Effect of Endothelin Receptor Antagonist Bosentan on Chronic Hypoxia-Induced Inflammation and Chemoafferent Neuron Adaptation in Rat Carotid Body

Xuemei Liu, Liang He, Bruce Dinger, Larry Stensaas, and Salvatore Fidone

Abstract

Liu, Xuemei, Liang He, Bruce Dinger, Larry Stensaas, and Salvatore Fidone. Effect of endothelin receptor antagonist bosentan on chronic hypoxia-induced inflammation and chemoafferent neuron adaptation in rat carotid body. *High Alt Med Biol.* 13:209–216, 2012.— Chronic hypoxia (CH) induces an inflammatory response in rat carotid body that is characterized by immune cell invasion and the expression of pro-inflammatory cytokines. In the present study, we have investigated the role of type-A endothelin (ET-A) receptors in the development of CH-induced inflammation. After 7 days of CH (380 Torr), double-label immunofluorescence studies demonstrated elevated levels of ET-A receptor and tyrosine hydroxylase (TH) in O₂-sensitive type I cells. Following CH, ET-A receptors were also expressed on resident and invasive CD45+ immune cells distributed in tissue surrounding chemosensory cell lobules. Immunofluorescence and quantitative PCR studies showed that concurrent treatment with the ET-A/B receptor antagonist, bosentan (200 mg/kg/day), blocked CH-induced ED-1+ macrophage invasion and the upregulation of cytokines, including interleukin-1 β (IL-1 β), interleukin-6 (IL-6), tumor necrosis factor α (TNF α), and monocyte chemoattractant protein-1 (MCP-1). Moreover, bosentan treatment blocked the CH-induced increases in expression of acid-sensitive ion channels (ASICs) in chemoafferent neurons in the petrosal ganglion (PG). Our findings are consistent with the hypothesis that CH-induced inflammation involves the upregulation and release of ET-1 from type I cells. ET-1 may act in an autocrine/paracrine mechanism via ET-A receptors on chemosensory type I cells and immune cells to promote an inflammatory response.

Key Words: gene expression, cytokines, macrophages, immunocytochemistry, sensory adaptation

Introduction

CAROTID BODY CHEMORECEPTORS continuously monitor arterial PO₂ (P_AO₂), and provide the principal ventilatory drive during hypoxia. The fundamental components of chemoreceptors are comprised by specialized type I cells and synaptically apposed chemoafferent nerve terminals. Hypoxia depolarizes the oxygen-sensitive type I cells, resulting in Ca²⁺ entry, the release of multiple neurotransmitters, and a rapid increase in CSN impulse activity (Fidone et al., 1995; Gonzalez et al., 1994). Chemoafferent impulse activity in the CSN reflexively elevates ventilation via central synaptic terminations on respiratory control neurons located in the nucleus tractus solitarius (NTS) (Finley and Katz, 1992). Numerous studies have elucidated the fundamental mecha-

nisms of O₂-chemotransduction and neurotransmitter action that occur in type I cells in response to acute hypoxia (Nurse, 2010; Peers et al., 2010).

Less well understood is chemoreceptor adaptation, in which sustained exposure to chronic hypoxia (CH) elicits increasing sensitivity to low O₂. The CH-induced elevated CSN discharge, which in rats occurs gradually over a period of 3–14 days (Chen et al., 2002a), results in ventilatory acclimatization to hypoxia (VAH), an adjustment that partially mitigates the adverse effects of CH (Powell et al., 1998). Multiple studies have demonstrated CH-induced functional changes in type I cells that are consistent with increased hypoxic sensitivity. These include 1), altered expression of ion channels known to participate in cell depolarization and Ca²⁺-entry, and 2), upregulation of neurotransmitter agents

which generate and regulate nerve impulse activity (Prabhakar et al., 2009). However, recent experiments in our laboratory have documented a robust inflammatory response in the chemoreceptor tissue following CH. Moreover, concurrent treatment with common anti-inflammatory drugs completely blocks chemoreceptor adaptation (Liu et al., 2009). These novel findings strongly suggest that immune cell invasion and the production of inflammatory cytokines are essential features of the adaptive process. Yet, virtually nothing is known about the cellular mechanisms which initiate inflammatory activity in the carotid body.

In view of their exquisite sensitivity to hypoxia, type I cells are possible candidates as initiators of adaptation. These highly specialized cells synthesize and release multiple conventional neurotransmitters (i.e., acetylcholine, catecholamines, serotonin), as well as neuro- and vaso-active peptides (substance P, met-enkephalin, atrial natriuretic peptide, endothelin), and ATP (Lahiri et al., 2006). In addition, the gaseous agents, nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H₂S) participate in the generation and regulation of chemoreceptor activity (Lahiri et al., 2006; Peng et al., 2010). Although many, if not all, of these numerous agents are known activators and/or modulators of immune cells (Reyes-Garcia and Garcia-Tamayo, 2009), there is one prominent and noteworthy candidate: namely, the 21-amino acid vasoactive peptide, endothelin-1 (ET-1), which has been shown to be particularly important for CH-induced adaptation (Chen et al., 2002a; Chen et al., 2007). First characterized as a potent vasoconstrictor agent, ET-1 was later shown to possess mitogenic and chemotactic activity (Barnes, 1994; Teder and Noble, 2000). In another O₂-sensitive tissue, the lung, ET-1 has been implicated in leukocyte invasion and the development of pulmonary hypertension during CH (Stenmark et al., 2005). Upregulation of ET-1 involves the transcription factor, hypoxia inducible factor-1 α (HIF-1 α), which controls the expression of numerous hypoxia-sensitive genes (Hu et al., 1998; Yamashita et al., 2001). ET-1 acts via ET_A and ET_B receptor subtypes in many tissues, and multiple ET_A receptor antagonists have been shown to block CH-induced pulmonary hypertension and immune cell invasion in lung (Chen et al., 1995; Oparil et al., 1995). Likewise, in a previous study, we showed that the excess chemoreceptor activity evoked by an acute hypoxic challenge following CH is blocked by the application of an ET_A-specific receptor antagonist (BQ-123) (Chen et al., 2002a). Moreover, we reported that concurrent treatment of CH rats with an orally-active mixed ET_{A/B} antagonist, bosentan, partially blocked CH-induced mitosis in the carotid body, as well as chemoreceptor adaptation (Chen et al., 2007). Importantly, these findings are consistent with the known CH-induced upregulation of ET-1 and ET_A receptor in rat carotid body (Chen et al., 2002a; Chen et al., 2002b).

In the current study, we have tested the hypothesis that ET and ET-receptors play critical roles in initiating inflammation and phenotypic changes in the chemoafferent pathway during CH. We have employed immunocytochemical techniques to localize ET_A receptors in carotid body tissue following CH, as well as to assess the effect of concurrent treatment with bosentan on CH-induced immune cell invasion. Conventional quantitative PCR (qPCR) assays have been used to evaluate the effect of bosentan on expression of the inflammatory cytokines, interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF α), and interleukin-6 (IL6), as well as the chemokine,

monocyte chemoattractant protein-1 (MCP-1). In a recent study, we showed that CH induces inflammation-dependent upregulation of acid-sensitive ion channels (ASIC) in chemoafferent neuron cell bodies located in the petrosal ganglion (PG) of the IXth cranial nerve (Liu et al., 2011). Consequently, we have also evaluated the effect of bosentan treatment on the expression of ASIC in PG.

Methods

Animals and exposure to chronic hypoxia

Eighteen rats exposed in a hypobaric chamber were housed in standard rodent cages with food and water. Pressures were reduced from ambient B_P at the University of Utah (i.e., B_P ~ 630 Torr; 1500 m) until a selected pressure equivalent to ~ 5500 m (380 Torr) was reached, and maintained for a selected period. Eight of the hypoxic rats were treated daily with the orally active ET-A/B receptor antagonist, bosentan (200 mg/kg/day). Artificially flavored strawberry gelatin was prepared according to package directions, and bosentan (provided as a gift from Dr. Marc Iglarz, Actelion Pharmaceuticals) was dissolved into the mixture before congealing. Prior to experiments, selected animals were trained to eat an ~ 8 cc cube of gelatin each morning after an overnight fast. Rats habituated to this routine within 3–4 days. The hypobaric chamber was opened for <1 h every day to accomplish ingestion of the gelatin. Normal rat chow was then returned to the feeding rack, the chamber was then sealed, and hypoxic exposure resumed. Control, normal animals ($n=14$; 7 received bosentan), were maintained outside the chamber in ambient conditions. Animal protocols were approved by the University of Utah Institutional Animal Care and Use Committee.

Immunocytochemistry

Twelve rats were divided into groups and treated as normal ($n=2$); normal plus bosentan ($n=2$); 3 days of CH ($n=3$); 3 days of CH plus bosentan ($n=3$); and 7 days of CH ($n=2$). Animals were anesthetized with ketamine (10 mg/100 g) and xylazine (0.9 mg/100 g), and 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 h, rinsed in 20% sucrose/PBS for 2 h, and stored at 4°C in 30% sucrose/PBS for 1 h. Four to six cryostat sections (6 μ m) from each organ 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 either the macrophage marker, ED-1 or ET_A receptor; or ET_A receptor and CD45 (diluted 1:2000 for anti-TH (Chemicon); 1:100 for anti-ED1 and CD45 (Serotec); 1:2000 for ET_A receptor [Maine Biotechnology]) 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 h 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

nonspecific staining of the secondary fluorescent antibodies. Specimens were viewed in a Zeiss Model M30 laser scanning confocal microscope.

Quantitative reverse transcriptase-polymerase chain reaction (real-time qPCR)

As previously described (Liu et al., 2011), carotid bodies and petrosal ganglia (PG) were harvested from rats anesthetized with a mixture of ketamine plus xylazine. Tissues were rapidly 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). Following removal of surrounding connective tissue, organs were 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 experimental group. Following removal of contaminating DNA (DNase treatment), first strand complementary DNA was synthesized from 1 µg 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, TNF α , IL-1 β , and IL-6) and ASIC. 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 (Sallmann et al., 2000), and data are normalized to 18S rRNA expression. In a previous study, we reported that 18S rRNA varies less than 10% between carotid bodies harvested from normal versus CH rats (Liu et al., 2009). Amplification of samples not treated with RETROscript were performed to exclude possible contamination with genomic DNA.

Results

In a previous study, we localized ET_A receptors in carotid body using a conventional immunohistochemical technique that resulted in a fine granular reaction product in groups of ovoid cells which displayed morphology consistent with type I cells (Chen et al., 2002a). In the present study, we have applied a double-antibody immunofluorescent method in order to distinguish between ET_A+ cells that also express either tyrosine hydroxylase (TH), a signature marker for type I cells, or CD45, an antigen expressed universally by active immune cells. Figure 1 shows typical tissue sections from normal (Figs. 1A–1C) versus 7-day CH (Figs. 1D–1F) carotid bodies. The moderate TH staining in normal tissue (Fig. 1A) is markedly enhanced following CH (Fig. 1D). In these preparations, the very low staining for ET_A in normal tissue (Fig. 1B), was likewise greatly elevated by CH (Fig. 1E). The merged images in Figures 1C and 1F, clearly indicate that CH substantially elevated both TH and ET_A expression in type I cells.

Figure 2 shows data for co-expression of ET_A and CD45 in a typical section from a CH carotid body. CD45 staining

FIG. 1. Effect of chronic hypoxia (CH) on expression of tyrosine hydroxylase (TH; green immunofluorescence) and type-A endothelin receptor (ETA; red immunofluorescence) in rat carotid body. (A) Normal carotid body showing TH expression in oxygen-sensitive type I cells; (D) shows that 7 days of CH substantially enhances TH expression. (B) and (E) Expression of ETA in normal and CH tissue, respectively. Staining in normal carotid body suggests minimal or no ETA expression. CH induces robust upregulation of receptor protein. (C) and (F) Merged images from A–B and D–E, indicating co-expression of TH and ETA in type I cells; red cells may represent ETA expression in vascular endothelial cells or smooth muscle. Scale bar = 30 µm.

FIG. 2. Co-localization of CD45 and ETA in rat carotid body following a 7-day exposure to hypobaric hypoxia (380 Torr). **(A)** Green immunofluorescence showing distribution of CD45+ immune cells in connective tissue matrix which surrounds blood vessels and groups of type I cells. **(B)** Red immunofluorescence indicating expression of ETA receptor protein in lobules of ovoid type I cells (large arrows). Note absence of staining in nuclei of type I cells (dark centers). Small arrows indicate ETA expression in presumptive vascular endothelial cells. **(C)** Combined images of **A** and **B**. Lower panel (**D–F**) shows expanded view of area bounded by white box in **C**, and corresponding areas in **A** and **B**. Scale bars = 40 μ m (**A,B,C**); 10 μ m (**D,E,F**).

(Fig. 2A) reveals immune cells widely distributed throughout the O₂-sensitive tissue. Most of the CD45+ cells are located within the connective tissue matrix that surrounds blood vessels and lobules of type I cells. The proximity of the immune cells to type I cells is indicated when images for CD45 and ET_A (red) are merged in Figure 2C. The expanded inset in the lower panel of Figure 2 shows co-expression of ET_A and CD45 in individual cells. ET_A appears to be robustly expressed in virtually all (yellow; Fig. 2F) CD45+ cells in the CH tissue.

The data in Figure 3 show the effect of bosentan on CH-induced upregulation of inflammatory cytokines in rat carotid body. Two groups of 5 rats were exposed to CH for 7 days; one group was fed the orally active mixed ET_{A/B}-receptor antagonist (200 mg/kg/day), and the other group was given vehicle (strawberry flavored gelatin; see Methods). Two groups of normoxic control rats were similarly treated with or without bosentan for 7 days. In agreement with our previous report (Liu et al., 2009), a 7-day exposure at 380 Torr induced increased expression of the inflammatory cytokines IL-1 β , IL6, TNF α , and the chemokine, MCP-1, in carotid body. Also in accord with our previous findings, we observed that delivering vehicle to 7-day CH rats resulted in an approximate 2.5-fold increase in IL6 expression versus animals exposed only to CH (Liu et al., 2009). Normal rats fed vehicle did not increase carotid body cytokine expression, when compared to animals that did not eat gelatin. These results are consistent with the notion that daily recompression/decompression in the hypobaric chamber elicits an additional physiological stress. Nonetheless, upregulation of the inflammatory genes following 7 days of CH is completely blocked in animals treated with the ET-receptor antagonist (plus daily recompression/decompression). Note that in normal animals, bosentan treatment resulted in nonsignificant reductions in the expression of 3 of 4 genes, suggesting that constitutive ET

production promotes cytokine expression; thus, basal cytokine production may be slightly increased due to altitude exposure (~1400 m) at the University of Utah.

In a parallel study, a double antibody immunofluorescence method was used to assess the invasion of ED1+ macrophages into carotid bodies from rats exposed to CH for 3 days. The merged confocal images in Figure 4 display ED1+ cells as green, whereas staining for TH is red. The increased incidence of small ED-1+ positive (green) cells (Figs. 4A versus 4C) is substantially diminished in a CH animal concurrently fed bosentan (Fig. 4D). Bosentan fed to a normal animal (Fig. 4B) did not noticeably alter the number of ED1+ macrophages in the O₂-sensitive tissue.

In a recent study (Liu et al., 2011), we showed that CH increases expression of 5 isoforms of ASIC (ASIC1a, ASIC1b, ASIC2a, ASIC2b, and ASIC3) in the petrosal ganglion (PG). This ganglion contains chemoafferent neuron cell bodies, along with baroreceptor neurons, which terminate in the carotid sinus, as well as gustatory and mechanoreceptor neurons innervating the tongue. We also demonstrated that concurrent treatment with ibuprofen blocked CH-induced upregulation of the ASIC gene expression. The quantitative PCR data in Figure 5 show that bosentan, likewise, completely blocked CH-induced ASIC expression in PG. Moreover, bosentan significantly lowered expression of four ASIC isoforms in PG from control animals that breathed room air.

Discussion

The current data, obtained using a double-antibody immunofluorescence technique, confirm and expand upon our previous findings by showing that CH upregulates expression of ET_A receptors in TH+ type I cells (Chen et al., 2002a). Moreover, ET_A receptors are also expressed by numerous CD45+ immune cells that are located primarily within

FIG. 3. Effect of $ET_{A/B}$ -receptor antagonist, bosentan, on expression of inflammatory genes in normal and 7-day CH rat carotid body. IL1b, interleukin-1 β ; IL6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; TNFa, tumor necrosis factor- α . CH, 7-day chronic hypoxia; CH+B, 7 day chronic hypoxia + bosentan. N, normal; N+B, normal + bosentan. *, **, and ***, indicate $p < 0.05$, 0.01 , and 0.001 , respectively, *vs.* normal. +, ++, and +++, indicate $p < 0.05$, 0.01 , and 0.001 , respectively, *vs.* CH.

FIG. 4. Effect of bosentan (4 mg/kg/day) on CH-induced immune cell activity in rat carotid body. Green cells are immunostained for macrophage antigen ED1; red indicates TH. (A) Normal carotid body contains a few ED1+ immune cells. (B) Treatment of a normoxic animal with bosentan for 3 days does not alter the incidence of ED1+ cells. (C) Following 3 days of CH (380 Torr), the tissue contains numerous ED1+ invasive macrophages. (D) In 3-day CH animals treated with bosentan, the incidence of ED1+ cells is similar to normal. Scale bar = 30 μ m.

FIG. 5. Effect of bosentan on normal expression, and CH-induced expression of 5 ASIC isoforms in rat petrosal ganglion. N, N+B, CH and CH+B, as in Figure 3 legend. *, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively, *vs.* normal. +++ indicates $p < 0.001$ *vs.* CH.

connective tissue that surrounds the dense capillary network and lobules of type I cells in the carotid body. Cells expressing CD45 are derived from bone marrow, and they constitute resident, circulating, and invasive immune cells. Such cells are associated with local inflammation and tissue remodeling (Frid et al., 2009; Stenmark et al., 2005). In lung, CH-induced pulmonary hypertension elicits thickening of arterial wall and increased vascular resistance. Recent evidence suggests that hypoxia-induced upregulation of chemoattractant molecules in pulmonary artery is a critical step leading to the invasion of circulating monocytes into the mesenchyme of the arterial wall (Frid et al., 2009). The resulting expression of inflammatory cytokines and growth factors appears to mediate specific remodeling of the vascular tissue. Importantly, ET-1 acting via ET_A receptors on circulating monocytes has been implicated as a primary chemoattractant in this process (Barnes, 1994; Dawes et al., 1994; Peacock et al., 1992).

Bosentan, as well as a specific ET_A receptor antagonist (BQ-123), block CH-induced pulmonary hypertension (Chen et al., 1995; Dicarolo et al., 1995). Our data demonstrate that bosentan blocks CH-induced immune cell invasion and expression of inflammatory cytokines in rat carotid body. These findings, along with our previous demonstration that bosentan partially blocks upregulated mitotic activity and chemoreceptor hypersensitivity, further implicate ET-1 and ET receptors as critical participants in the adaptive process (Chen et al., 2007). A role for ET-1 and inflammation in adaptation is further indicated by the observation that bosentan prevents the CH-induced increase in the expression of ASIC in PG neurons.

An important aspect of our observations is the degree to which bosentan, compared to ibuprofen and dexamethasone, inhibits CH-induced chemoreceptor hypersensitivity. The ET_{A/B} receptor antagonist reduces the excess in hypoxia-evoked CSN discharge by ~50% (Chen et al.), whereas similar concurrent treatment with the conventional anti-inflammatory drugs completely eliminates chemoreceptor adaptation (Liu et al., 2009). In view of the fact that all three drugs fully inhibit CH-induced immune cell invasion as well as the upregulation of inflammatory cytokines, our findings

suggest that other hypoxia-sensitive mechanisms can mediate adaptation in the absence of a cytokine-mediated inflammatory response. Previous studies suggest that hypoxia inducible factor-1 α (HIF-1 α), a transcription factor that promotes the expression of numerous hypoxia-sensitive genes, is involved in carotid body adaptation. Kline et al. (2002) showed that partial HIF-1 α gene deficiency (i.e., HIF-1 α ^{+/−}) results in a severely reduced ventilatory response to acute hypoxia, and an absence of VAH following CH. Multiple HIF-1 α -regulated genes are expressed in carotid body, including ET-1, VEGF, and both inducible and neuronal nitric oxide synthase (iNOS and nNOS) (Powell and Fu, 2008; Prabhakar and Jacono, 2005).

Interestingly, in cultured endothelial cells, 1 or 3 hours of hypoxia upregulates HIF-1 α , as well as nuclear factor- κ B (NF- κ B) (Lukiw et al., 2003). Moreover, both transcription factors were demonstrated to bind to specific sequences of the upstream promoter region of cyclo-oxygenase2 (COX2) DNA, consistent with the demonstrated low-O₂ induced expression of COX2 mRNA, and the production of the prostaglandin, PGE₂ (Lukiw et al., 2003). These findings in cultured cells strongly suggest that hypoxia can induce the production of inflammatory molecules independent of elevated levels of IL-1 β , IL-6, TNF α , and MCP-1. Cytokine-independent upregulation of these transcription factors by hypoxia, along with their demonstrated ability to induce COX2, could explain the partial adaptation in the presence of bosentan. In such a scenario, COX2 induction and the synthesis of downstream inflammatory mediators (e.g., prostaglandins) may be the final common pathway in the adaptive process. This notion is consistent with the ability of the COX inhibitor, ibuprofen, to completely block chemoreceptor adaptation (Liu et al., 2009).

Unlike its effect on CH-induced chemoreceptor hypersensitivity, bosentan completely blocked the upregulation of ASIC in PG. This finding supports the hypothesis that cytokine levels play an essential role in elevated ASIC expression (Liu et al., 2011; Voilley et al., 2001). Bosentan also reduced ASIC expression in PG from normal rats. This finding may reflect the effect of mild hypoxia associated with altitude at the University of Utah (~1400 m).

Alternatively, reduction of ASIC expression in normoxia could indicate a non-anti-inflammatory effect of the drug. In CH, reduced ASIC expression corresponds to less hypersensitivity, a result that agrees with our previous observation that the specific ASIC antagonist (A-317567) blocks the excess CSN discharge following CH (Liu et al., 2011). In addition, we demonstrated that blocking CH-induced ASIC expression with ibuprofen or dexamethasone fully eliminates chemoreceptor adaptation (Liu et al., 2009; Liu et al., 2011). However, these results do not preclude the participation of other sensory transduction molecules in CH-induced adaptation of chemoafferent nerve terminals. In models of chronic inflammatory pain, multiple studies have implicated a variety of ion channel-forming molecules in nociceptor terminals as mediators of hyperalgesia and allodynia. These include in addition to ASIC (Mamet et al., 2002; Voilley et al., 2001), purinergic receptors (P2X_N) (Barclay et al., 2002; Jarvis, 2010), and the multimodal transient receptor potential vallerinoid-1 (TRPV1) receptor, which is sensitive to temperature, pH, and selected chemical stimuli (Stucky et al., 2009). A recent study by Light and his colleagues (Light et al., 2008) suggest that maximal responses in muscle afferent neurons require the co-participation of ASIC, P2X_N, and TRPV1. Moreover, selective knock-out of Na_v1.7, an α -subunit of voltage-gated sodium channels, in a subpopulation of these small diameter neurons resulted in decreased thermal and mechanical hypersensitivity induced by inflammation, suggesting that specific subsets of Na⁺-channels can alter the threshold for impulse generation (Nassar et al., 2004).

In summary, CH induces expression of ET_A receptors on type I cells and CD45+ immune cells in rat carotid body. CH also elicits invasion of ED1+ macrophages and elevated expression of inflammatory cytokines in this chemosensory tissue. Like ibuprofen and dexamethasone, treatment with the mixed ET_{A/B} receptor antagonist, bosentan, eliminates immune cell invasion and cytokine expression in the tissue, as well as upregulation of ASIC transcript in PG. However, bosentan only partially inhibited the development of chemoreceptor hypersensitivity (Chen et al., 2007). Collectively, the effects of bosentan versus ibuprofen and dexamethasone suggest the presence of both cytokine-dependent and cytokine-independent adaptive mechanisms. Additional studies are required to determine whether these diverse mechanisms converge on COX2 and prostaglandin production as the final common downstream mediators of the adaptive process.

Disclosure Statement

This study was supported by USPHS Grant HL-086508. No competing financial interests exist.

References

- Barclay J, Patel S, Dorn G, Wotherspoon G, Moffatt S, Eunson L, Abdel'al S, Natt F, Hall J, Bevan S, Wishart W, Fox A, and Ganju P. (2002). Functional downregulation of P2X3 receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. *J Neurosci* 22:8139–8147.
- Barnes PJ. (1994). Endothelins and pulmonary diseases. *J Appl Physiol* 77:1051–1059.
- Chen J, He L, Dinger B, Stensaas L, and Fidone S. (2002). Role of endothelin and endothelin A-type receptor in physiological adaptation of the carotid body during chronic hypoxia. *Am J Physiol Lung Cell Molec Physiol* 282:L1314–L1323.
- Chen J, He L, Liu X, Dinger B, Stensaas L, and Fidone S. (2007). Effect of the endothelin receptor antagonist bosentan on chronic hypoxia-induced morphological and physiological changes in rat carotid body. *Am J Physiol Lung Cell Mol Physiol* 292:L1257–L1262.
- Chen S-J, Chen Y-F, Meng QC, Durand J, Dicarolo VS, and Oparil S. (1995). Endothelin-receptor antagonist bosentan prevents and reverses hypoxic pulmonary hypertension in rats. *J Appl Physiol* 79: 2122–2131.
- Chen Y, Tipoe GL, Liong E, Leung S, Lam SY, Iwase R, Tjong YW, and Fung ML. (2002b). Chronic hypoxia enhances endothelin-1-induced intracellular calcium elevation in rat carotid body chemoreceptors and upregulates ETA receptor expression. *Pflugers Arch* 443:565–573.
- Dawes KE, Peacock AJ, Gray AJ, Bishop JE, and Laurent GJ. (1994). Characterization of fibroblast mitogens and chemoattractants produced by endothelial cells exposed to hypoxia. *Am J Respir Cell Mol Biol* 10:552–559.
- Dicarolo VS, Chen S-J, Meng QC, Durand J, Yano M, Chen Y-F, and Oparil S. (1995). ET_A-receptor antagonist prevents and reverses chronic hypoxia-induced pulmonary hypertension in rat. *Am J Physiol* 269:L690–L697.
- Fidone S, Dinger BG, and Gonzalez C. (1995). Mechanisms of carotid body chemoreception. In *The Lung Biology in Health Disease, Vol. X, The Regulation of Breathing*, eds. Dempsey JA, Pack AI, Marcel Dekker, Inc., New York, pp. 391–471.
- Finley JCW and Katz DM. (1992). The central organization of carotid body afferent projections to the brainstem of the rat. *Brain Res* 572:108–116.
- Frid MG, Li M, Gnanasekharan M, Burke DL, Frago M, Strassheim D, Sylman JL, and Stenmark KR. (2009). Sustained hypoxia leads to the emergence of cells with enhanced growth, migratory, and prometogenic potentials within the distal pulmonary artery wall. *Am J Physiol Lung Cell Mol Physiol* 297:L1059–L1072.
- Gonzalez C, Almaraz L, Obeso A, and Rigual R. (1994). Carotid body chemoreceptors: From natural stimuli to sensory discharges. *Physiol Rev* 74:829–898.
- Hu J, Sischer DJ, Bishopric NH, and Webster KA. (1998). Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand. *Biochem Biophys Res Comm* 245:894–899.
- Jarvis MF. (2010). The neural-glia purinergic receptor ensemble in chronic pain states. *Trends Neurosci* 33:48–57.
- Kline DD, Peng YJ, Manalo DJ, Semenza GL, and Prabhakar NR. (2002). Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1 alpha. *Proc Natl Acad Sci USA* 99:821–826.
- Lahiri S, Roy A, Baby SM, Hoshi T, Semenza GL, and Prabhakar NR. (2006). Oxygen sensing in the body. *Prog Biophys Mol Biol* 91:249–286.
- Light AR, Huguen RW, Zhang J, Rainier J, Liu Z, and Lee J. (2008). Dorsal root ganglion neurons innervating skeletal muscle respond to physiological combinations of protons, ATP, and lactate mediated by ASIC, P2X, and TRPV1. *J Neurophysiol* 100:1184–1201.
- Liu X, He L, Dinger B, and Fidone SJ. (2011). Chronic hypoxia-induced acid-sensitive ion channel expression in chemoafferent neurons contributes to chemoreceptor hypersensitivity. *Am J Physiol Lung Cell Mol Physiol* 301:L985–L992.

- Liu X, He L, Stensaa L, Dinger B, and Fidone S. (2009). Adaptation to chronic hypoxia involves immune cell invasion and increased expression of inflammatory cytokines in rat carotid body. *Am J Physiol Lung Cell Mol Physiol* 296:L158–L166.
- Lukiw WJ, Ottlecz A, Lambrou G, Grueninger M, Finley J, Thompson HW, and Bazan NG. (2003). Coordinate activation of HIF-1 and NF-kappaB DNA binding and COX-2 and VEGF expression in retinal cells by hypoxia. *Invest Ophthalmol Vis Sci* 44:4163–4170.
- Mamet J, Baron A, Lazdunski M, and Voilley N. (2002). Proinflammatory mediators, stimulators of sensory neuron excitability via the expression of acid-sensing ion channels. *J Neurosci* 22:10662–10670.
- Nassar MA, Stirling LC, Forlani G, Baker MD, Matthews EA, Dickenson AH, and Wood JN. (2004). Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. *Proc Natl Acad Sci USA* 101:12706–12711.
- Nurse CA. (2010). Neurotransmitter and neuromodulatory mechanisms at peripheral arterial chemoreceptors. *Exp Physiol* 95:657–667.
- Oparil S, Chen S-J, Meng QC, Elton TS, Yano M, and Chen Y-F. (1995). Endothelin-A receptor antagonist prevents acute hypoxia-induced pulmonary hypertension in the rat. *Am J Physiol* 268:L95–L100.
- Peacock AJ, Dawes KE, Shock A, Gray AJ, Reeves JT, and Laurent GJ. (1992). Endothelin-1 and endothelin-3 induce chemotaxis and replication of pulmonary artery fibroblasts. *Am J Respir Cell Mol Biol* 7:492–499.
- Peers C, Wyatt CN, and Evans AM. (2010). Mechanisms for acute oxygen sensing in the carotid body. *Respir Physiol Neurobiol* 174:292–298.
- Peng YJ, Nanduri J, Raghuraman G, Souvannakitti D, Gadalla MM, Kumar GK, Snyder SH, and Prabhakar NR. (2010). H2S mediates O2 sensing in the carotid body. *Proc Natl Acad Sci USA* 107:10719–10724.
- Powell FL and Fu Z. (2008). HIF-1 and ventilatory acclimatization to chronic hypoxia. *Respir Physiol Neurobiol* 164:282–287.
- Powell FL, Milsom WK, and Mitchell GS. (1998). Time domains of the hypoxic ventilatory response. *Respir Physiol* 112:123–134.
- Prabhakar NR and Jacono FJ. (2005). Cellular and molecular mechanisms associated with carotid body adaptations to chronic hypoxia. *High Alt Med Biol* 6:112–120.
- Prabhakar NR, Peng YJ, Kumar GK, Nanduri J, Di GC, and Lahiri S. (2009). Long-term regulation of carotid body function: Acclimatization and adaptation—Invited article. *Adv Exp Med Biol* 648:307–317.
- Reyes-Garcia MG and Garcia-Tamayo F. (2009). A neurotransmitter system that regulates macrophage pro-inflammatory functions. *J Neuroimmunol* 216:20–31.
- Sallmann S, Jüttler E, Prinz S, Petersen N, Knopf U, Weiser T, and Schwaninger M. (2000). Induction of interleukin-6 by depolarization of neurons. *J Neurosci* 20:8637–8642.
- Stenmark KR, Davie NJ, Reeves JT, and Frid MG. (2005). Hypoxia, leukocytes, and the pulmonary circulation. *J Appl Physiol* 98:715–721.
- Stucky CL, Dubin AE, Jeske NA, Malin SA, McKemy DD, and Story GM. (2009). Roles of transient receptor potential channels in pain. *Brain Res Rev* 60:2–23.
- Teder P and Noble PW. (2000). A cytokine reborn? Endothelin-1 in pulmonary inflammation and fibrosis. *Am J Respir Cell Mol Biol* 23:7–10.
- Voilley N, de WJ, Mamet J, and Lazdunski M. (2001). Nonsteroid anti-inflammatory drugs inhibit both the activity and the inflammation-induced expression of acid-sensing ion channels in nociceptors. *J Neurosci* 21:8026–8033.
- Yamashita K, Discher DJ, Hu J, Bishopric NH, and Webster KA. (2001). Molecular regulation of the endothelin-1 gene by hypoxia. *J Biol Chem* 276:12645–12653.

Address correspondence to:
Bruce Dinger, Ph.D.

Department of Physiology
University of Utah School of Medicine
420 Chipeta Way
Salt Lake City, UT 84108

E-mail: b.dinger@utah.edu

Received January 31, 2012;
accepted in final form March 27, 2012