Role of endothelin and endothelin A-type receptor in adaptation of the carotid body to chronic hypoxia

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Chen, J., L. He, B. Dinger, L. Stensaas, and S. Fidone. Role of endothelin and endothelin A-type receptor in adaptation of the carotid body to chronic hypoxia. Am J Physiol Lung Cell Mol Physiol 282: L1314–L1323, 2002; 10.1152/ajplung.00454.2001.—Chronic exposure in a low-PO2 environment (i.e., chronic hypoxia, CH) elicits an elevated hypoxic ventilatory response and increased hypoxic chemosensitivity in arterial chemoreceptors in the carotid body. In the present study, we examine the hypothesis that changes in chemosensitivity are mediated by endothelin (ET), a 21-amino-acid peptide, and ETα receptors, both of which are normally expressed by O2-sensitive type I cells. Immunocytochemical staining showed incremental increases in ET and ETα expression in type I cells after 3, 7, and 14 days of CH (380 Torr). Peptide and receptor upregulation was confirmed in quantitative RT-PCR assays conducted after 14 days of CH. In vitro recordings of carotid sinus nerve activity after in vivo exposure to CH for 1–16 days demonstrated a time-dependent increase in chemoreceptor activity evoked by acute hypoxia. In normal carotid body, the specific ETα antagonist BQ-123 (5 μM) inhibited 11% of the nerve discharge elicited by hypoxia, and after 3 days of CH the drug diminished the hypoxia-evoked discharge by 20% (P < 0.01). This inhibitory effect progressed to 45% at day 9 of CH and to nearly 50% after 12, 14, and 16 days of CH. Furthermore, in the presence of BQ-123, the magnitude of the activity evoked by hypoxia did not differ in normal vs. CH preparations, indicating that the increased activity was the result of endogenous ET acting on an increasing number of ETα. Collectively, our data suggest that ET and ETα autoreceptors on O2-sensitive type I cells play a critical role in CH-induced increased chemosensitivity in the rat carotid body.

chemoreceptor; chemosensitivity; chemotransduction; hypoxic ventilatory response; ventilatory acclimatization to hypoxia

EXPOSURE TO LOW AMBIENT O2 elicits a number of molecular, cellular, and systemic adjustments that collectively mitigate hypoxia and promote homeostasis (40). An increase in ventilation is the earliest and most prominent of the adaptive changes elicited by acute hypoxia. However, chronic exposure to low O2 (i.e., chronic hypoxia, CH) evokes an additional time-dependent increase in minute volume known as ventilatory acclimatization to hypoxia (VAH; see Ref. 5). VAH has been observed in humans during sojourns to high altitude and in animals exposed in controlled low-O2 environments. VAH is associated with an increased hypoxic ventilatory response (HVR), an index of hypoxic ventilatory drive that is assessed by exposure to an acute hypoxic challenge (39). Enhanced hypoxic chemosensitivity in the carotid body, which is manifest as an elevated hypoxia-evoked carotid sinus nerve (CSN) response, is an important physiological mechanism underlying changes in ventilatory function during chronic exposure (3, 5, 38).

Chemotransduction in the carotid body occurs in specialized O2-sensitive type I cells. Current views suggest that hypoxia evokes a cascade of events in type I cells, including membrane depolarization, Ca2+ influx, and the release of multiple biogenic amine and neuropeptide neurotransmitters that excite synaptic terminals of the CSN (16). Previous efforts to explain the CH-induced increase in chemosensitivity have been focused primarily on alterations in neurotransmitter actions (reviewed in Ref. 6). These efforts have identified important changes in the synthesis, storage, and turnover of the numerous endogenous neuroactive agents present in type I cells (e.g., dopamine, norepinephrine, ACh, serotonin, and substance P), but attempts to demonstrate direct involvement of particular neurotransmitters and/or their receptors in increased chemosensitivity have produced negative results and/or conflicting sets of data (e.g., see Refs. 23, 24, and 37).

On the other hand, recent studies in other O2-sensitive tissues, namely the lung and heart, have shown that the vasoactive peptide endothelin-1 (ET-1) and its receptor (ETα) are critically involved in physiological and morphological adjustments in these tissues elicited by sustained exposure to low O2. ET-1 and ETα are substantially upregulated during CH (27, 28), and, most importantly, specific ETα antagonists are able to prevent CH-induced vascular wall thickening, hypertrophy of the right heart, and pulmonary hypertension induced by exposure to CH (7, 10, 12, 13, 33).

In a previous communication, we reported that ET- and peptide-like immunoreactivity is present in rat carotid body type I cells and that exposure to CH enhances ET immunostaining in these cells (18). The

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present study confirms and extends these immunocytochemical findings and provides a quantitative evaluation of ET-1 and ET_\text{A} gene expression. Correlative electrophysiological and pharmacological experiments demonstrate that ET is involved in increased carotid body chemosensitivity elicited by CH. Our data not only show that CH elicits an upregulation of ET-1 peptide and ET_\text{A} protein in type I cells but that these changes are also correlated with an enhancement of the hypoxia-evoked chemoreceptor discharge. Furthermore, between days 3 and 9 of CH, the elevated hypoxia-evoked chemoreceptor nerve activity becomes increasingly sensitive to inhibition by the specific ET_\text{A} antagonist BQ-123.

**MATERIALS AND METHODS**

*Animals and exposure to hypobaric hypoxia.* Sixty-two adult male albino rats (180–200 g; Sprague-Dawley derived; Simonsen, Gilroy, CA) were housed in standard rodent cages with 24-h access to pellet food and water. Cages containing two to four rats were placed in a hypobaric chamber where pressures were reduced incrementally from ambient (~640 Torr at Salt Lake City, 1,400 m) over a 24- to 36-h period and then were maintained at 380 Torr, equivalent to 5,500 m. The chamber was continuously flushed with fresh room air, and the internal temperature was maintained at 20–22°C. The hypobaric chamber was opened every 2 days to replenish food and water. All animals exposed to the hypobaric environment survived for up to 16 days without signs of discomfort. Age-matched control male rats were similarly housed outside the chamber.

*Immunocytochemical localization of ET peptides and ET_\text{A} protein.* Normal (n = 4) and CH (n = 12; 4 each at 3, 7, and 14 days) rats were anesthetized with ketamine (10 mg/100 g body weight). The hypobaric chamber was opened every 2 days to replenish food and water. All animals exposed to the hypobaric environment survived for up to 16 days without signs of discomfort. Age-matched control male rats were similarly housed outside the chamber.

**Electrophysiological recording of CSN activity.** Under ketamine/xylazine anesthesia, and with the aid of a dissecting microscope, the carotid artery bifurcations containing the carotid bodies were located and removed from 25 rats after exposure to CH for 0–16 days. The excised tissue was placed in a lucite chamber containing 100% O\textsubscript{2}-equilibrated modified Tyrode solution at 0–4°C (in mM: 112 NaCl, 4.7 KCl, 2.2 CaCl\textsubscript{2}, 1.1 MgCl\textsubscript{2}, 42 sodium glutamate, 5 HEPES buffer, and 5.6 glucose; pH = 7.4). 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 superfusion 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 μm ID) of a glass suction electrode for monopolar recording of chemoreceptor activity. Sufficient suction was applied to seal the electrode tip against the connective tissue surrounding the carotid body and CSN. The bath was grounded with a Ag/AgCl\textsubscript{2} wire,
and 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 analog-to-digital/digital-to-analog converter for display of frequency histograms on a personal computer monitor. After neural recording, the CSN was carefully removed, and carotid body wet weight was determined in a Cahn electrobalance equipped with a humidified weighing chamber. Data were expressed as impulses per second and were analyzed using ANOVA with the Bonferroni multiple comparison post-tests or paired t-tests.

RESULTS

Localization of ET-like immunoreactivity in normal and chronically hypoxic rat carotid body. Figure 1 shows the effects of CH on ET immunostaining in the rat carotid body. In accord with previous studies that reported that exposure to CH for 14 days elicited a marked increase in ET immunoreactivity in O2-sensitive type I cells (18), the present results confirm this increase and show that the hypoxia-induced elevation of ET peptide expression is recognizable in most type I chemosensory cells after only 3 days of CH exposure, during the period of incremental pressure reduction to 380 Torr (see MATERIALS AND METHODS). Although the intensity of ET immunostaining was similar in carotid bodies after 3 vs. 7 days of CH exposure, ET immunostaining was markedly enhanced in type I cells after 14 days of CH, indicating additional peptide production and storage. By contrast, the levels of reaction product were substantially lower in normoxic animals. In all conditions, staining occurred in virtually all type I cells as a fine granular precipitate throughout the cytoplasm, whereas the large ovoid nuclei of these cells remained unstained. Importantly, ET immunoreactivity appeared after CH in many reactive endothelial cells whose somata protruded into the lumen of dilated sinusoidal blood vessels. However, other tissue components, including nerve fibers, fibroblasts, type II cells, and other vascular endothelial cells of arteries and veins, were not stained.

ETA localization in normal and chronically hypoxic rat carotid body. In normal carotid bodies, ETA immunoreactivity occurred in nearly all type I cells as a fine granular reaction product throughout the cytoplasm. In Figure 1, ET-like immunoreactivity is localized to cell cytoplasm in type I cells in normal carotid body. Incremental increases in immunostaining intensity occur after 3 (B), 7 (C), and 14 (D) days of chronic hypoxia (380 Torr). Arrows in D indicate large prominent endothelial cells protruding into vascular lumen. Scale bar = 20 µm.

Fig. 1. Endothelin immunoreactivity in normal and chronically hypoxic rat carotid body. A: immunostaining is localized to cell cytoplasm in type I cells in normal carotid body. Incremental increases in immunostaining intensity occur after 3 (B), 7 (C), and 14 (D) days of chronic hypoxia (380 Torr). Arrows in D indicate large prominent endothelial cells protruding into vascular lumen. Scale bar = 20 µm.
Fig. 2. Immunoreactivity for endothelin receptor (ET\(_{A}\)) protein in normal and chronically hypoxic rat carotid body. A: in normal tissue, ET\(_{A}\) is expressed in chemosensory type I cells. Immunostaining intensity is noticeably increased after days 3 (B) and 7 (C) of CH and is markedly elevated after 14 days of CH (D). Scale bar = 20 \(\mu\)m.

(Fig. 2A). After 3 and 7 days of CH (Fig. 2, B and C), there were slight to moderate elevations in ET\(_{A}\) immunostaining. However, after 14 days of CH, receptor immunoreactivity in type I cells was substantially elevated (Fig. 2D). In all preparations, no ET\(_{A}\) immunoreactivity was found in type II cells or in nerve fibers, Schwann cells, fibroblasts, and blood vessels surrounding the lobules.

Expression of ET-1 peptide and ET\(_{A}\) genes in normal and chronically hypoxic carotid bodies. The elevated levels of ET peptide and ET\(_{A}\) protein immunostaining observed after CH suggest possible increased expression of respective peptide- and protein-specific genes. Figures 3 and 4 present analyses of quantitative RT-PCR assays for mRNAs coding for the ET-1 precursor molecule, pre-pro-ET-1, and ET\(_{A}\) protein, respectively. The marked \(x\)-intercepts in Fig. 3 indicate estimated amounts of pre-pro-ET-1 cDNA (corresponding to tissue mRNA) in normal (Fig. 3A) and CH (Fig. 3B) carotid bodies. These data, when expressed per milligram of protein in tissue extracts, indicate a 180-fold increase in the level of pre-pro-ET-1 mRNA in carotid bodies after 14 days of CH. A replicate of this experiment in a second group of four normal and four CH rats similarly indicated a 170-fold increase in the expression of the pre-pro-ET-1 transcript. Figure 4 shows a similar evaluation in rat carotid body of ET\(_{A}\) gene expression. In this experiment, 14 days of CH resulted in a 14-fold increase in ET\(_{A}\) transcript levels. In three replicate experiments, the mean relative increase in ET\(_{A}\) mRNA was 15.1 \pm 1.97-fold (mean \pm SE, \(n = 4\); \(P = 0.0056\) vs. hypothetical mean of 1.0).

Effect of CH on resting and stimulus-evoked CSN activity. CH in the rat induces VAH and an elevated HVR, but adaptive changes in chemoreceptor nerve activity in this species have not been documented. We evaluated basal (normoxia) and hypoxia-evoked CSN chemoreceptor activity in vitro after in vivo exposure to CH for selected periods lasting up to 16 days. Figure 5 summarizes basal nerve activity recorded in solutions...
equilibrated at PO2 /H11005 450 Torr and after reducing bath PO2 to 120 Torr for 150 s (acute hypoxia), which elicited submaximal increases in chemoreceptor activity. Recordings from multiple preparations were highly reproducible (SE 10%), and the data show that CSN activity at both PO2 levels progressively increases after exposure to CH. Significant changes are first observed after 3 days of CH, and both basal nerve activity and the response to acute hypoxia continued to increase up to day 9 of CH exposure. No further increases were observed in preparations from animals exposed to CH for 12, 14, and 16 days. In normal carotid body/CSN preparations, basal nerve activity was 15.53 ± 1.39 (SE) impulses/s, and this value was elevated to 95.10 ± 7.54 impulses/s after 9 days of CH (P < 0.001). The 150-s averaged nerve discharge rate during superfusion at PO2 = 120 Torr was 186.4 ± 12.8 impulses/s in normal vs. 390.6 ± 30.2 impulses/s in 9-day CH preparations (P < 0.001).

Effect of the ETA antagonist BQ-123 on CSN activity. The participation of endogenous ET peptide in the generation of chemoreceptor nerve discharge was evaluated using the specific ETA antagonist BQ-123. Figure 6 shows examples of integrated CSN activity in normal and chronic hypoxia (CH) preparations.

Fig. 3. Quantitative assessment of endothelin (ET)-1 mRNA transcript level in normal (A) and in 14-day chronic hypoxia (CH; B) rat carotid body. X-axis: initial concentration of mimic DNA molecule (internal standard) that contains primer sequence identical to ET-1 target transcript. Y-axis: ratio of target-to-mimic concentration in reaction mix after 35 cycles of PCR. Marked intercepts indicate equal concentrations of target and mimic molecules. Transcript ratio: concentration of ET-1 transcript in hypoxic/normoxic carotid bodies expressed per mg protein. Transcript ratio in replicate experiment indicated 170.7-fold increase in tissue levels of ET-1 mRNA after 14 days of CH. cpm, Counts/min.

Fig. 4. Effect of 14 days of CH on ETA mRNA levels in rat carotid body. Details as in Fig. 3. In 3 replicate experiments, the mean CH-induced increase in ETA transcript was 15.1 ± 1.97-fold (mean ± SE, P = 0.0056).

Fig. 5. Effect of CH on basal (normoxic) and acute hypoxia-stimulated carotid sinus nerve (CSN) activity. Basal nerve activity evaluated in superfusion solutions equilibrated at bath PO2 = 450 Torr. CSN responses to hypoxia are expressed as impulses (imp/s) and averaged over a 150-s period of acute hypoxia at bath PO2 = 120 Torr. *P < 0.05 and ***P < 0.001 vs. activity in normal (i.e., 0 days CH) preparations (ANOVA).

Fig. 6. Effect of CH on the sensitivity of chemoreceptor nerve discharge to BQ-123. Left: 3 superimposed traces of integrated CSN activity; separate trace indicates changes in bath PO2. Basal- and hypoxia-stimulated nerve activity are minimally altered in the presence of 5 μM BQ-123. After 3 days of CH (right), basal nerve activity is marginally affected by the ETA antagonist. However, nerve activity evoked by hypoxia is substantially reduced in the presence of the drug.
normal (left) and 3-day CH (right) carotid body/CSN preparations. In each experiment, after establishing the basal rate of nerve discharge, we lowered the bath Po2 to ~120 Torr (see Po2 trace, Fig. 6) for 150 s to evoke the “control” hypoxic discharge. This was followed by a 2.5-min superfusion with solution at Po2 = 450 Torr containing 5 µM BQ-123, a drug concentration sufficient to saturate ET_A (41, 42). A second hypoxic stimulus involved superfusion with 5 µM BQ-123 as the bath Po2 was again lowered to 120 Torr for 150 s. After a 15- to 20-min wash in the absence of the antagonist (superfusion solution equilibrated at Po2 = 450 Torr), a third hypoxic stimulus (Po2 = 120 Torr) evaluated “recovery” of the response. The effect of BQ-123 on CSN discharge is shown in Fig. 6, where after 3 days of CH, the control and recovery responses to hypoxia were larger than normal (see also Fig. 5), and in the presence of BQ-123 the response to hypoxia was reduced by ~20%.

The experimental protocol described above was employed to evaluate the effects of BQ-123 on CSN activity elicited by superfusion with low-O2 solution (Po2 = 120 Torr) after animal exposure to varying periods of CH up to 16 days. The data summarized in Fig. 7A demonstrate that the antagonist reduced the hypoxia-evoked (i.e., stimulus minus basal) CSN activity by 10.9 ± 1.8% (mean ± SE; n = 13) in preparations from normal animals. However, after 3 days of CH, BQ-123 exposure diminished the hypoxia-evoked discharge by ~20%, and this effect progressed to 45% at day 9 of CH and to nearly 50% after 12, 14, and 16 days of CH.

The effect of CH on carotid body size is a potentially important factor for interpretation of these data in our in vitro superfused preparations. Increased organ mass may significantly steepen O2 gradients, which could result in an enhanced hypoxic stimulus and potentiation of endogenous peptide release. However, the progressive increase in carotid body wet weight (Fig. 8) after exposure to CH suggests that increased organ size does not directly account for the inhibitory effect of BQ-123. In this regard, it is important to note that, although significantly enhanced sensitivity to the drug is evident on days 3 and 5 of CH, the size of the carotid body has not yet increased. Furthermore, a substantial increase in organ wet weight occurs between days 9 and 12 of CH, but this change is not accompanied by a parallel change in the effect of BQ-123. The wet weight data indicate that organ size is increased three- to fourfold after 12–16 days of hypoxia, consistent with prior studies employing quantitative morphological techniques (26).

The analysis of the CH nerve recording data presented in Fig. 8 compares the averaged CSN activity evoked (stimulus minus basal) by hypoxia (Po2 = 120 Torr) in the presence of 5 µM BQ-123 with nerve activity evoked in normal preparations (i.e., 0 days CH) exposed to the drug. In CH preparations, the mean evoked nerve activity in the presence of the ET_A antagonist was always greater than activity evoked in normal preparations. However, a one-way ANOVA of these data, combined with Bonferroni multiple comparison posttests, suggests that significant differences do not exist between normal and CH groups, with the exception of the 12-day CH group vs. normal (P < 0.05). In this series of experiments, most data were obtained from CH groups consisting of only three or four preparations, a statistical minimum.
15 preparations examined after 5 days of CH (i.e., preparations demonstrating increased chemosensitivity before CH-induced organ growth), the data demonstrate levels of evoked nerve activity in the presence of BQ-123 that are indistinguishable from normal ($P > 0.05$; at $\alpha = 0.05$ the statistical test has a power of 40% to detect a 22% difference in the mean).

The effectiveness of the ETA antagonist was further explored in normal ($n = 10$) vs. 5-day CH ($n = 11$) preparations exposed to the full range of bath $P_{O_2}$ levels (40–450 Torr). The data presented in Fig. 9 show that, in normal preparations, the percent depression of CSN activity by BQ-123 is increased with decreasing $P_{O_2}$. Furthermore, in 5-day CH preparations exposed to $P_{O_2}$ 180, 120, 100, and 40 Torr, the drug was significantly more effective at reducing the nerve discharge rate. At higher bath $P_{O_2}$ levels (i.e., 200 and 450 Torr), the drug caused only a marginal depression of nerve activity that did not differ between normal and 5-day CH preparations.

The failure of BQ-123 to completely block the CH-induced increase in CSN discharge when bath $P_{O_2}$ levels are relatively high is further documented in Fig. 10, which presents data from preparations superfused at $P_{O_2} = 450$ Torr, after exposure to CH for 0–16 days. The receptor-saturating dose of BQ-123 (5 $\mu$M) caused a significant reduction in nerve activity in both normal and CH preparations. However, unlike the complete occlusion of the CH-induced increase in chemoreceptor activity observed at $P_{O_2}$ of 120 or lower, BQ-123 only partially depressed the elevated nerve activity in preparations superfused at 450 Torr, after exposure to CH for 5–16 days. (Nerve activity in the presence of BQ-123 was not significantly different in 0-day vs. 3-day preparations.)

The present study was designed to elucidate the physiological role of endogenous ET in the chemoreceptor response of the normal carotid body and to evaluate its involvement in the dynamic physiological adjustments during exposure to CH. McQueen and colleagues (30) were the first to demonstrate that intravenous injection of ET peptide elevates respiratory minute volume and elicits CSN excitation in rat carotid body. Of particular interest was the observation that these effects were blocked by the specific ETA antagonist FR-139317. Autoradiographic studies using $^{125}$I-labeled ET peptides further demonstrated specific ET binding sites both in carotid body lobules and in surrounding microvascular elements (30, 36). Chen et al. (8, 9) reported that ET peptides potentiated hypoxia-evoked nerve activity when applied to rat and rabbit carotid body/CSN preparations superfused in vitro, where the potent vascular effects of ET-1 are eliminated. This effect of ET-1 is blocked by BQ-123 but not by the ETB antagonist IRL-1038. Additional studies have shown that incubation of intact rat carotid body in ET-1 increases cAMP levels in type I cells (9). Moreover, in dissociated type I cells from rabbit, ET-1 potentiates hypoxia-evoked intracellular $Ca^{2+}$ responses and voltage-gated $Ca^{2+}$ currents (8). Thus the pharmacological effects of ET appear to be mediated by the following dual mechanisms: on the one hand they involve cAMP- and $Ca^{2+}$-dependent mechanisms in type I cells during hypoxia (8, 9), and, on the other, they activate hypotensive and pressor effects, which may occur independently of arterial $P_{O_2}$ when peptide is administered intravenously (30).

The present immunocytochemical findings confirm earlier studies that demonstrated ET peptide in type I cells and increased levels of peptide expression after 2 wk of CH (18). These data further demonstrate that peptide content is noticeably elevated after only 3 days
of low-O₂ exposure and that levels in type I cells continue to increase, resulting in substantially enhanced immunostaining after 14 days of CH. However, the presence of ET in certain large prominent endothelial cells at 14 days in the largest of the dilated sinusoidal capillaries indicates a belated vascular effect that is restricted to the final stage of remodeling when typical capillaries are entirely absent in the carotid body. It is noteworthy that studies in other tissues have revealed that ET peptide levels are regulated primarily via gene transcription (25). Thus the presence of high levels of ET peptide in type I cells is corroborated by studies of pre-pro-ET gene expression using the RT-PCR technique with an internal standard mimic molecule. These data indicate that transcript levels for the precursor molecule are elevated >100-fold on day 14 of CH exposure. Smaller effects have been reported in rat lung, where conventional mRNA hybridization techniques indicated a three- to fourfold increase in ET gene expression after 28 days of 10% O₂ breathing (27, 28). Interestingly, analysis of pre-pro-ET gene structure has shown that the proximal promoter region contains an active binding site for hypoxia-inducible factor-1 and that mutations in this site prevent hypoxia-induced ET expression in cultured vascular endothelial cells (20). Moreover, in transgenic mice expressing a pre-pro-ET-1-luciferase gene construct, exposure to 10% O₂ for 24 h elicits a sixfold increase in promoter activity in lung tissue (2).

Less is known about regulation of the ETA gene. Studies in the heart and lung have shown that CH induces increased receptor transcript levels (27, 28), and our data for 14-day CH indicate a 15-fold increase in ETA mRNA in the carotid body. This elevated transcript level agrees with the marked increase in immunostaining intensity for ETA protein on CH day 14, with smaller changes observed after 3 and 7 days of CH. Importantly, in all experimental conditions, ETA immunoreactivity is localized exclusively in type I cells. The colocalization of ET peptide and the A-type receptor in type I cells indicates that this endogenous peptide acts via an autocrine or paracrine mechanism. The suggestion that it has no direct effects on afferent chemoreceptor nerve terminals is supported by the finding that ETA protein immunoreactivity is not present in nerve fibers in the carotid body. This is also consonant with the reports of McQueen and colleagues (30), who showed that the specific ETA antagonist FR-139317 does not displace 125I-ET binding sites in nodose ganglion, a structure known to contain a subpopulation of sensory neurons that innervate arterial chemoreceptors in the aortic bodies near the heart (30).

Endogenous ET peptide and ETA appear to participate minimally in the generation of chemoreceptor nerve activity in normal preparations, where receptor saturating concentrations of BQ-123 depress the hypoxia-evoked CSN discharge by <11%. However, after 3 days of CH, ~20% of the evoked nerve activity is sensitive to the antagonist, and this effect is incrementally increased in preparations exposed up to 9 days of CH, when 45% of the evoked discharge is blocked. This gradual emergence of sensitivity to the ETA blocker is paralleled by an increase in the nerve response to a standardized hypoxic stimulus. Conversely, these changes in drug sensitivity and nerve activity are not correlated with the time course of carotid body enlargement induced by CH. Interestingly, in an early study of ventilatory acclimatization induced in the rat by exposure at 433 Torr (less severe than the 380 Torr used here), Olson and Dempsey (32) showed that the progressive increase in minute volume occurs over the first 4 days, a period corresponding to the steepest phase of developing BQ-123 sensitivity in our preparations. In addition, a significant portion of the progressive increase in hypoxia-evoked CSN activity likewise develops within the first 3 days of CH.

Our nerve recording data also indicate that basal chemoreceptor activity is increased after CH. These changes were first observed on day 3, with subsequent incremental increases up to day 9 of exposure. Increased resting CSN discharge after CH has not been reported in any species (31), but previous studies have demonstrated a persistent hyperventilation upon returning to normoxia after CH in animals and humans (11). However, this phenomenon appears to be present even after 1 day of hypoxia in rats exposed at 433 Torr (32), whereas our data indicate that basal CSN activity is unchanged after 24 h of exposure at 380 Torr. Nonetheless, the elevated nerve activity that develops after 3 days in low O₂ is likely to support the continuation of hyperventilation in normoxia. In any case, an important mechanism for altered basal nerve activity appears to involve the upregulation of both endogenous ET levels and the number of ETA because bath application of 5 μM BQ-123 partially inhibits the increase in resting CSN discharge in CH preparations. The partial effectiveness of the receptor antagonist under normoxic/hypoxic conditions suggests that the CH-induced increase in resting nerve activity involves both ET-dependent and -independent mechanisms. Earlier observations suggested the existence of different mechanisms governing basal vs. hypoxia-evoked neurotransmitter release in type I cells, where in the presence of 0 mM Ca²⁺ and 2.1 mM Mg²⁺, dopamine release evoked by hypoxia is almost completely abolished, whereas basal dopamine release is unaltered (15). Such findings are in accord with classical studies that showed that, although 0 mM Ca²⁺ and high Mg²⁺ fully inhibit evoked ACh release from motor nerve terminals, these conditions do not alter the frequency and amplitude of spontaneous miniature end-plate potentials, suggesting that transmitter release at the resting synapse is the result of Ca²⁺-independent (random) fusion of secretory vesicles with the plasma membrane (14). It is unknown whether CH increases the rate of Ca²⁺-independent vesicle fusion. However, it is noteworthy that previous ultrastructural studies of rat type I cells have shown that the volume density of vesicles decreases after 1 wk of CH but returned to normal values after 2 or 3 wk of hypoxia (19).

The inability of BQ-123 to completely block the elevated resting nerve activity after CH differs from the
effect of this drug during moderate to severe hypoxia, where the CH-induced increase in chemoreceptor discharge appears to be quantitatively excluded by the ETA antagonist. This latter finding suggests that increased levels of endogenous ET acting at ETA may account for the increased chemoreceptor discharge evoked by acute hypoxia in CH preparations. These data strongly suggest that ET peptides and ETA are essential for induction of enhanced carotid body chemosensitivity by CH. However, our data do not exclude the involvement of other neuroactive agents in the adaptation of the chemoresponse. The basic functional components of the carotid body, namely type I cells and chemoafferent nerve terminals, comprise a highly complex neurochemical apparatus containing multiple competing excitatory and inhibitory neuroactive agents (1, 17, 35). Thus the dynamic adjustments induced by CH likely involve a complex interplay between competing endogenous transmitter systems that act in concert to regulate the functional output of the carotid body. In such a scheme, the blockade of ETA by BQ-123 may, in addition to blocking the purely excitatory effects of endogenous ET peptide, influence the synthesis, release, and actions of competing agents. Although our data strongly support important roles for ET peptide and ETA, the complete reversal of increased chemosensitivity by BQ-123 could, nonetheless, be the fortuitous consequence of interference in one of several highly interactive and integrated signaling cascades, resulting in a change in nerve discharge suggestive of an unrealistically simple underlying mechanism. The possible involvement of other mechanisms may be indicated in 12-day CH preparations, where BQ-123 failed to completely block the CH-induced increase in stimulus-evoked activity (Fig. 8). Indeed, numerous studies have demonstrated significant CH-induced alterations in the synthesis, storage, and turnover of multiple candidate neurotransmitters and receptors in type I cells and chemosensory afferent neurons, suggesting their participation in altered chemosensitivity (see Ref. 6). Involvement of these factors in adaptive mechanisms will require further detailed investigations.

In conclusion, CH-induced upregulation of ET peptide and ETA in O2-sensitive type I cells occurs concurrently with a time-dependent increase in carotid body chemosensitivity, an adaptive phenomenon that is blocked by the specific ETA antagonist BQ-123. Our findings indicate that endogenous ET mediates enhanced type I cell activity and that elevated CSN activity may result from effects of ET that modulate the actions of other neurotransmitter(s) at synapses between type I cells and chemoafferent nerve terminals. This role of ET in the moment-to-moment function of the carotid body may occur in addition to long-term actions of this interesting peptide. Indeed, ET is known to act as a mitogenic agent involved in tissue remodeling and reshaping in the lung and heart during CH. Thus high levels of ET peptide and ETA could also participate in hypertrophy and mitotic activity, which occur in type I cells during sustained exposure to low ambient O2 (4, 29, 34).

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REFERENCES


