A CHRONIC PAIN: INFLAMMATION-DEPENDENT CHEMORECEPTOR ADAPTATION IN RAT CAROTID BODY

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Running head: Inflammation and Chemoreceptor Adaptation

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Abstract

Experiments in recent years have revealed labile electrophysiological and neurochemical phenotypes in primary afferent neurons exposed to specific stimulus conditions associated with the development of chronic pain. These studies collectively demonstrate that the mechanisms responsible for functional plasticity are primarily mediated by novel neuroimmune interactions involving circulating and resident immune cells and their secretory products, which together induce hyperexcitability in the primary sensory neurons. In another peripheral sensory modality, namely the arterial chemoreceptors, sustained stimulation in the form of chronic hypoxia (CH) elicits increased chemoafferent excitability from the mammalian carotid body. Previous studies which focused on functional changes in oxygen-sensitive type I cells in this organ have only partially elucidated the molecular and cellular mechanisms which initiate and control this adaptive response. Recent studies in our laboratory indicate a unique role for the immune system in regulating the chemo-adaptive response of the carotid body to physiologically relevant levels of hypoxia.

Key words: Cytokines, Macrophages, Hypoxia, Voltage-gated Sodium Channels, Neuroplasticity

Introduction

Oxygen chemoreceptors in the carotid body continuously monitor arterial blood PO_2 as part of a complex autonomic mechanism that matches lung ventilation and cardiac output to systemic tissue oxidative metabolism. Acute hypoxia excites oxygen-sensitive type I cells in carotid body and elevates impulse traffic in the carotid sinus nerve (CSN) which reflexly increases ventilation. Under controlled conditions, CSN responses to repeated acute hypoxic challenges are highly reproducible. However, a peculiar aspect of carotid body function is that following chronic hypoxia (CH), chemoreceptor activity evoked by an acute challenge is substantially enhanced. This increased activity has been shown to underlie ventilatory acclimatization to hypoxia (VAH), a phenomenon in mammals characterized by a gradual increase in ventilation which occurs over a period of days-to-weeks at high altitude and in hypobaric hypoxia(Powell, Milsom et al. 1998). The cellular and molecular mechanisms underlying CH-induced chemoreceptor adaptation are only partially understood. The current review highlights the role of unique neuroimmune mechanisms in the adaptive process. Available data suggest that chemoreceptor plasticity is analogous to adjustments induced in the peripheral nervous system during chronic inflammatory pain.

The Carotid Body and Adaptation to Chronic Hypoxia

The carotid body consists of integrated units of chemoreceptor, neural, glial and vascular cells surrounded by connective tissue, which collectively constitute a highly adaptive chemosensory organ. Stimulus transduction occurs in paracrine type I (chemoreceptor) cells which release multiple neuro-active agents in response to hypoxia, hypercapnia and acidosis. Primary afferent neurons in the petrosal ganglia (PG) project axons through the carotid sinus nerve (CSN) to form synaptic terminals on type I cells, while glial-like type II cells envelop the type I cells and terminal axon enlargements, forming lobules which are embedded in connective tissue penetrated by a microvascular network of highly permeable sinusoidal capillaries. Fibroblasts, resident macrophages, and a small number of mast cells are also present in the tissue, together with post-ganglionic parasympathetic and sympathetic axons(McDonald ,1981). According to the current view of low O_2-

transduction, specialized K^+-channels close when ambient O_2 decreases, initiating Ca^{2+} entry and excitatory neurotransmitter(s) release by type I cells. This leads to activation of chemoafferent neurons projecting to the nucleus tractus solitarius (NTS) of the medulla (Buckler ,1999;Gonzalez, ~~Almaraz~~ et al. 1994).

Adaptation to CH involves remarkable morphological and physiological adjustments in the rat carotid body, including altered morphology, gene expression and increased chemosensitivity in the initial 1-3 days of exposure to hypobaric hypoxia (380 Torr)(Chen, ~~He~~ et al. 2002a;Chen, ~~He~~ et al. 2002b;Dinger, ~~He~~ et al. 2003). Such changes subsequently include elevated expression of vasoactive agents: vascular endothelial growth factor (VEGF), endothelin (ET), atrial natriuretic peptide (ANP) and nitric oxide (NO), which contribute to tissue remodeling and/or modified chemosensitivity (Chen, ~~He, Dinger, Stensaas, and Fidone~~ et al. ,2002a;Chen, He, Dinger, Stensaas, and Fidone ,2002b;He, Chen et al. 1998;He, Dinger et al. 2000;Dinger, He, Chen, Stensaas, and Fidone ,2003). Moreover, rat type I cells have been shown to increase Na^+-channel (Na_V 1.1) expression in CH, an adaptation which may contribute to enhanced cell depolarization and neurotransmitter release(Caceres, Obeso et al. 2007). Adaptive changes to CH are also observed in chemoafferent neurons of the PG, which involve the expression of cholinergic receptors, and altered purinergic and cholinergic chemotransmission between type I cells and chemoafferent neurons (He, Dinger et al. 2005;He, Chen et al. 2006). In addition, we have documented elevated expression of the gap-junction-forming protein, connexin-43 in type I cells and PG neurons (Chen, He, Dinger, Stensaas, and Fidone ,2002b).

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Given their exquisite sensitivity to oxygen, it has long been assumed that type I cells initiate and regulate adaptive processes via the autocrine and paracrine action of their secretory products. Consistent with this assumption, studies of these cells following CH have demonstrated phenotypic changes consistent with increased excitability(Bisgard ,2000;Wang and Bisgard ,2002). However, the alternate possibility that CH-induced adaptation involves other cell types in the carotid body has been virtually ignored.

Moreover, little consideration has been given to the hypothesis that chemoreceptor adaptation is dependent on major changes in the excitability of chemoafferent neurons.

The Chronic Pain Model of Sensory Neuron Adaptation

Amongst sensory systems, altered sensitivity in response to chronic stimulation is not unique to the carotid body. In fact, it has long been known that chronic inflammation or neuropathy induces chronic pain states characterized by hyperalgesia and allodynia. Within the last decade it has become widely recognized that nociceptor hyperexcitability involves unique neuroimmune mechanisms(Watkins and Maier ,2002). Numerous studies of chronic pain have demonstrated that invading macrophages and neutrophils, as well as resident mast cells and dendritic cells induce remodeling and hypersensitivity of primary nociceptor neurons (Watkins and Maier ,2002). In addition, mechanoreceptor neurons that do not normally signal pain initiate the production of "pain neurotransmitters" consequent to the action of inflammatory mediators (Neumann, Doubell et al. 1996). Even cells which are not commonly associated with immune function may participate in the alteration of peripheral nerve function. For example, fibroblasts become a source of chemoattractant chemokine molecules [e.g., macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1)] that recruit circulating immune cells (primarily neutrophils and macrophages); this is followed by the production and secretion of proinflammatory cytokines(Watkins and Maier ,2002). Numerous studies of chronic inflammatory and neuropathic pain consistently demonstrate up-regulation of three cytokines: interleukin-1β (Il-1β), interleukin-6 (Il-6) and tumor necrosis factor-α (TNF-α), along with the chemokine, MCP-1(Watkins and Maier ,2002).

The growing body of evidence that supports a connection between immune cells and the development of altered tissue structure and hyperexcitability in chronic pain appears to provide a model for the role of immune cells and cytokines in altered carotid body structure, and the increased chemosensitivity in CH. In an effort to follow this lead we have set forth to determine 1), whether CH induces an inflammatory condition in the carotid body, 2), the effect of common anti-inflammatory drugs on chemoreceptor

adaptation, and 3), indications of CH/inflammation-induced phenotypic changes that are consistent with increased excitability in chemoafferent PG neurons.

CH-Induced Immune Cell Invasion and Cytokine Expression in Carotid Body

It is well known that immune cells express an isoform of the enzyme NADPH oxidase (Nox2) which uses O_2 and the cofactor NADPH to produce reactive oxygen species (ROS) as part of a killing mechanism in tissues invaded by infectious microbes. Experiments in our laboratory established that Nox2 and Nox4 are increased in carotid body in CH(He, Liu et al. 2010). These findings may reflect increased Nox expression by resident cells in the organ and/or they may be due to invasion of activated immune cells, primarily monocytes and neutrophils. Circulating monocytes commonly differentiate into ED1+ macrophages following diapedesis. We used fluorescent immunocytochemistry to establish the time-course of ED1+ cell activity in carotid body following 0, 1, 3 and 7 days of CH @ 380 Torr(Liu, He et al. 2009). Figure 1 shows that ED1+ cells are rare in normal carotid body, and that their presence is only slightly increased following 1 day of CH. However, following 3 days of CH the population of ED1+ cells is substantially elevated. After 7 days the incidence of macrophages remains high, but somewhat less than in organs from 3 day CH rats.

The increased presence of immune cells in the carotid body is correlated with elevated expression of inflammatory cytokines(Liu, He, Stensaas, Dinger, and Fidone ,2009). Quantitative PCR studies (see figure 2) showed that 24 hr of hypoxia elicited a 2-3-fold increase in MCP-1, IL-1β and TNFα. The lattermost cytokine remained at similar levels following 3 and 7 days of CH, whereas IL-1β was equally elevated following 3 days, but fully recovered after 7 days. In contrast the chemoattractant chemokine, MCP-1 was further elevated beyond 6-fold after 3 days, and it remained at a similar level on day 7. CH induced a more gradual increase in IL-6 which was measured at ~3-fold and ~2-fold following 3 and 7 days of CH, respectively. Interestingly, following 28 days of hypoxia the expression of inflammatory molecules was normal, with the exception of IL-6 which was some 5.5-fold above normal, suggesting that this latter cytokine may play a maintenance role in chemoreceptor adaptation. Support for the validity of the qPCR

assays was provided by assessments of the mRNA transcript level of tyrosine hydroxylase (TH), which is known to increase in type I cells during CH (figure 2).

Additional studies in our laboratory showed that cytokine production was not limited to immune cells(Liu, He, Stensaas, Dinger, and Fidone ,2009). Indeed, *in situ* hybridization experiments showed that IL-6 expression during CH is increased to high levels in putative type II cells, while a smaller but substantial elevation in probe signal was localized to cell clusters displaying morphology consistent with type I cells. We also used amplified RNA (aRNA) technology to demonstrate that 24 hr of *in vivo* hypoxia elevated MCP-1, IL-1β, TNFα and IL-6 in type I cells isolated in culture. These findings are in agreement with Lam et al.(Lam, Tipoe et al. 2008), who used immunocytochemical techniques to show elevated expression of inflammatory cytokines in type I cells following 7 days of CH. These studies also support the concept that virtually all cells are capable of cytokine production(Oppenheim J J and Feldmann M ,2000).

Effect of Anti-Inflammatory Drugs on Chemoreceptor Adaptation

Studies of inflammatory pain in rats have shown that small doses of the common anti-inflammatory drugs ibuprofen and dexamethasone prevent phenotypic changes associated with hyperexcitability in DRG neurons(O'Rielly and Loomis ,2008;Huang, Hsu et al. 2010;Voilley, de et al. 2001;Teixeira, Oliveira et al. 2010). Ibuprofen is a well established non-selective inhibitor of cyclooxygenase 1 and 2 (COX1 and COX2)(Rainsford ,2003). In addition, this drug has been shown to block the nuclear translocation of the transcription factor, nuclear factor kappa beta (NF-κβ), which mediates cytokine production including IL-1β, TNFα and IL-6(Scheuren, Bang et al. 1998). A potentially important physical property of ibuprofen is its tendency to partition into low pH compartments, such as local areas of inflammation(Brune and Lanz ,1985;Brune, Graf et al. 1976). The steroid, dexamethasone, operates via nuclear receptors to block the cascade of cytokine synthesis and release. Studies have shown that dexamethasone blocks the early inflammation-induced release of IL-1β and TNFα(Haynes ,1990). IL-6 production is also suppressed at least in part due to its dependence on the actions of IL-1β and TNFα(Haynes ,1990).

Figure 3 shows the effects of these drugs on hypoxia-evoked chemoreceptor discharge recorded in carotid body-CSN preparations superfused *in vitro*. The upper panel demonstrates the effect of CH on carotid body excitability in a side-by-side comparison of integrated nerve activity in a normal versus 9-day CH preparation. Following CH, the activity evoked by a standardized acute hypoxic stimulus (bath PO2 recorded via separate trace) is approximately doubled, as indicated in the summary histogram of 4 preparations. The middle and lower panels show the effect of concurrent administration of ibuprofen (4 mg/kg/day, i.p.) or dexamethasone (0.1 mg/kg/day, i.p.) in non-hypoxic (normal) and CH animals. The results indicate that treatment with the anti-inflammatory drugs during CH completely blocks chemoreceptor adaptation.

Parallel studies evaluated the effect of these anti-inflammatory drugs on cytokine expression in the carotid body. The data in figure 4A indicate that ibuprofen inhibits more than 50% of the increase in expression of MCP-1, TNFα and IL-6 induced by 7 days of CH. As shown in figure 2, IL-1β expression is fully recovered at this time point, and the presence of ibuprofen had no significant effect on the expression of this cytokine. It is also noteworthy that ibuprofen did not significantly alter CH-induced up-regulation of TH mRNA, suggesting that this phenomenon occurs independently of the inflammatory response. In contrast, the increase in TH expression was enhanced during CH in animals treated with dexamethasone (figure 4B), a finding which concurs with previously demonstrated effects of this drug on normal carotid body(Hanbauer ,1976). Moreover, this steroid potently blocked expression of all cytokines, even lowering IL-1β to a level slightly below normal.

Confirmation of the drug effects on immune cell activity was achieved by evaluating the inmmunostaining intensity of immune specific antigen CD45 in carotid body. Virtually all immune cells express this antigen which is known to be increased in inflamed tissue. The images in figure 5 show that the low CD45 intensity (green) in normal carotid body is substantially enhanced in tissue from a 3-day CH rat. Immunostaining for TH (red) is also greatly elevated in the CH tissue. Treatment with ibuprofen (4 mg/kg/day) or

dexamethasone (0.1 mg/kg/day) lowered CD45 expression in normal tissue, and eliminated the increase induced by CH. Consistent with qPCR assays, increased immunostaining for TH following CH was not altered by ibuprofen, but it appeared to be slightly elevated by treatment with dexamethasone in both normal and CH tissue.

Inflammation and Primary Sensory Neuron Excitability

Numerous studies have shown that inflammation in the terminal fields of peripheral nerves induces phenotypic changes in primary sensory neurons, leading to enhanced excitability. Behavioral assessments have consistently demonstrated lowered mechanical and thermal stimulus thresholds and decreased latencies for reflexes elicited from inflamed peripheral fields. These changes have been correlated with altered gene and/or protein expression in corresponding DRG sensory neurons. Amongst the up-regulated genes are those coding for specialized transduction/receptor molecules, including ATP-gated purinergic receptors ($P2X_N$)(Barclay, Patel et al. 2002;Jarvis ,2010), acid sensitive ion channels (ASICs)(Mamet, Baron et al. 2002;Voilley, de, Mamet, and Lazdunski ,2001), and the multimodal transient receptor potential vallinoid-1 (TRPV1) receptor, which is sensitive to temperature, pH and selected chemical stimuli(Stucky, Dubin et al. 2009). Importantly, pharmacological manipulations which block inflammation-induced expression of transducer molecules, also decrease the associated hyperalgesia and allodynia(Voilley, de, Mamet, and Lazdunski ,2001;Barclay, Patel, Dorn, Wotherspoon, Moffatt, Eunson, Abdel'al, Natt, Hall, Winter, Bevan, Wishart, Fox, and Ganju ,2002).

In addition to the altered expression of transducer molecules, numerous studies by Waxman and his colleagues(Cummins, Sheets et al. 2007) have shown that inflammation induces altered expression of selected proteins that comprise voltage-gated Na^+-channels (VGSC or Na_V), which are generally responsible for the rapid up-stroke of the action potential in peripheral axons. VGSC consist of α- and β-subunits, with the latter serving a supporting role as stabilizers and modulators of α-subunit function. Thus specific channel properties, including gating, kinetics and conductance are determined primarily by 9

isoforms of the α-subunit found in mammals(Cummins, Sheets, and Waxman ,2007). Molecular analysis has revealed that primary sensory neurons in DRG express at least 5 α-subtypes including the tetrodotoxin (TTX)-sensitive $Na_V1.1$, $Na_V1.6$, $Na_V1.7$; and the TTX-resistant $Na_V1.8$ and $Na_V1.9$(Waxman, Cummins et al. 2000). Models of neuropathic pain (e.g., peripheral axotomy; nerve constriction) have shown down-regulation of $Na_v1.8$ and $Na_v1.9$, and the induced expression of a previously silent TTX-sensitive (TTX-S) channel, $Na_V1.3$(Waxman, Cummins, Dib-Hajj, and Black ,2000). In contrast, a recent study of inflammatory pain (subcutaneous carrageenan injection) revealed a different pattern of Na^+-channel expression in which $Na_V1.3$, $Na_V1.7$ and $Na_V1.8$ are selectively increased in small (<30μm) DRG neurons(Black, Liu et al. 2004). Electrophysiological assessments of sensory neurons have demonstrated altered action potential characteristics consistent with increased excitability, suggesting that modified expression of Na^+-channels is a critical factor in hyperalgesia(Waxman, Cummins, Dib-Hajj, and Black ,2000). The fact that administration of nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF) prevent hyperexcitability following nerve injury supports the hypothesis that altered channel expression is due to the failure of retrograde axonal transport of growth factors in damaged axons (Waxman, Cummins, Dib-Hajj, and Black ,2000;Leffler, Cummins et al. 2002). On the other hand, phenotypic changes in Na^+-channel expression in inflammation may be due to elevated levels of NGF and other mediators released near inflamed nerve terminals(Black, Liu, Tanaka, Cummins, and Waxman ,2004;Frostick, Yin et al. 1998). A particularly relevant observation is that concurrent treatment with ibuprofen reduces inflammation-induced hyperalgesia as well as expression of $Na_V1.7$ and $Na_V1.8$(Gould, III, England et al. 2004).

Donnelly and his colleagues explored the properties of voltage-sensitive Na^+-currents in rat PG neurons which were back-labeled with a fluorescent dye applied to the carotid body(Cummins, Dib-Hajj et al. 2002). These authors reported that in ~80% of chemoafferent neurons >95% of the Na^+-current was TTX-sensitive. Moreover, detailed analysis of current kinetics indicated the involvement of $Na_V1.7$, along with $Na_V1.1$ and/or $Na_V1.6$. These findings concur with a previous study showing that spontaneous

activity generated in the terminals of rat carotid chemoreceptors is highly sensitive to TTX(Donnelly, Panisello et al. 1998).

We recently initiated a preliminary study of the effect of CH on Na^+-channel expression, and voltage-sensitive Na^+-current in PG neurons. Consistent with reports that inflammation up-regulates Na_V expression(Black, Liu, Tanaka, Cummins, and Waxman ,2004), our immunocytochemical data shown in figure 6 demonstrate increased Na_V staining intensity in PG neurons following 3 days of CH. These images were produced using a pan-specific antibody which recognizes all α-isoforms. Indication of specific Na_V involvement is suggested in figure 7, which shows that 3 days of CH elicits an approximately 16-fold increase in expression of $Na_V1.7$ transcript in the PG. Note that the transcript level was normal following 1 day of hypoxia, consistent with the possibility that increased expression involves retrograde signaling from

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In a 2004 study, Black, et al., demonstrated that hyperalgesia associated with inflammation is correlated with increased Na^+-current density in small diameter (≤ 25 μm) DRG neurons(Black, Liu, Tanaka, Cummins, and Waxman ,2004). In similarly sized PG neurons we have found that Na^+-currents are likewise increased following 4-5 days of CH. The family of membrane current traces shown in figure 8 were obtained using a holding potential of -120mV, and currents were evoked by 20 msec voltage steps (5mV) to potentials between -80mV and +40mV. Measurement of peak current amplitude indicates a 50% increase following CH (fig. 8, lower panel). In view of these findings it is intriguing that Waxman and his colleagues proposed that the unique kinetic properties of $Na_V1.7$ make it a candidate for activation in response to the slow depolarization elicited by chemical, mechanical and/or thermal stimuli in sensory nerve terminals(Cummins, Howe et al. 1998;Cummins, Sheets, and Waxman ,2007). Moreover, selective knock-out of $Na_V1.7$ in a subpopulation of small diameter neurons resulted in decreased thermal and mechanical hypersensitivity induced by inflammation(Nassar, Stirling et al. 2004). Consequently, increased expression of this isoform may, in part, underlie the observed hyperexcitability in nociceptors, as well as chemoreceptors, following inflammation. A more complete understanding of the role of inflammation in

altered Na^+-channel expression will come with an evaluation of the effect of anti-inflammatory drugs on Na_V transcript levels and Na^+-currents in chemoafferent neurons. In addition, a role for Na^+-currents may be further defined by examining the effects of CH on chemoreceptor adaptation in selected Na_V gene-deleted animals.

The Chemoafferent Pathway and Inflammation: Future Directions

Our recent studies have expanded upon earlier work which demonstrated that oxygen-sensitive type I cells in the carotid body express cytokine receptors, and that CH elicits cytokine expression in the organ(Shu, Wang et al. 2007;Wang, Wang et al. 2002;Wang, Zhang et al. 2006;Lam, Tipoe, Liong, and Fung ,2008;Fan, Zhang et al. 2009). The extent of neuroimmune involvement in CH was further elucidated by documenting immune cell invasion, and examining the effects of anti-inflammatory drugs on adaptation. These findings may have implications for the use of common anti-inflammatory agents in the treatment of COPD and chronic heart failure, in addition to their use at high altitude.

Major unresolved issues remain for future investigations of inflammation-mediated chemoreceptor adaptation. At this writing virtually nothing is known about the cellular mechanisms which trigger immune cell invasion and the up-regulation of cytokines. Because of their exquisite sensitivity to hypoxia, it is likely that type I cells are involved in signaling the immune response. As noted earlier, type I cells are highly adaptive to CH; it is likely that following the initial immune response, cytokines and their receptors participate in altering the functional status of type I cells. In this regard, it is important to note that previous studies of type I cell adaptation have demonstrated the involvement of hypoxia inducible factor-1 (HIF-1), a transcription factor known to be up-regulated by cytokines in multiple cell types(Hellwig-Burgel, Stiehl et al. 2005).

In addition, it appears that the full extent of adaptation involves significant changes in the functional status of primary chemoafferent neurons, particularly the expression of specific receptor molecules in nerve terminals. Moreover, animal models of chronic pain

indicate that plasticity of the neuronal phenotype is influenced by local and retrograde signaling of neurotrophic factors produced in inflamed targets(Malin, Molliver et al. 2006;Cummins, Sheets, and Waxman ,2007). Therefore, important regulatory mechanisms may be elucidated by investigating the production and dynamics of selected factors in the inflamed carotid body. Finally, it is well established that inflammation and neuropathy initiate complex changes within the spinal cord, including the up-regulation of inflammatory cytokines. The resulting central sensitization is critical for the full development of hyperalgesia and allodynia(Woolf ,2010). Thus the studies of Powell and his colleagues (in press, this issue Resp. Physiol. Neurobiol.), which show that central amplification of chemoreflex signaling following CH is blocked by ibuprofen, further implicate the global involvement of unique neuroimmune mechanisms in the control of breathing.

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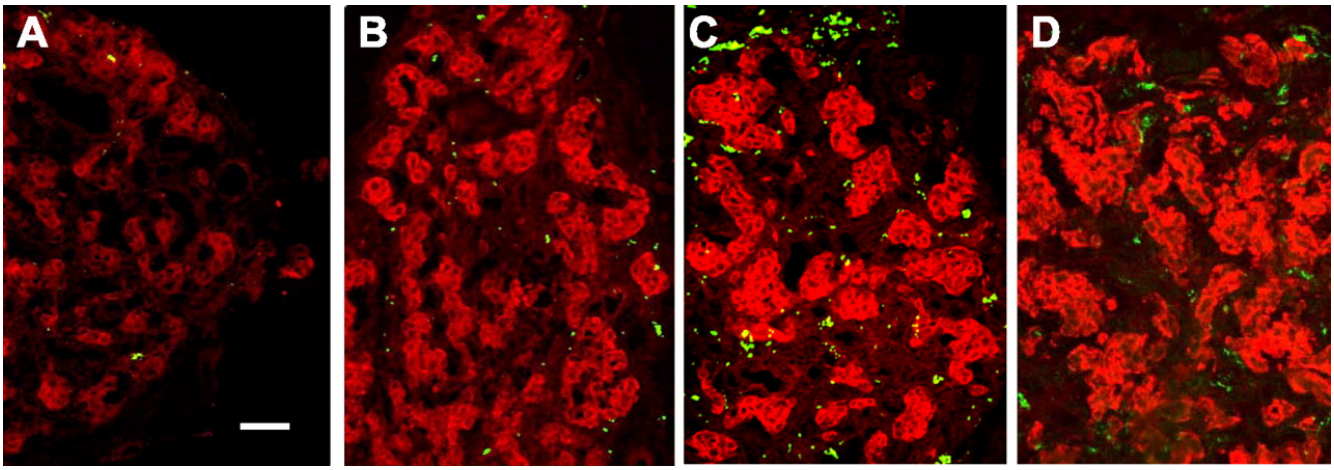

Figure 1. Immunofluorescence in rat carotid body of type I cell marker tyrosine hydroxylase (red, TH), and immune cell antigen ED1 (green). A: normal; B, C, and D:1, 3 and 7 days of CH, respectively. Scale bar: 50 um. (From Liu, He et al. 2009)

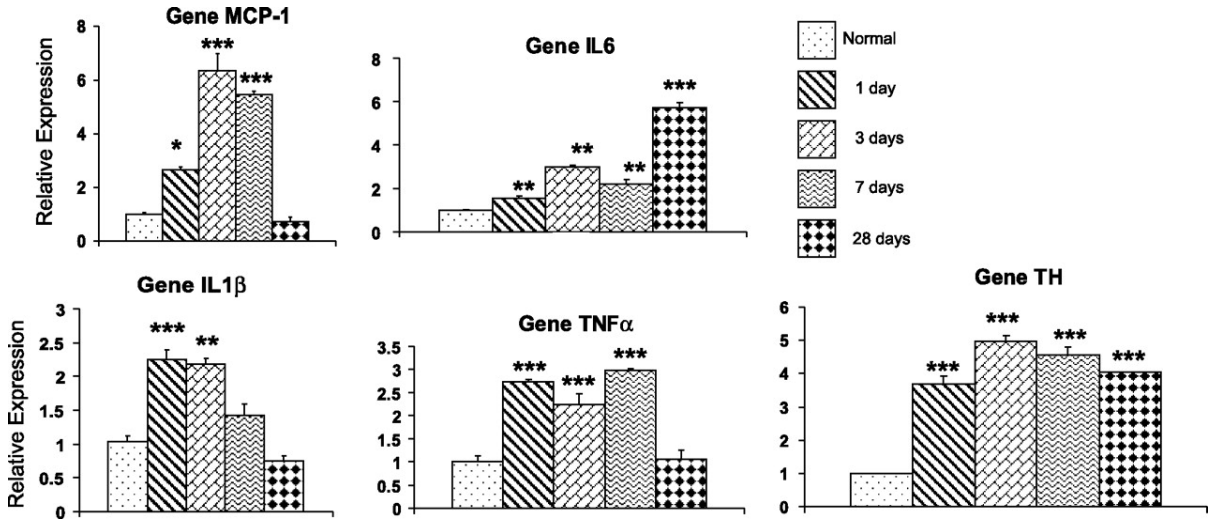

Figure 2. Time course of chronic hypoxia (CH)-induced inflammatory cytokine, and tyrosine hydroxylase (TH) gene expression in rat carotid body. Quantitative PCR data normalized to 18sRNA and expressed relative to mRNA levels in normal tissue. MCP-1: monocyte chemoattractant protein-1; IL6: interleukin-6; IL 1b: interleukin-1β; TNFa: tumor necrosis factor-α. *, **, and *** indicate $p<0.05$, 0.01 and 0.001, respectively. CH: exposure at 380 Torr for time indicated. (From Liu, He et al. 2009)

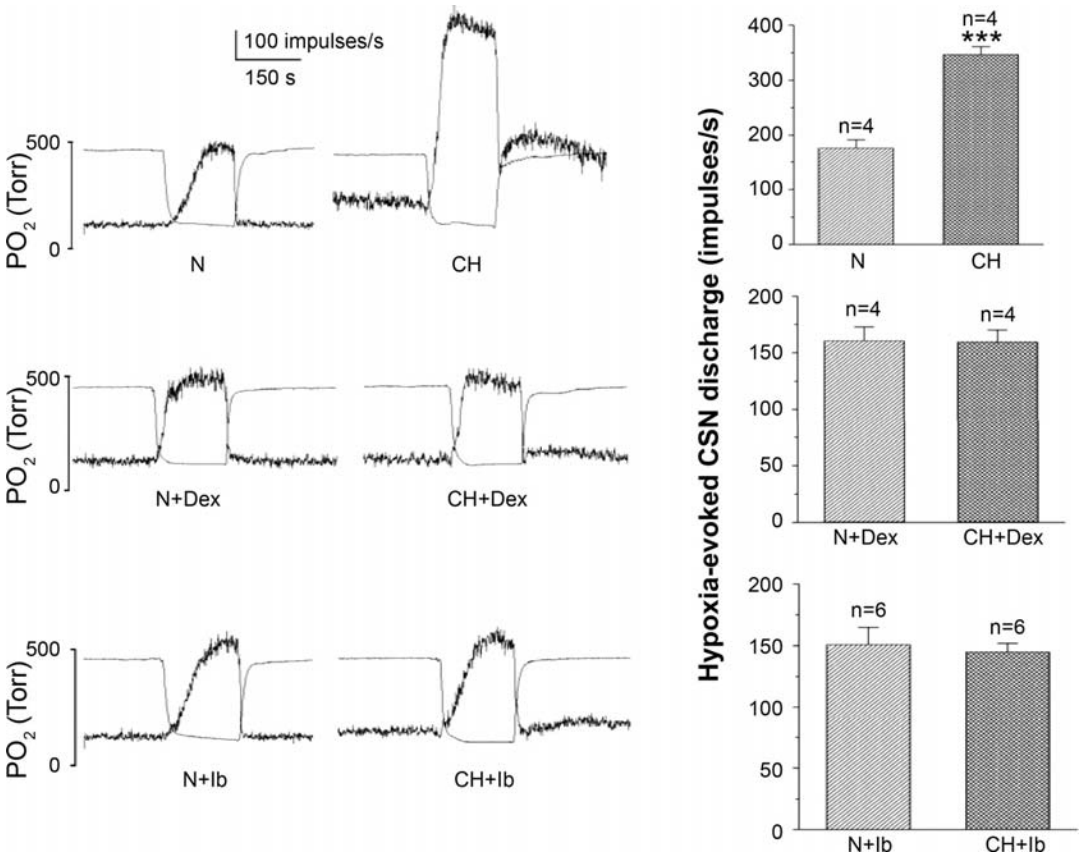

Figure 3. *Left panel*: Carotid sinus nerve activity evoked by a standard hypoxic stimulus (indicated by separate trace of bath PO2) in normal (N) vs. 8-10 day chronic hypoxia (CH) preparations. CH elicits a robust increase in hypoxic sensitivity and basal resting activity; however, note that hypersensitivity to hypoxic challenge is absent in CH animals concurrently treated with dexamethasone (Dex; 0.1 mg/kg/day), or ibuprofen (Ib; 4 mg/kg/day). *Right panel*: Summary data (averaged evoked impulses/sec) from 4 or 6 preparations in each group: drug free (upper) or concurrently treated with either dexamethasone or ibuprofen (middle and lower). *** indicates p<0,001 vs. normal. (From Liu, He et al. 2009)

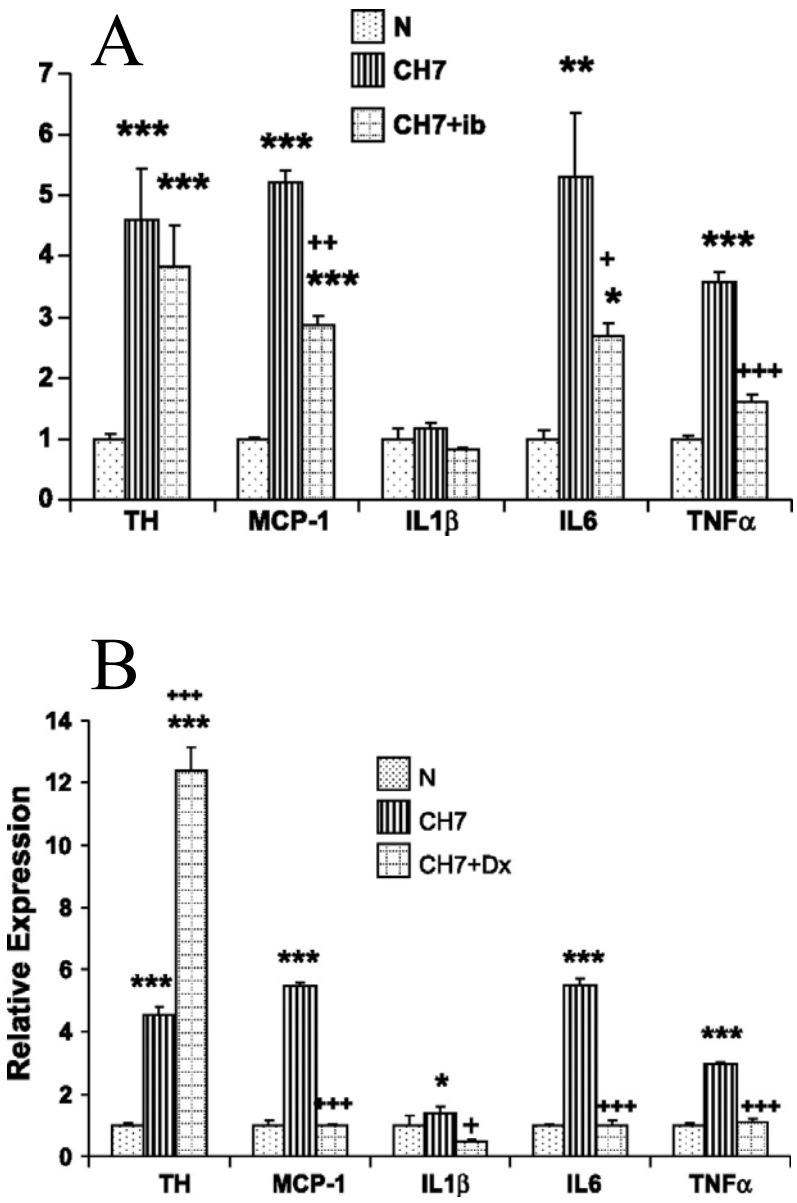

Figure 4. Effect of ibuprofen, A, (ib; 4 mg/kg/day) or dexamethasone, B, (DX; 0.1 mg/kg/day) on 7 day CH-induced gene expression of tyrosine hydroxylase and cytokines in rat carotid body. Abbreviations as in figure 2. *, **, and *** indicate p< 0.05, 0.01, and 0.001, respectively, vs. normal; +, ++, and +++, p< 0.05, 0.01, and 0.001, respectively, vs. 7 days CH. (Adapted from Liu, He et al. 2009)

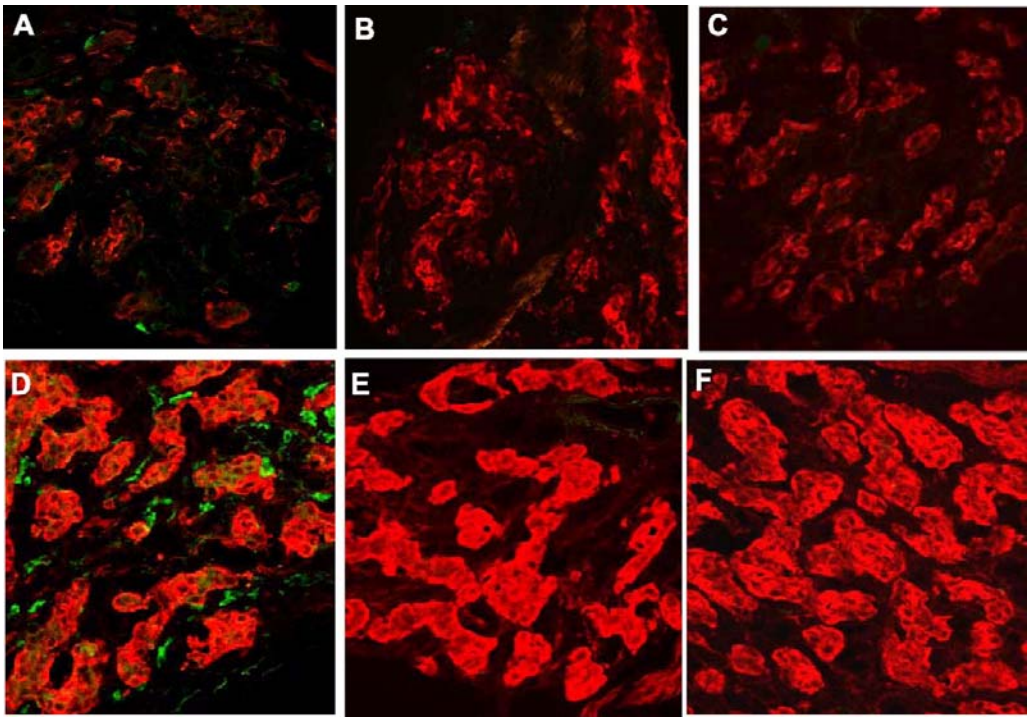

Figure 5. Effect of anti-inflammatory drugs on CH-induced immune cell activity in rat carotid body. Green cells are immunostained for CD45, a universal leukocyte marker; red indicates TH. (**A**) Normal carotid body contains a few immune cells that weakly express CD45. Following 3 days of CH (D) the tissue contains numerous cells which express higher levels of CD45. In normoxic carotid bodies from animals treated with dexamethasone (0.1 mg/kg/day; **B**) or ibuprofen (4 mg/kg/day; **C**) few or no detectable CD45+ immune cells are visible. Likewise, in CH animals treated with dexamethasone (**E**) or ibuprofen (**F**), CD45+ cells are virtually absent. Notice that TH fluorescence is increased in animals treated with dexamethasone. (From Liu, He et al. 2009)

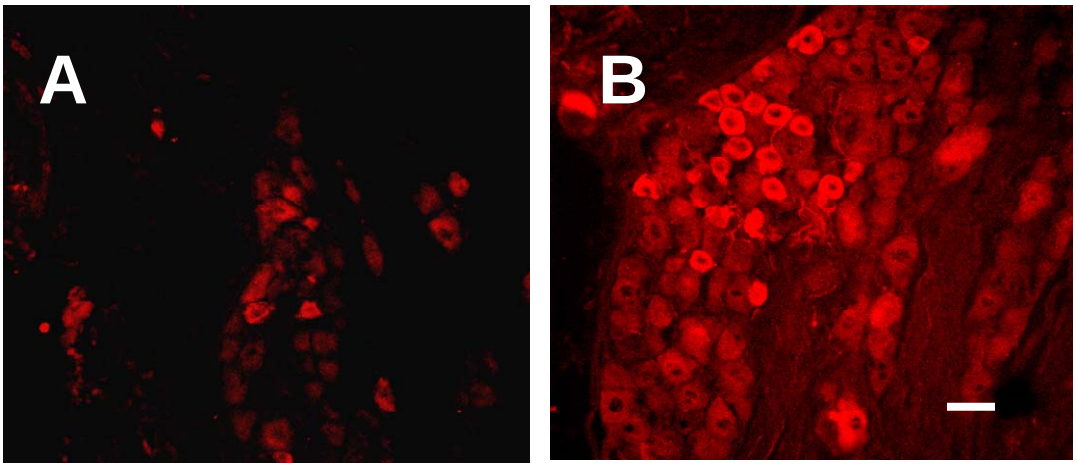

Figure 6. Immunofluorescent assessment of Na+-channel expression in petrosal (IXth nerve) ganglion from normal (A) and 3 day CH rats. Formaldehyde-fixed frozen sections were immunostained with pan-specific antibody (Sigma, St. Louis, MO; product #S6936) which recognizes all α-Na_V subunits. Scale bar, 30 um.

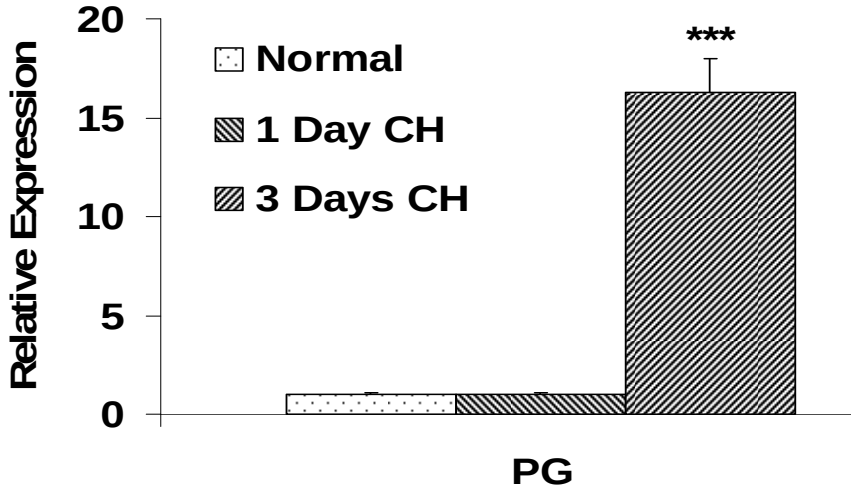

Figure 7. Time course of chronic hypoxia (CH)-induced up-regulation of $Na_V1.7$ α-subunit of voltage gated Na^+-channel. Quantitative PCR data normalized to 18sRNA and expressed relative to mRNA levels in normal tissue. *** indicates $p< 0.001$. CH: exposure at 380 Torr for time indicated.

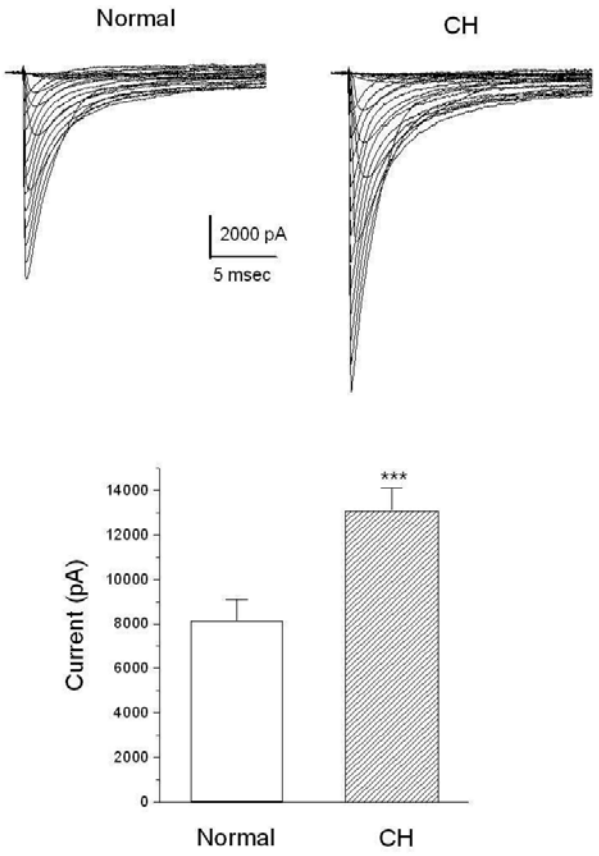

Figure 8. *Upper panel*: Whole-cell patch clamp recording of family of Na^+-currents in petrosal ganglion neurons (≤ 25 μm) harvested from normal (left) and 5 day CH (380 Torr; right) adult male rats. Currents were evoked from a holding potential of -120mV by 20 msec steps (5mV) to potentials between -80mV and +40mV. *Lower panel*: Plot of peak current amplitude indicates a 50% increase following CH.