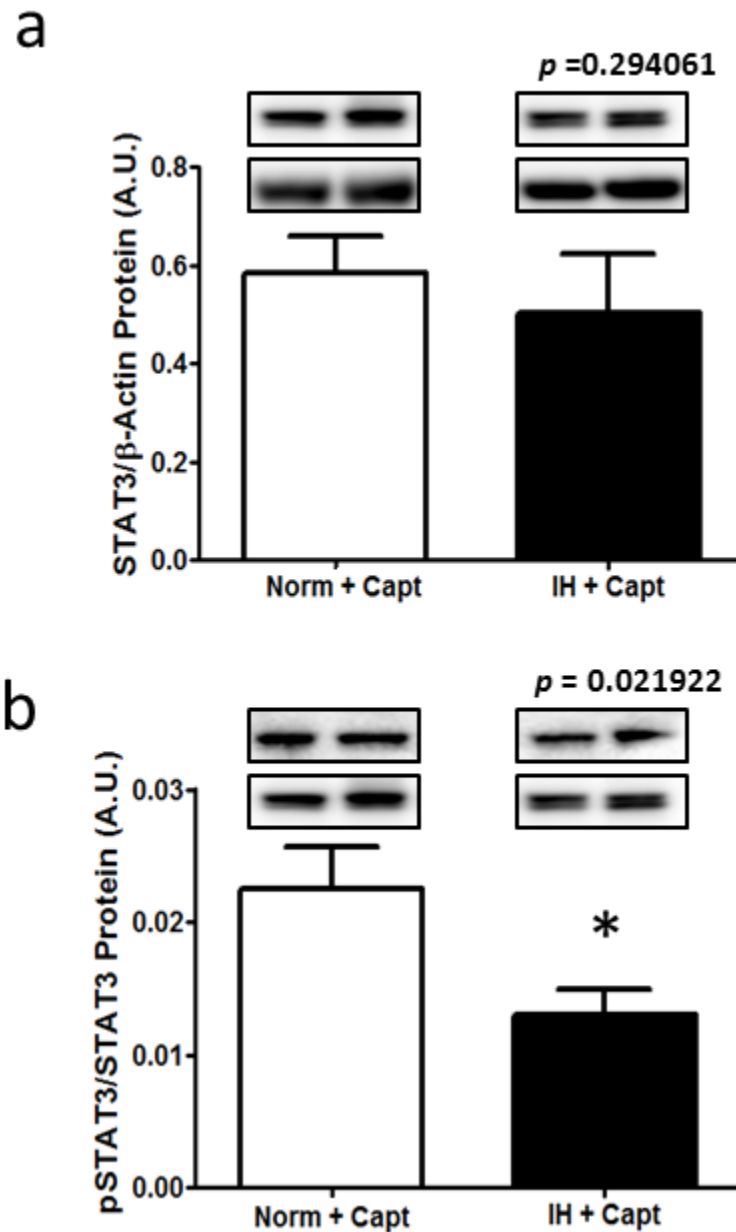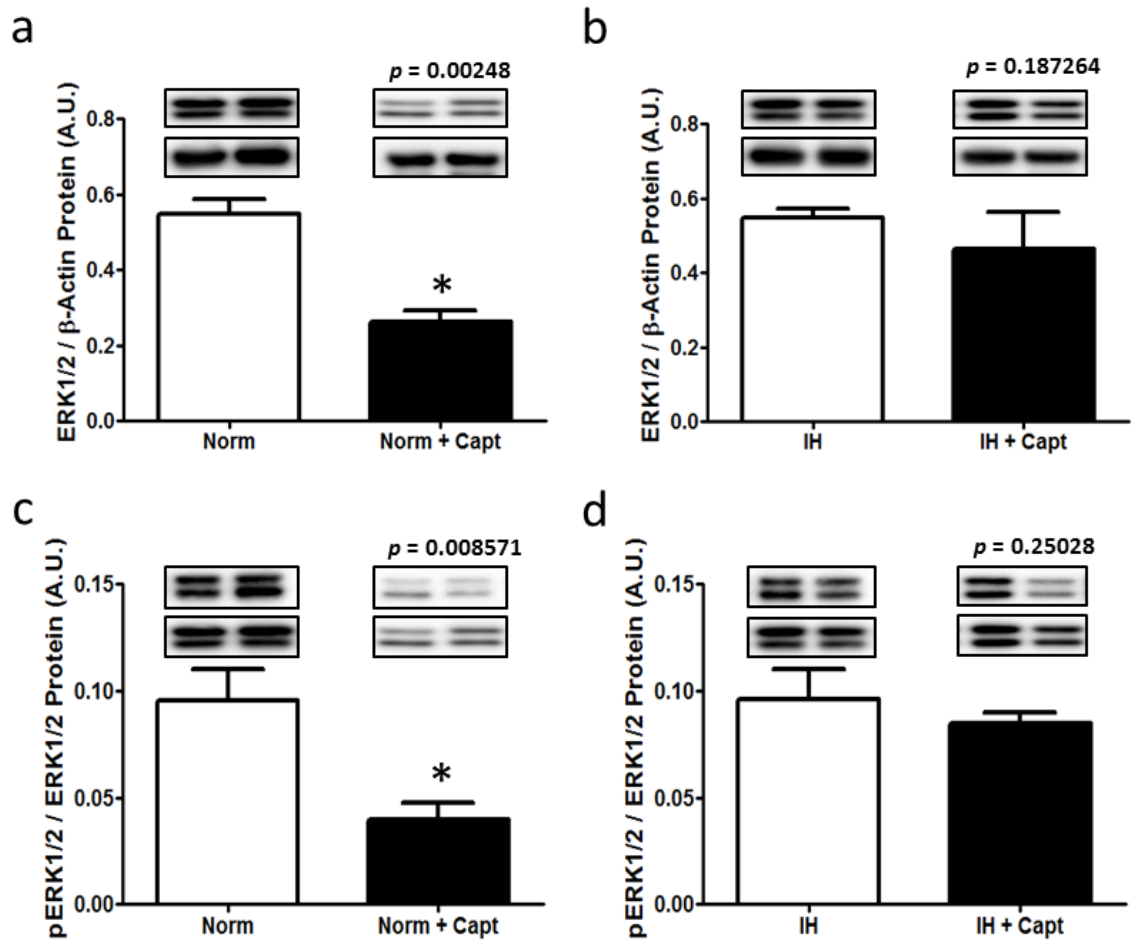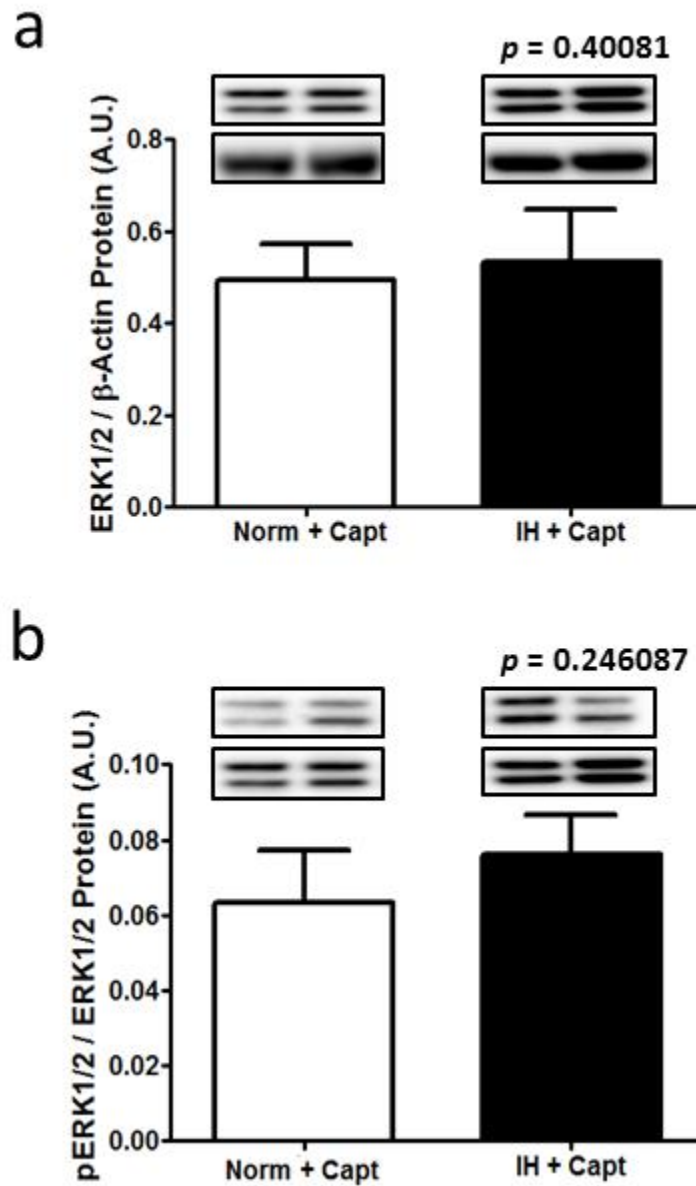
Figure 5.11. Western blots showing changes in ERK 1/2 (a) and pERK 1/2 (b) protein within the carotid body following captopril treatment in both the normoxic and IH groups. Note that the protein levels were not altered in either group treated with captopril. n=7.

5.4   Discussion

These data provide the first direct evidence that ANG II is important in the regulation of leptin not only within the carotid body, but also within the circulation, and that ANG II contributes to changes in downstream leptin signaling within the carotid body during IH. These conclusions are based on the findings that within the carotid body, cells that expressed leptin or the OB-Rb also expressed the AT_1R and AT_2R. Additionally, inhibition of ANG II by blocking ACE decreased leptin not only within the carotid body, but also the plasma levels of leptin during IH. Furthermore, the decrease in available leptin to the carotid body was accompanied by a decrease in the OB-Rb in captopril treated animals, which suggests a dampening of the entire leptin signaling system within the carotid body. This suggestion is consistent with the observation that captopril treatment resulted in a decrease in the activation of STAT3 into pSTAT3, supporting the notion of a reduction in OB-Rb signaling. Finally, contrary to the decrease in OB-Rb protein expression levels following captopril treatment, OB-R_{100} protein levels in the carotid body were increased suggesting an either an increase in transport of leptin out of cells or an increase in leptin degradation within the cells. Taken together, these data can be interpreted to suggest that ANG II effects on carotid body chemosensitivity may be mediated in part through the carotid body leptin system, and that ANG II signaling is critical for the maintenance of circulating levels of leptin in normoxia and during IH. This suggestion is consistent with the finding that AT1R blockade produces decreased BMI and food intake, but has no effect in obese Zucker rats with a genetic mutation in the leptin receptor (Muller-Fielitz et al., 2011). In addition, it has been shown

that the brain RAS facilitates renal and brown adipose tissue sympathetic nerve responses to leptin (Hilzendeger et al., 2012).

The finding of a decrease in circulating leptin following ANG II inhibition is consistent with earlier data suggesting that ANG II signaling stimulates not only leptin production, but also leptin secretion (Cassis et al., 2004; Premaratna et al., 2012). Considering that the main production/secretion site of leptin into the plasma is adipose tissue (Lonnquist et al., 1996; Caro et al., 1996), this study provides further evidence suggesting that the leptin system may be in part under the control of ANG II. This latter suggestion is consistent with the observation in a mouse model deficient in the AT1Ra isoform, in which plasma leptin concentrations were found to be reduced when compared to wild-type (Kaneko et al., 2011).

Carotid body leptin levels were also found to be decreased in animals treated with captopril during both normoxia and IH conditions. This finding is similar to that observed within adipocytes taken from rats treated with captopril, in which both circulating leptin and leptin release was decreased (Cassis et al., 2004). Additionally, incubation of adipocytes with ANG II resulted in an up-regulation of leptin mRNA, as well as leptin secretion (Cassis et al., 2004) while in ventricular myocytes, ANG II promotes *de novo* synthesis and secretion of leptin (Rajapurohitam et al., 2006). Furthermore, blocking OB-R's in these cardiomyocytes prevented the hypertrophic effects ANG II normally has on these cells suggesting that leptin may mediate effects normally attributed to direct ANG II action (Rajapurohitam et al., 2012). Finally, in human vascular smooth muscle cells, it has been previously found that increased ANG II results in increased leptin protein and mRNA expression (Shyu et al., 2012). Thus, it is not unreasonable to suggest that leptin

may be involved in mediating some of the changes in carotid body chemosensitivity normally attributed to ANG II (Gomez-nino et al., 1990; Eyzaguirre and Zapata, 1968; Bock, 1980). Interestingly, the increased leptin protein and mRNA expression observed in human vascular smooth muscle cells as a result of the elevated ANG II has been attributed to increases in reactive oxygen species (Shyu et al., 2012). The IH induced changes in the carotid body chemosensitivity have been suggested to be related to an enhanced reactive oxygen species production (Pawar et al. 2009; Iturriaga et al., 2009). In addition, the IH effects associated with increases in superoxide have been shown to be blocked by losartan, a specific AT1R blocker (Marcus et al., 2010). Therefore, taken together, these data suggest a possible mechanism by which ANG II signaling may modulate ROS activity and subsequently leptin activity within the carotid body.

Captopril treatment in both normoxia and after IH reduced protein levels of OB-Rb compared to their respective controls. This finding suggests that ANG II plays an important role in the regulation of Ob-Rb although, due to the low levels of available leptin both locally and from the circulation following captopril treatment, ligand-induced endocytosis seems unlikely (Bennett et al., 1998; Uotani et al., 1999). The short-form leptin receptor displayed increased protein expression in the carotid bodies following captopril-treated animals in both the normoxic and IH conditions. Generally, OB-Ra is believed to be involved in leptin transport, specifically across the blood-brain barrier (Banks et al., 1996; Golden et al., 1997; Bjorbaek et al., 1998). One potential explanation may be that the reduced leptin availability stimulates OB-Ra protein expression to aid in leptin secretion from glomus cells. Additionally, the OB-Ra has been suggested to function in the degradation process of leptin (Iida et al., 1996; Merabet et al., 1997;

Sharma et al., 1997) and may provide one mechanism through which the decreases seen in carotid bodies occurs. However, the functional significance of ANG II effects on Ob-Ra is unknown.

The decreased protein expression of leptin and OB-Rb in the carotid bodies after captopril treatment is consistent with the decreases in activation of STAT3. In normoxic and IH conditions, captopril treatment reduced pSTAT3 levels, a downstream signaler involved in OB-Rb activation (Elmquist et al., 1997; Fruhbeck, 2006; Wang et al., 1998; Huo et al., 2006, 2007). The down regulation of both the ligand and the receptor would be expected to result in a decrease in activation of the signaling pathway (Elmquist et al., 1997; Fruhbeck, 2006; Wang et al., 1998; Huo et al., 2006, 2007). One other potential explanation for the decrease in pSTAT3 levels found in captopril treated animals is the decrease in ANG II levels as ANG II signaling, through AT_1R, can activate the JAK/STAT pathway resulting in phosphorylation of STAT3 (Ji et al., 2012; Omura et al., 2001). However, it has also been shown that leptin injections resulted in up-regulation of pSTAT3 protein expression (Messenger et al., 2012), and thus this decrease after ANG II inhibition most likely occurs at least in part through decreased leptin signaling.

Consistent with the decrease in pSTAT3 is the concomitant rise in SOCS3 levels. SOCS3 acts as a negative feedback loop on JAK/STAT signaling (Bjorbaek et al., 2000; Fruhbeck et al., 2006). The decrease in pSTAT3 accompanied by the SOCS3 rise would be expected in the IH condition, which has previously been shown to stimulate the JAK/STAT pathway (Messenger et al., 2012; Messenger & Ciriello, 2012). In some cell types, ANG II has been known to stimulate the JAK/STAT pathway which results in increased SOCS3 expression (Calegari et al., 2003; Ji et al., 2012; Omura et al., 2001).

However, as to the reason why such a profound increase was found in both the normoxic and IH condition is unknown.

In addition to the JAK/STAT pathway, OB-Rb signaling can also activate the MAPK cascade (Bjorbaek et al., 1997; Banks et al., 2000) and thus activation of ERK1/2 is an indicator of pathway activation. In normoxic animals, captopril treatment resulted in a decrease in pERK1/2 protein levels, however no changes were observed in the IH animals. This finding suggests during IH, other mechanisms in addition to leptin (Bjorbaek et al., 1997; Banks et al., 2000) or ANG II (Li et al., 1998; Nakai et al., 2012) may activate ERK1/2.

In summary, this study has demonstrated that the RAS modulates the leptin system not only in the circulation, but also within chemosensitive carotid body. Inhibition of ANG II by ACE results in a dampening effect on circulating leptin levels as well as local carotid body leptin protein levels. There is also a decrease in the OB-Rb and downstream indicators of OB-Rb activation. Angiotensin signaling through the AT_1R is thought to be critical for chemosensitivity by the carotid bodies (Marcus et al., 2010), and given its co-localization with OB-Rb and leptin within the glomus cells suggests that AT_1R has a stimulatory effect on the carotid body leptin system and ANG II chemosensitive role may be mediated in part through leptin signaling.

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Chapter 6

6   Conclusions

6.1   Conclusions Chapter 2

- Carotid bodies, afferents in carotid sinus nerve and petrosal/nodose ganglia express long form of leptin receptor (Ob-Rb).

- Intermittent hypoxia (IH) in conscious animal elicits an increase in plasma levels of leptin.

- IH induces pSTAT3 and fos/Fra-1 proteins expression in carotid body cells of these animals.

- IH induces immediately early gene fos/Fra-1 expression in petrosal and nodose ganglion cells.

- Systemic injections of leptin in anesthetized animals induces pSTAT3 and fos/Fra-1 protein expression in carotid body cells, and fos/fra-1 in petrosal/nodose ganglia cells.

- These data suggest that leptin released during IH may modulate the activity of peripheral chemoreceptor reflex.

6.2   Conclusions Chapter 3

- Carotid bodies express leptin, and the long form (Ob-Rb) and short form (Ob-R$_{100}$) leptin receptors.

- Intermittent hypoxia (IH) in conscious animal elicits an increase in plasma levels of leptin.

- IH induces leptin, Fra-1, Ob-R$_{100}$, pSTAT3, ERK 1/2 and SOCS3 protein expression in carotid body glomus cells, while down-regulating Ob-Rb.

- Intravenous injections of leptin induce leptin and Fra-1 expression in carotid body cells.

- Data suggest that leptin released during IH may modulate the activity of carotid body glomus cells and the carotid chemoreceptor reflex.

6.3   Conclusions Chapter 4

- Carotid bodies express leptin, and the long form (OB-Rb) and short form (Ob-R100) leptin receptors.

- Conscious animals were exposed to short term (7 days) and long term (95 days) chronic intermittent hypoxia (CIH).

- CIH induced changes in leptin, OB-Rb, pSTAT3, SOCS3 and pERK 1/2 protein expression in carotid body glomus cells.

- Data suggest that leptin, through its receptor and downstream signaling proteins, may contribute to alterations in carotid chemoreceptor sensitivity.

6.4   Conclusions Chapter 5

- Carotid bodies express the angiotensin type-1 receptor (AT$_1$R) which is co-expressed in cells expressing leptin and the long-form leptin receptor (OB-Rb).

- Angiotensin converting enzyme (ACE) inhibition decreases circulating and carotid body leptin protein expression in normal animals and in IH-exposed animals.

- ACE inhibition affects downstream signaling proteins of OB-Rb signaling.

- Data suggest angiotensin signaling modulates carotid body leptin signaling activity.

6.5 Overall Conclusion

Taken together, the data presented describe an active leptin-signaling system within the carotid body which may play an important role in modulating the carotid chemoreceptor reflex and describe a potential angiotensin-related mechanism by which these changes come about. The data provide evidence for conditions during IH where both circulating and local, carotid body-produced leptin are increased and may influence the activity of type-I glomus cells of the carotid body. Additionally, the leptin produced by type-I cells may act both pre- and post-synaptically to signal in an autocrine/paracrine fashion in the carotid body or signal within the CNS to exert an action on the carotid chemoreceptor reflex.

Exposure to IH for differing amounts of time has varying effects on downstream indicators of leptin signaling within the carotid body which may be due to activation of homeostatic mechanisms that change circulating factors, such as catecholamines, angiotensin II and leptin itself. Additionally, the sympathetic efferent innervation of the

carotid body may play a role in the leptin system as it is well known to affect leptin production and secretion in tissue such as adipocytes and may be of interest in future studies.

The finding of an active leptin system within the carotid body has major implications in conditions of increased circulating leptin including OSA and obesity. The findings provide a pathway by which SNA and thus arterial pressure may be elevated in these cases, specifically during daytime normoxia in OSA patients. Further studies are required to disover the exact role of leptin within the carotid chemoreceptor reflex, specifically whether it alters glomus cell activity only, altering sensitivity of the cells or inducing transmitter release, or whether it can act directly on PG nerve terminals and directly signal the reflex.

Future studies should involve the use of OB-Rb-deficient animals, leptin deficient animals and administration of a leptin antagonist to confirm that the activation of downstream OB-Rb signalers are indeed due to leptin signaling and not from other potential mechanisms, such as angiotensin signaling. Electrophysiological (patch clamp) studies using type-I cells in culture would be critical in determining the effect of leptin on both the function of type-I cells as well as on petrosal ganglion neurons or carotid sinus nerve afferents. Furthermore, Ca^{2+} imaging of glomus cells may provide evidence of leptin's cellular action on chemoreceptor cell sensitivity to PO_2. Coupled with the Ca^{2+} imaging, the media in which these cells are cultured should also be investigated to determine which, if any, peptides are secreted as a result of leptin application, including leptin itself. Additionally, ANG II application to these carotid body cells in culture may help support the idea of ANG II modulates the leptin system.

Many of the studies presented in this manuscript would benefit from qPCR techniques. One criticism of the results, specifically leptin levels in the carotid body, is the fact it is not directly known how the changes come about. The increases or decreases in leptin levels may come about by changes in production, secretion or degradation and mRNA levels may give a better indication of which mechanism is occurring.

Finally, using an *in vivo* approach, physiological measurements including blood pressure, sympathetic and phrenic nerve activity would be critical in elucidating the function of leptin in the carotid body to chemoreception. Particularly useful would be analyzing physiological measurements of chemosensitivty using the OB-Rb-deficient Zucker rat or a leptin-deficient animal model.

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Curriculum Vitae

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Graduate Teaching Assistantship   **2011-2013**

Ontario Graduate Scholarship   **2012-2013**

Refereed Publications

Messenger SA, Moreau JM, Ciriello J, 2012. Intermittent Hypoxia and Systemic Leptin Administration Induces pSTAT3 and Fos/Fra-1 in the Carotid Body. *Brain Res*. 1446: 56-70.

Figure 5c Selected for cover of *Brain Research* Issue 1446:March *from* **Messenger SA**, Moreau JM, Ciriello J, 2012. Intermittent Hypoxia and Systemic Leptin Administration Induces pSTAT3 and Fos/Fra-1 in the Carotid Body. *Brain Res*. 1446: 56-70.

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Professional Affiliations

Canadian Obesity Network, Member, 2010

Society for Neuroscience, Student Member, 2011

American Physiological Society, Student Member, 2011-2013

Ontario Ginseng Innovation and Research Consortium, Member, 2009

Professional Conferences Attended

Neuroscience 2011, Society for Neuroscience, Washington D.C.; November 12-16, 2011.

Experimental Biology 2012, FASEB, San Diego CA; April 21-25, 2012.