ADAPTATION TO CHRONIC HYPOXIA INVOLVES IMMUNE CELL INVASION AND INCREASED EXPRESSION OF INFLAMMATORY CYTOKINES IN RAT CAROTID BODY

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Running head: Chronic hypoxia-induced inflammation in carotid body

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Exposure to chronic hypoxia (CH: 3-28 days @ 380 Torr) induces adaptation in mammalian carotid body such that following CH an acute hypoxic challenge elicits an abnormally large increase in carotid sinus nerve (CSN) impulse activity. The current study examines the hypothesis that CH initiates an immune response in the carotid body, and that chemoreceptor hyperexcitability is dependent on the expression and action of inflammatory cytokines. CH resulted in a robust invasion of ED1+ macrophages which peaked on day 3 of exposure. Gene expression of pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα), and the chemokine, monocyte chemoattractant protein-1 (MCP-1), was increased more than 2-fold after 1 day of hypoxia, followed by a 2-fold+ increase in interleukin-6 (IL) on day 3. After 28 days of CH, IL-6 remained elevated >5-fold, whereas expression of other cytokines recovered to normal levels. Cytokine expression was not restricted to immune cells. Studies of cultured type I cells harvested following 1 day of in vivo hypoxia showed elevated transcript levels of inflammatory cytokines. In situ hybridization studies confirmed expression of IL-6 in type I cells, and also showed that CH induces IL-6 expression in supporting type II cells. Concurrent treatment of CH rats with anti-inflammatory drugs (ibuprofen or dexamethasone) blocked immune cell invasion and severely reduced CH-induced cytokine expression in carotid body. Drug treatment also blocked the development of chemoreceptor hypersensitivity in CH animals. Our findings indicate that chemoreceptor adaptation involves novel neuroimmune mechanisms which may alter the functional phenotypes of type I cells and chemoafferent neurons.
INTRODUCTION

The mammalian 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.

Prolonged and continuous exposure of mammals to a low-PO$_2$ environment (i.e., chronic hypoxia, CH) elicits adaptation in the carotid body involving remarkable morphological and physiological adjustments. These include altered gene expression and increased chemosensitivity in the initial 1-3 days of exposure to hypobaric hypoxia (3; 4; 9). In the rat, exposure to hypobaric hypoxia (380 Torr) for 9-14 days approximately doubles stimulus-evoked CSN activity in response to a standardized acute hypoxic challenge. Moreover, the resting nerve activity in these preparations is substantially elevated(3). 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. Indeed, numerous studies have documented altered expression of multiple excitatory, as well as inhibitory agents in the chemosensory tissue following CH(2; 27; 41).

Given the proven critical role of type I cells in the chemosensory process, it is not surprising that virtually no attention has been given to the possibility that increased excitability in CH is the consequence of changes initiated by the actions of other cells in the carotid body parenchyma. Interestingly, multiple recent studies have demonstrated that chronic pain in humans, and neuropathic and inflammatory pain in animal models, involve immune cell-induced hyperexcitability of primary sensory neurons(42). It is now well documented that invading macrophages and neutrophils, as well as resident mast cells and dendritic cells induce...
remodeling and hypersensitivity of primary nociceptor neurons (42). In addition, mechanoreceptor neurons that do not normally signal pain initiate the production of “pain neurotransmitters” subsequent to the action of inflammatory mediators (25). 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 and monocyte chemoattractant protein-1] that recruit circulating immune cells (primarily neutrophils and macrophages); this is followed by the production and secretion of proinflammatory cytokines (42). 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), in addition to the chemokine, monocyte chemoattractant protein-1 (MCP-1) (42).

The growing body of evidence that supports a connection between immune cells, inflammatory cytokines and the development of hyperexcitability in chronic pain appears to provide a model for the development of altered carotid body structure, and increased chemosensitivity in CH. The present study uses real-time quantitative PCR (qPCR) and immunofluorescence techniques to demonstrate that CH induces an inflammatory condition in rat carotid body. Furthermore, amplification of RNA from dissociated cell preparations, and in situ hybridization histochemistry have been employed to explore whether cytokine production occurs in type I cells, in addition to invading immune cells. Finally, common anti-inflammatory drugs, ibuprofen and dexamethasone, have been used to test the hypothesis that inflammation is a conditional requirement for the development of CH-induced chemosensory adaptation.

METHODS

Animals and Exposure to Chronic Hypoxia

Eighty seven rats exposed in a hypobaric chamber were housed in standard rodent cages with food and water. Pressures were reduced from ambient Bp at the University of Utah (i.e., Bp~630 Torr; 1500 m) until a selected pressure equivalent to ~2560m (565 Torr), ~3350m (515 Torr), or ~5500m (380 Torr) was reached, and maintained for a selected period (up to 28 days). The chamber was opened every one or two days to replenish food and water and change litter.
Control, normal animals (32 rats), were maintained outside the chamber in ambient conditions. Animal protocols were approved by the University of Utah Institutional Animal Care and Use Committee.

**Quantitative Reverse Transcriptase-Polymerase Chain Reaction (Real-Time qPCR)**

Carotid bodies were harvested from rats anesthetized with a mixture of ketamine (10mg/100g) plus xylazine (0.9mg/100g). Carotid artery bifurcations were located, excised and placed in a lucite chamber containing 100% O₂-equilibrated modified Tyrode solution at 0-4 °C (in mM: NaCl, 112; KCl, 4.7; CaCl₂, 2.2; MgCl₂, 1.1; sodium glutamate 42; HEPES buffer, 5; glucose, 5.6; pH = 7.4). Each carotid body was carefully dissected from the artery and cleaned of surrounding connective tissue. Tissues were then immediately frozen on Al-foil on dry ice. In accord with the kit instructions (RNAqueous-Micro, Ambion, Austin, TX) total RNA was extracted from homogenized tissue samples pooled from groups of 5 rats for each experiment. Following removal of contaminating DNA (DNase treatment), first strand complementary DNA was synthesized from 1μg total RNA (quantified with a NanoDrop ND-1000 spectrophotometer) using RETROscript (Ambion). Aliquots of cDNA corresponding to 2 ng of total RNA were introduced into a SybrGreen reaction mix (25 μl; Qiagen) containing ‘upstream’ and ‘downstream’ primers for selected cytokines (MCP-1, TNFα, IL-1β and IL-6). All primer pairs were “blasted” against known rat gene sequences. qPCR was conducted in an MJ Research PTC-200 equipped with a Chromo4 detector. From each pooled group of cDNA 3-5 PCR reactions were initiated at 95°C for 15 min followed by 40 cycles consisting of 30 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C, with the final cycle extended to 5 min at 72 °C. Product purity was evaluated by determination of the melting curve, after which samples were stabilized at 4 °C. Sample comparisons were based on the relative standard curve method (29) and data are normalized to 18S rRNA expression. In preliminary studies using cDNA aliquots equivalent to equal amounts of total RNA we found that 18S rRNA varies less than 10% in CH versus normal samples with p>0.24. Amplifications of samples not treated with RETROscript were performed to exclude possible contamination with genomic DNA.
Amplified RNA (aRNA)

Carotid bodies were dissected free of surrounding connective tissue and transferred to Ham's F-12 medium (Ca\(^{2+}\) and Mg\(^{2+}\)-free) containing 0.2% collagenase and 0.2% trypsin. Each organ was cut into 6 to 12 pieces and incubated for 40 min in a CO\(_2\) incubator (5% CO\(_2\), 95% air) at 36.5°C. Tissue fragments were rinsed (2 x 10 min, room temperature) in F-12 medium (Ca\(^{2+}\) and Mg\(^{2+}\)-free), transferred to poly-l-lysine coated glass coverslips, and triturated in a small volume of medium, plus 10% fetal calf serum and 5 μg/ml insulin. Dissociated single type I cells collected using a patch-clamp pipet were immersed in buffer provided in a PicoPure™ RNA isolation kit (Microgenomics/Arcturus), and total RNA was isolated according to kit directions. The RNA was further purified with RNeasy MinElute Cleanup kit (Qiagen). mRNA was amplified according to directions in the MessageBOOSTER cDNA Synthesis kit (Epicentre Biotechnologies).

Immunocytochemistry

Anesthetized rats were perfused intracardially with ice-cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Carotid bodies were removed, cleaned of surrounding connective tissue and immersed in the same fixative for 1 hour, rinsed in 20% sucrose/PBS for 2 hours and stored at 4°C in 30% sucrose/PBS for 1 hr. Cryostat sections (6 μm) were thaw-mounted onto gelatin subbed slides. Sections were treated for 20 min with 5% goat serum in PBS plus 0.1% TritonX-100 and then incubated at 4°C overnight in primary antibodies for tyrosine hydroxylase (TH) and the macrophage marker, ED-1 or the universal leucocyte marker, CD45, diluted (1:2000 for anti-TH [Chemicon]; 1:100 for anti-ED1 and anti-CD45 [Serotec]) in PBS containing 2% goat serum and 0.1% Triton X-100. Sections were then rinsed in PBS at room temperature, and incubated for 1 hr with selected secondary antibodies (diluted 1:200 to 1:400) conjugated with fluorescein or rhodamine in 2% goat serum plus 0.1% Triton X-100, and then rinsed in PBS for 20 min. In all experiments, normal versus experimental tissue samples and frozen sections were processed simultaneously, and all incubation and reaction conditions were identical. In selected sections the primary antibody was omitted to assess non-specific staining of the secondary fluorescent antibodies. Specimens are viewed in a Zeiss Model M30 laser scanning confocal microscope.
In situ Hybridization Histochemistry

Tissues harvested from rats were quick-frozen in OCT, sectioned (5-8 μm), mounted on gelatin subbed glass slides, fixed with 4% paraformaldehyde, dehydrated in an ascending series of ethanols, and stored at room temperature. Sections were rehydrated in a descending series of ethanols, incubated in proteinase K (10μg/ml; 37 °C, 9 min), washed in PBS, treated with triethanolamine (100μM, 2 min) and acetylated in 0.25% acetic anhydride (10 min). Following a wash in PBS, sections were dehydrated. A 463 base riboprobe for IL-6 was constructed from specified PCR products, along with a ligated restriction sites, and inserted into a pBluescript II KS+ (Stratagene). Restriction enzyme digests were linearized, followed by in vitro transcription incorporating the digoxigenin label (DIG RNA Labeling Kit, Roche Diagnostics, Germany). The probe was purified on a Micro Bio-Spin 30 column (BioRad). The hybridization solution consisted of 50% formamide, 600 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1X Denhardt’s solution, 0.25% SDS, 10% dextran sulfate, and 200μg/ml yeast tRNA. Solution was heated to 85 °C for 10 min; incubation at 85 °C continued for 3 min following addition of riboprobe. Sections were incubated overnight in a chamber humidified with 50% formamide @ 55°C. Slides were washed 2X in SSC (3M NaCl, 0.3M sodium citrate; pH 7.0) containing 50% formamide @ 60 °C for 30 min, followed by washes in 2X SSC (20 min, 60 °C) and 0.2 SSC (2X 20 min, 60 °C). Blocking proceeded in 10% normal sheep serum for 1 hr at room temperature, followed by treatment with alkaline phosphatase conjugated anti-digoxigenin antibody (1:1000) overnight at 4 °C. After washing, sections were incubated overnight in nitroblue tetrazolium salt (NBT, 0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate toludinium salt (BCIP, 0.16 mg/ml), in a buffer containing 0.1 M NaCl, 0.1 M Tris and 5 mM MgCl₂ (pH 9.5)(14).

Electrophysiological Recording of Carotid Sinus Nerve (CSN) Activity

As has been described previously(3), the carotid bifurcations were excised from rats under Ketamine/Xylazine anaesthesia and placed in a lucite chamber containing 100% O₂-equilibrated modified Tyrode solution at 0-4 °C. Each carotid body along with its attached nerve was carefully dissected from the artery and cleaned of surrounding connective tissue. Preparations were then placed in a conventional flow chamber where the carotid body was continuously superfused (up to 4 h) with modified Tyrode solution maintained at 37 °C and equilibrated with a selected gas mixture. The CSN was drawn up into the tip (~100 um, i.d.) of a glass suction
electrode for monopolar recording of chemoreceptor activity. Basal neural activity was established in superfusates maintained at $PO_2 = 450$ Torr. The $PO_2$ was lowered to 120 Torr in superfusates equilibrated with air to provide a moderately hypoxic stimulus. Neural activity was led to an AC-coupled preamplifier, filtered and transferred to a window discriminator and a frequency to voltage converter. Signals were processed by an AD/DA converter for display of frequency histograms on a PC computer monitor. Data were expressed as impulses per second, and analyzed using Student’s t-test and ANOVA.

RESULTS

CH-induced immune cell invasion and cytokine gene expression in carotid body

Immunofluorescence (IF) assessments indicate that increased numbers of invasive ED1+ macrophages(20) are present in the carotid body following hypoxia. In figure 1, type I cells immunostained for tyrosine hydroxylase (TH) are red, and macrophages expressing the ED1 antigen green. Macrophages are rare in normal carotid body, but their incidence increased noticeably after 1 day of CH. Moreover, 3 days of CH elicited a marked elevation in the incidence of ED1+ cells in perivascular and connective tissue, consistent with extravasation and differentiation of monocytes, the circulating precursors of macrophages. In addition, TH IF is elevated following 1 and 3 days of hypoxia, in accord with previous reports of CH-induced up-regulation of this catecholaminergic enzyme(6). After 7 days of CH, TH levels remain high in type I cells, but the incidence of macrophages, while above normal, has markedly receded.

The data in figure 2 show the effects of 1, 3, 7 and 28 days of CH (380 Torr) on the expression of the chemokine, MCP-1, and principal inflammatory cytokines, IL-6, IL-1β and TNFα. All values are expressed relative to levels measured in carotid bodies from control animals maintained at the University of Utah (1500m, ~630 Torr). A particularly prominent finding is the early elevation in carotid body after only 1 day of hypoxia, of transcript for the inflammatory mediators. These indicators of inflammation are also significantly increased on day 3; and 3 of the 4 cytokines remain elevated after 7 days of CH. However, following a 28 day exposure, only IL-6 remains at high levels; expression of MCP-1, IL-1β and TNFα is similar to normal in these long-exposure animals. Data in figure 2 also show a similar analysis of the effects of CH on expression of tyrosine hydroxylase (TH) in the carotid body. These findings
confirm a previous study which showed that TH expression increases ~3-4-fold following 24-48 hours of hypoxia(6), and in addition they demonstrate that unlike the inflammatory cytokines, TH levels remain at elevated levels for up to 4 weeks of continuous exposure.

Figure 3 compares the effects of less severe hypoxia with that evoked by 380 Torr on cytokine and TH expression in carotid body. Exposure for 7 days at 565 Torr (~equivalent to 2560m or ~8400 ft) did not evoke increases in the expression of tyrosine hydroxylase and the inflammatory cytokines IL-6 and TNFα, as well as the chemokine, MCP-1. It is noteworthy that 565 Torr is only slightly lower than the lowest pressure (575 Torr) allowed by the United States Federal Aviation Administration in commercial airliner cabins(8). Thus, it may not be surprising that this modest stimulus evoked a significant increase in IL-1β, but not other cytokines, nor of TH. CH at 515 Torr (~3350m, 11,000ft) evoked significant increases in expression of all inflammatory cytokines, in addition to TH. Quantitatively, these changes were similar to expression at 380 Torr, with the exception that levels of the chemokine, MCP-1, were significantly lower at 515 Torr vs. 380 Torr, and they were even lower at 565 Torr, indicating a graded effect of hypoxia. Expression of IL-1β was similar at all levels of hypoxia following 7 days of CH. Data presented in figure 2 show the level of IL-1β peaked at 3 days, and recovered to near normal on CH day 7. Thus, more robust changes in IL-1β may be observed following shorter exposures to 515 Torr.

The production of high levels of IL-1β and TNFα on day 1 of hypoxia appears to conflict with the IF data showing relatively low levels of ED1+ macrophages at this time. However, because virtually all cells are capable of producing cytokines(26), an important question is whether non-immune cells participate in the inflammatory response. Figure 4 presents data using the amplified RNA (aRNA) technique. Originally developed by Eberwine and his colleagues(36), aRNA produces large amounts of mRNA from a few cells while maintaining the proportionality between the expression of different genes. We harvested mRNA from dissociated carotid body cells which were identified based on size and morphological characteristics commonly used in our laboratory to select cells for examination of O2-sensitive K+-currents(17; 18). Following amplification, mRNA was converted to cDNA and subjected to qPCR for selected genes. The
data show that following 1 day of in vivo hypoxia, expression of TH is elevated over 3-fold. In addition, expression of IL-1β, IL-6 and TNFα were significantly increased. The short hypoxic exposure did not alter expression of MCP-1. Complementary results using the in situ hybridization technique (figure 5) show that after 3 days of CH, IL-6 expression is up-regulated in slender cellular processes consistent with the morphology of sustentacular type II cells; there also appears to be a lesser but substantial increase in IL-6 mRNA in cell lobules, consistent with type I cells. These findings suggest that both type I as well as type II cells participate in the initial phase of cytokine production. A recent report likewise indicated that CH up-regulates production of inflammatory cytokines in type I cells(22).

**Effect of anti-inflammatory drugs on CH-induced inflammation and chemoreceptor adaptation**

The possibility that inflammation plays a role in CH-induced chemoreceptor adaptation was examined in CH animals concurrently treated with common anti-inflammatory drugs ibuprofen and dexamethasone. Data presented in the upper left panel of figure 6 show that CH elicits a marked hypersensitivity as indicated by CSN responses to a standardized acute hypoxic challenge after 8 days of CH @ 380 Torr. Summary data (right) show that CH induces a doubling of the averaged hypoxia-evoked nerve discharge. Separate groups of animals received ibuprofen (4 mg/kg/day) or dexamethasone (0.1mg/kg.day) during exposure to either CH or normoxia for 8-10 days (middle and lower panels). Drug doses were chosen based on their ability to suppress inflammation induced phenotypic changes in rat primary sensory neurons(38). In normoxic rats drug treatment did not affect basal or hypoxia-evoked CSN activity. However, typical records of basal and stimulus evoked nerve activity show that CH did not induce increased chemosensitivity in rats treated with the anti-inflammatory agents. Summary histograms (right) show the averaged impulses/sec evoked over the 150 sec of hypoxia from each of 6 or 4 preparations treated with ibuprofen or dexamethasone, respectively, indicating that adaptation does not occur in the CH-drug-treated groups.

Data in figure 7 show immunofluorescence images of carotid body stained for TH (red) and the universal leukocyte antigen, CD45 (green). Figure7A shows the presence of a few CD45+ cells in normal carotid body. Exposure at 380 Torr for 3 days elicits an enhanced incidence of
CD45+ cells (fig. 7B). Following hypoxia, type I cells show a robust increase in TH immunostaining intensity. In addition, individual CD45+ cells display a remarkable increase in fluorescence, consistent with the previous demonstrations of elevated expression of this antigen in inflamed tissue(31). CH (380 Torr) and concurrent treatment with dexamethasone (0.1 mg/kg/day; fig. 7E) or ibuprofen (4 mg/kg/day; fig. 7F) virtually eliminated expression of CD45 in carotid body, and likewise, few CD45+ immune cells were present in normoxic carotid bodies treated with the anti-inflammatory drugs (fig. 7B and 7C).

Data from separate real-time PCR experiments shown in figure 8 indicate that the anti-inflammatory drugs inhibit expression of proinflammatory cytokines. A 7 day course of ibuprofen (4mg/kg/day) concurrent with CH (380 Torr) inhibited more that 50% of the hypoxia-induced up-regulation of the inflammatory genes TNFα and IL-6 in carotid body. Expression of the chemokine, monocyte chemo-attractant protein-1 (MCP-1) is also substantially less in the presence of ibuprofen. Note that after 7 days @ 380 Torr, expression of IL-1β had returned to normal levels (see figure 2), and that treatment with ibuprofen did not cause any further decrease in the expression of this cytokine. Importantly, CH-induced expression of TH was not significantly hampered in ibuprofen treated animals, suggesting that increased expression of this gene is not dependent on inflammation. This latter finding is consistent with the immunocytochemical data presented in figure 7.

In separate experiments we examined the effects of dexamethasone on inflammatory gene expression following 3 and 7 days of CH (380 Torr). Data in figure 9A demonstrate that the corticosteroid depresses expression of all 4 inflammatory genes, including IL-1β following 3 days of CH, when both the incidence of immune cells and cytokine expression are at high levels in carotid body. Moreover, TH expression is elevated in the presence of dexamethasone, in accord with previously demonstrated effects of this drug on carotid body(15). TH expression is further enhanced following 7 days of CH in the presence of dexamethasone (figure 9B), but expression of the proinflammatory genes remains at levels comparable to normal.
DISCUSSION

Most previous studies of carotid body adaptation have implicitly assumed that the adaptive process is a primary and exclusive function of type I cells. Because of their exquisite sensitivity to O₂ and ability to secret multiple neuroactive agents, these cells have been viewed as the master regulators of chemosensitivity in both acute and chronic hypoxia. Our data for the first time suggest that CH-induced increased chemoreceptor excitability is a process that is heavily influenced by a local immune response in the carotid body. The importance of inflammation is strongly supported by the finding that low doses of ibuprofen or dexamethasone prevent increased cytokine production, as well as chemosensory adaptation in CH. In addition, the up-regulation of cytokines begins within the first 24 hours of hypoxia, and the tissue is invaded by numerous macrophages within 72 hours. In a previous study we established that increased CSN sensitivity is detectable after 3 days of CH, but not following 24 hours(3), a time-course consistent with the development of inflammation. A particularly interesting finding is that with the exception of IL-6, levels of cytokine expression recede after 7 days of CH and are fully recovered on day 28. A caveat to our measurements is that gene expression is not necessarily quantitatively linked to protein production. It is intriguing that in a study of the temporal course of cytokine expression following peripheral nerve injury, Winkelstein, et al.(45), similarly showed that spinal cord IL-6 expression remained above normal for up to 2 weeks, whereas expression of TNFα spiked on day 1 and returned to normal on day 3. Importantly, these authors showed that allodynia and thermal hyperalgesia persisted throughout the course of the study, even while expression and protein levels of cytokines had recovered. Thus, in accord with the physiological hypersensitivity developed in nociceptors in response to chronic pain(42), our data indicate that increased responsiveness of arterial chemoreceptors following CH likewise involves unique neuroimmune mechanisms.

Dvorakova, et al.(10) first demonstrated the presence of a small population of resident ED1+ macrophages in the normal carotid body. Our immunocytochemical results confirm these findings, and show in addition that CH elicits a progressive increase in the number of ED1+ cells consistent with extravasation and differentiation of circulating monocytes. Activated macrophages are sources of inflammatory cytokines IL-1β, IL-6 and TNFα. However, our data indicate that cytokine production also occurs in non-immune cells in the carotid body.
parenchyma, including O₂-sensing type I and neuroglia-like type II cells. Contemporary studies have now firmly established that virtually all cells are capable of producing cytokines and are not the exclusive domain of immune cells (26). Peripheral nerve damage or the introduction of inflammatory agents elicit local cytokine production in Schwann cells and fibroblasts(32; 42). Moreover, prolonged depolarization induces IL-6 production in chromaffin-derived pheochromcytoma (PC12) cells(29), which are considered functional analogs of type I cells because they contain high levels of catecholamine and express O₂-sensitive K⁺-channels(7; 21; 47). Our data from type I cells indicate a robust increase in IL-1β and TNFα after only 1 day of hypoxia, a time when the incidence of ED1+ macrophages is low, suggesting that the up-regulation of cytokine production in type I cells may not require the presence of immune cells. Collectively, our single-cell PCR data and in situ hybridization findings demonstrate the participation of type I and type II cells in the production of cytokines, thus further implicating these cells as key components of the adaptive mechanism. In accord with cytokine action, recent studies have demonstrated receptors for IL-1, IL-6 and TNFα on type I cells(22; 39; 40). Moreover, the notion that primary sensory chemoafferent neurons may be affected by cytokines is supported by studies which documented expression of specific cytokine receptors on primary sensory dorsal root neurons(34; 44). Moreover, the administration of NSAIDs has been shown to block inflammation-induced phenotypic changes in DRG neurons(38).

Our finding that cytokine expression occurs in type I cells following CH is also in accord with a recent report by Lam et al.(22). However, our respective observations differ on several important points. First, Lam et al. did not report the presence of invading immune cells in their preparations. This difference may be due to the small size of immune cells relative to type I cells which were immunostained for pro-inflammatory cytokines. Moreover, our studies utilized antibodies directed against ED1 and CD45, antigens expressed specifically by leukocytes. Second, Lam, et al. reported that breathing 10% O₂ for 7 days elicited increases of inflammatory cytokines of only 15-30%. At a similar level of hypoxia we have documented a more robust increase in expression of IL-6 (~200%) and TNFα (~300%) following 7 days of exposure. An explanation for these discrepancies may lie in the choice of internal control genes for normalization of PCR data. Using qPCR assays we have found that expression of β-actin (the control gene used by Lam, et al.) is increased by ~250% following 3 and 7 days of CH. In
contrast, our data indicate that 18sRNA varies by less than 10% in CH versus normal samples. A final point is that with the exception of IL-6, expression of inflammatory cytokines recovers to normal levels following 28 days of CH. Using an assessment of tissue area occupied by positive immunocytotoxic staining, Lam et al., estimated that expression of all cytokines remained above normal on day 28 of exposure. However, previous studies demonstrated a high incidence of mitotic activity in type I cells and an increased proportion of tissue occupied by these cells following CH(1; 5; 19). Without quantifying staining intensity in individual type I cells and/or assessment of gene expression in cells or tissues, it is difficult to ascertain whether physiologically meaningful changes in cytokine activity have occurred.

Inhibition of cytokine production in animals concurrently treated with ibuprofen or dexamethasone is consistent with the known anti-inflammatory effects of these agents. Ibuprofen was developed some 50 years ago; along with aspirin it was characterized as a non-selective inhibitor of cyclooxygenases 1 and 2 (COX1 and COX2)(28). The anti-inflammatory effects of ibuprofen and similar drugs were therefore attributed to blocking the production of pro-inflammatory prostanoids. However, studies conducted within the last decade have shown that in addition to COX inhibition, ibuprofen mediates COX-independent anti-inflammatory effects(35).

Perhaps most important is inhibition of nuclear translocaton of the transcription factor, nuclear factor kappa beta (NF-κβ), which mediates cytokine production, including TNFα, IL-1β and IL-6(30). Thus, the ibuprofen-mediated block of cytokine expression in CH carotid body suggests the possible involvement of NF-κβ in the adaptive process. In addition, cytokine mediated production of high levels of inflammatory prostanoids may also contribute to carotid body adaptation. In fact, numerous studies have demonstrated the involvement of selected prostaglandins in the development of nociceptor hyperexcitability in inflamed skin(37). It is relevant that previous studies of normal rabbit carotid body showed that acute hypoxia elicits release of prostaglandin E2 (PGE2), while inhibition of PGE2 synthesis with indomethacin augmented type I cell activity, as indicated by increased release of catecholamine(12; 13). These findings indicate that PGE2 promotes an inhibitory effect on type I cells in acute hypoxia. However, chronic local treatment with PGE2 has been shown to induce a hypersensitive state in nociceptors(11), suggesting the possibility of divergent short- versus long-term effects of
prostanoids. Moreover, prostanoid effects on type I cells may differ from those on chemoafferent nerve terminals.

Glucocorticoids, including dexamethasone, are known to inhibit the release of IL-1β and TNFα from activated macrophages(16). Given that IL-6 synthesis and release occur as a consequence of the actions of IL-1β and TNFα(16), it is not surprising that expression of this cytokine was decreased in dexamethasone/CH rats. Recent studies also indicate that dexamethasone inhibits gene expression of MCP-1, a chemokine that promotes leukocyte migration(46). Thus, our data are also consistent with the known ability of dexamethasone to inhibit the recruitment of monocytes and macrophages into affected tissue, and accounts for the decreased numbers of ED1+ and CD45+ cells in the carotid body of drug treated animals. In addition to their anti-inflammatory effects, glucocorticoids can suppress pituitary-adrenal function, and promote the induction of anabolic enzymes. The extent to which these latter processes may be involved in suppressing CH-induced chemoreceptor adaptation are unknown. However, the use of dexamethasone in humans indicates that non-anti-inflammatory effects require prolonged treatment (multiple weeks to months(16)), whereas our assessments of CSN activity were completed after 8-10 days.

Although the present findings strongly support a role for inflammation in resetting chemosensory sensitivity, they do not directly identify the targets of inflammatory mediators, nor the precise changes in cellular physiology which result in receptor hyperexcitability. Pro-inflammatory cytokines and downstream inflammatory mediators are known to alter multiple phenotypic properties of primary sensory neurons, including expression of ion channels and neurotransmitters(23; 42; 43). Moreover, recent studies of normal rat type I cells have demonstrated that IL-1β inhibits O2-sensitive K+-channels and evokes a transient rise in intracellular Ca2+, presumably via action at specific IL-1 receptors(33; 39). In CH, sustained high levels of specific cytokines may mediate short-term stimulatory effects, as well as long-term gene-induced phenotypic changes which contribute to the adaptive process. These actions may occur in multiple cell types in the chemoafferent pathway, including type I cells and primary sensory (chemoafferent) neurons. Collectively, our data suggest that novel neuroimmune interactions are a heretofore unrecognized feature of the regulation of chemoreceptor sensitivity.
Acknowledgements: Supported by USPHS Grants NS 12636, NS 07938 and HL 086508. We would like to thank Dr. Alan Light, University of Utah Department of Anesthesiology, for valuable discussions.
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Figure Legends

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.

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 1β: interleukin-1β; TNFα: tumor necrosis factor-α. *, **, and *** indicate p<0.05, 0.01 and 0.001, vs. normal, respectively. CH: exposure at 380 Torr for time indicated.

Figure 3. Effect of exposure to hypoxia at 565, 515 or 380 Torr for 7 days on expression of tyrosine hydroxylase (TH) and inflammatory cytokines in rat carotid body. Data expressed relative to normal non-hypoxic rats.* , **, and *** indicate p<0.05, 0.01 and 0.001, respectively vs. normal; +++ indicates p<0.001 vs. 515 Torr group.

Figure 4. Effect of 24h in vivo hypoxia on expression of tyrosine hydroxylase (TH), the chemokine, MCP-1, and inflammatory cytokines, IL-1β, IL-6 and TNFα in rat type I cells. Data obtained from 15 normal and 15 hypoxic cells using aRNA/qPCR technology. See text for details. *, **, and *** indicate p<0.05, 0.01 and 0.001, respectively vs. normal.

Figure 5. In situ hybridization histochemistry for cytokine IL-6 in normal (A), and 3 day CH (380 Torr; B) carotid body. CH induces increased IL-6 expression in slender cell processes, consistent with the morphology of type II cells (arrows in A verses B). Up-regulation of IL-6 expression is also indicated in type I cells (enveloped by type II cell processes). Incubation in “sense probe” (C) shows background staining. Scale bar = 20μm.

Figure 6. 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.

Figure 7. 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.

**Figure 8.** Effect of ibuprofen (ib; 4 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 (N); +, ++, and ++++, p< 0.05, 0.01, and 0.001, respectively, vs. 7 days CH (CH7).

**Figure 9.** Effect of dexamethasone (DX; 0.1 mg/kg/day) on CH-induced gene expression of tyrosine hydroxylase and cytokines in rat carotid body. A, 3 days CH (CH3); B, 7 days CH (CH7). 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. CH.
Relative expression

Normal

1 Day CH

TH  MCP-1  IL1b  IL6  TNFa

Relative expression

0  1  2  3  4  5  6

**  ***  ***  *
Hypoxia-evoked CSN discharge (imp/sec)

N + Ib

CH + Ib

N + Dex

CH + Dex

N

CH