Effect of the endothelin receptor antagonist bosentan on chronic hypoxia-induced morphological and physiological changes in rat carotid body

Chen J, He L, Liu X, Dinger B, Stensaas L, Fidone S. 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, 2007; doi:10.1152/ajplung.00419.2006.—Previous experiments have repeatedly demonstrated that exposure to chronic hypoxia (CH) elicits remarkable structural changes and chemosensory hypersensitivity in the mammalian carotid body. Moreover, recent studies have shown that CH upregulates the neuroactive peptide, endothelin (ET), in oxygen-sensitive type I cells. The present study examines the possible involvement of ET in adaptation by concurrently exposing rats to hypobaric CH (BP0.14-fold increase; CH plus bosentan: 1.92 ± 0.14-fold increase; P < 0.05). Morphometric studies revealed that bosentan substantially eliminated CH-induced hyperplasia of chemosensory cell lobules as well as expansion of the connective tissue matrix. Vascular dilation associated with CH was not altered by the drug. In untreated animals exposed to 3 days of CH, expression of proliferating cell nuclear antigen (PCNA), a marker of mitosis, was increased in lobules of oxygen-sensitive type I cells and in extralobular vascular and connective tissue cells. The incidence of PCNA expression was significantly (P < 0.05) reduced in bosentan-treated animals. In vitro assessments of carotid sinus nerve (CSN) activity showed that enhancement of basal and hypoxia-evoked chemosensory activity following 9 days of CH was significantly (P < 0.001) blunted by concurrent treatment with bosentan. Collectively, our data are consistent with the hypothesis that CH-induced adaptation in the carotid body is at least partially mediated by signaling pathways involving ET receptors.

MATERIALS AND METHODS

Animals, bosentan treatment, and exposure to CH. Animal protocols were approved by the University of Utah Institutional Animal Care and Use Committee. A total of 37 young adult CD IGS rats (150–200 g, Sprague-Dawley derived) were divided into four groups consisting of 1) normal, 2) normal plus bosentan, 3) CH, and 4) CH plus bosentan. Bosentan (provided as a gift from Dr. Martine Clozel, Actelion Pharmaceuticals) was fed to rats (200 mg·kg⁻¹·day⁻¹) in strawberry-flavored gelatin (Jell-O) once daily in the morning following an overnight fast.

Rats 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; pressure was gradually lowered from ambient (640 Torr at Salt Lake City, UT, 1,400 m) to 380 Torr (equivalent to 5,500 m). Animals were maintained in the hypobaric environment for 9 to 14 days. The chamber was opened briefly to replenish food/water and administer bosentan. Age-matched control male rats were similarly housed at ambient pressure outside the chamber.

Morphometric assessment of carotid body structure. Sixteen rats (4 per group) anesthetized with ketamine (100 mg/kg) plus xylazine...
(10 mg/kg) were perfused intracardially with ice-cold phosphate-buffered 1% glutaraldehyde plus 1% paraformaldehyde. Tissues were postfixed for 1 h in the same fixative at 4°C. Selected tissues, including dissected heart chambers and carotid bodies, were weighed following fixation. Four carotid bodies from each experimental group were embedded in Araldite, and semithin sections (0.5–1.0 μm) were sampled at three levels separated by 200–300 μm. Methylene blue-stained cross-sections of the entire carotid body were photographed using a ×40 objective and an Olympus model DP11 digital camera. Images were analyzed using ImageTool software calibrated with a stage micrometer, and morphometric data were collected on the area occupied by vessels, connective tissue, and lobules. The total area of carotid body in each section was also measured. Data were used to calculate volume density defined as the area occupied by a selected tissue element divided by the total area circumscribed by the connective tissue capsule bounding the carotid body. Data were normalized to the weight of each organ to obtain the relative volume percent occupied by vascular lumen, lobules of type I and type II cells, and interlobular nonchemoreceptor connective tissue components consisting of vascular endothelial cells, smooth muscle, fibroblasts, extracellular connective tissue, resident immune cells, and other miscellaneous cell types.

Immunocytochemical techniques: peroxidase immunostaining. Twelve anesthetized rats (3 per group) 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 h, rinsed in 20% sucrose/PBS for 2 h, and stored at 4°C in 30% sucrose/PBS for 1 h. Cryostat sections (4–8 μm) were thaw-mounted on gelatin-subbed slides. Sections were first exposed to avidin-biotin preblocking reagents (20 min; Vector) and incubated at 4°C overnight in primary antibody (anti-PCNA, Abcam) diluted 1:1,600 in PBS containing 0.3% Triton X-100. Sections were then rinsed in PBS at room temperature, incubated for 2 h in biotinylated goat anti-rabbit IgG (Vector), rinsed in PBS for 20 min, incubated in avidin-biotinylated horseradish peroxidase complex (2 h; Vector Elite kit), and treated with 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide. In all experiments, normal and CH tissue samples and frozen sections were processed simultaneously, and all incubation and reaction conditions were identical. In selected sections, the primary antibody was omitted; no immunostaining was observed in these specimens.

Immunofluorescence. Cryostat sections (6 μm) were thaw-mounted onto gelatin-subbed slides. Sections were treated for 40 min with 10% goat serum in PBS plus 0.1% Triton X-100 and then incubated at 4°C overnight in primary antibody [anti-tyrosine hydroxylase (TH; Chemicon), rabbit polyclonal diluted 1:1,000; anti-PCNA (Abcam), mouse monoclonal diluted 1:800] in PBS containing 5% goat serum and 0.1% Triton X-100. Sections were rinsed in PBS at room temperature and incubated for 1 h with selected secondary antibodies (fluorescein-conjugated goat anti-rabbit diluted 1:200 and rhodamine-conjugated goat anti-mouse diluted 1:200) in 5% goat serum plus 0.1% Triton X-100 and then rinsed in PBS for 20 min. In all experiments, normal vs. experimental tissue samples and frozen sections were processed simultaneously, and all incubation and reaction conditions were identical. In selected sections, the primary antibodies were omitted; no immunostaining was observed in these specimens. Specimens were viewed in a Zeiss model M30 laser-scanning confocal microscope. Images were analyzed using ImageTool software.

Electrophysiological recording of CSN activity. The carotid bifurcations with the carotid bodies were removed from nine rats (150–200 g; 3 rats each in normal, CH, and CH plus bosentan groups) and placed in a lucite chamber containing 100% O2-equilibrated modified Tyrode solution at 0–4°C. Each carotid body and its attached nerve were carefully removed from the artery and cleaned of surrounding connective tissue, and the preparation was 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 positioned in the tip (~200-μm inner diameter) of a glass suction electrode for monopolar recording of chemoreceptor activity. The bath was grounded with a Ag/AgCl 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 AD/DA converter for display of frequency histograms on a PC monitor. Bath PO2 was measured with a Diamond General model 760 needle electrode connected to a Harvard model 102 oxygen electrode amplifier. Basal (resting) CSN activity was established in solutions equilibrated with 100% O2, which results in a bath PO2 of ~450 Torr. The superfused preparations were stimulated in solutions equilibrated with air (PO2 ~120 Torr).

RESULTS

Previous studies (6, 9–11) of hypoxia-induced cardiac remodeling have documented substantial hypertrophy and hyperplasia in right heart following various periods of CH. In addition, several laboratories (9–11) have demonstrated a reduction of such changes following systemic treatment with ET receptor antagonists, including bosentan. To verify that current experimental conditions were in accord with those previous reports, we measured the ratio of right ventricular mass vs. the mass of the left ventricle plus septum, previously shown to indicate the responsiveness of the right heart to CH. Data in Fig. 1A show that a 14-day exposure to hypobaric hypoxia (380 Torr) elicits a significant increase in the mass of the right ventricle and that this effect was significantly mitigated in animals concurrently treated with bosentan. Although the ET receptor antagonist appeared to slightly decrease the ratio in normal hearts, this effect was not significant and may indicate a mild hypoxic effect due to the 1,400-m geographic elevation in Salt Lake City, UT.

Carotid body weight shown in Fig. 1B indicates a 2.5-fold increase in organ size following 14 days of CH. Concurrent treatment with bosentan significantly inhibited CH-induced
organ enlargement (1.9-fold enlargement; \( P < 0.05 \) vs. CH) but failed to significantly alter the size of the carotid body in normoxic animals.

Semithin sections of normal carotid body (Fig. 2A) reveal lobular groups (L) of chemoreceptor type I and sustentacular type II cells and a highly vascular tissue consisting of large venous sinusoids and capillaries (*). Vascular connective tissue also contains a few parasympathetic neurons, mast cells, and resident immune cells. Figure 2B from a normoxic rat treated for 14 days with bosentan shows little or no alteration from normal. Figure 2C shows that 14 days of CH produces marked dilation of small capillaries and sinusoidal vessels. In addition, the normal oval-to-rounded lobular structure is flattened and elongated. Following CH plus concurrent treatment with bosentan (Fig. 2D), connective tissue and lobular structure appear to be the same as that in CH tissue, but the dilated vessels are somewhat larger.

Volume density analysis of the normal carotid body is presented in Table 1. The vascular lumen in these perfusion-fixed specimens accounts for some 16% of the tissue, lobules containing type I and type II cells 30%, with the remaining (~54%) consisting of endothelial cells, connective tissue, and other vascular and nonvascular cell types and nerve fibers. A similar overall pattern is apparent in normoxic animals treated with bosentan. CH (14 days) induces a threefold increase in the vascular lumen component, whereas the volume density of chemoreceptor cell lobules is not significantly altered. Treatment with bosentan during CH produces a reduction in the chemoreceptor cell lobules but a corresponding increase in the volume density of vascular lumen.

The relative volume of measured tissue components in each of the four experimental conditions is shown in Fig. 3, which combines volume density data with changes in carotid body size (wet weight of each organ). Such calculations reveal the important effects of bosentan when the size of the normal carotid body is normalized to 100. Values for the vascular lumen (16.1%), parenchyma lobules (30.2%), and other tissue components (53.7%) tended to be smaller in normal animals treated with bosentan for 14 days (due to slight reduction in organ weight), but the changes were not significant. However, following CH, there was a significant expansion of the vascular lumen and parenchyma cell lobules, together with a substantial enlargement of tissue occupied by connective tissue and endothelial and other various cell types. In CH animals concurrently treated with the bosentan ET\(_{A/B}\) antagonist, vascular dilation remained a prominent feature to the extent that absolute volume of the lumen was slightly larger in the CH plus bosentan group vs. animals exposed to CH without drug. In contrast, bosentan treatment significantly inhibited expansion of the space occupied by parenchymal cells (\( P < 0.001 \) vs. CH) as well as other tissue components (\( P < 0.01 \) vs. CH). In fact, these components were of similar absolute volume in normal and bosentan-treated CH animals.

Figure 4 shows immunoperoxidase staining for PCNA, a protein cofactor necessary for activation of DNA polymerase. This marker of mitotic cells was sparsely expressed in the normal carotid body (Fig. 4A) and in chemosensory tissue harvested from normoxic rats treated with bosentan (Fig. 4B). Following 3 days of hypoxia (Fig. 4C), PCNA expression was markedly elevated, but the incidence of positive cells appeared to be substantially lower in 3-day hypoxic animals concurrently treated with bosentan (Fig. 4D). These data concur with

### Table 1. Volume density (%) of major tissue components in rat carotid body

<table>
<thead>
<tr>
<th>Component</th>
<th>Normal</th>
<th>Normal + Bosentan</th>
<th>CH</th>
<th>CH + Bosentan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemoreceptor Cell Lobules</td>
<td>30.20 ± 3.20</td>
<td>16.07 ± 1.55</td>
<td>14.77 ± 1.48</td>
<td>54.54 ± 4.65**</td>
</tr>
<tr>
<td>Vascular Lumen</td>
<td>14.77 ± 1.48</td>
<td>14.77 ± 1.48</td>
<td>39.96 ± 4.37**</td>
<td>30.12 ± 3.96*</td>
</tr>
<tr>
<td>Other Tissue</td>
<td>53.74 ± 6.68</td>
<td>53.15 ± 4.29</td>
<td>32.63 ± 4.14*</td>
<td>30.12 ± 3.96*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *, **, and *** \( P < 0.05 \), 0.01, and 0.001 vs. normal, respectively. * \( P < 0.05 \) vs. chronic hypoxia (CH).

**Fig. 2.** Effect of CH and treatment with bosentan on carotid body morphology. A: semithin section of normal carotid body shows a highly developed vascular network composed of sinusoidal vessels and capillaries (*). Lobules (L) of type I and type II cells are surrounded by a loose matrix of connective tissue. B: tissue from a normoxic rat treated with bosentan for 14 days shows microscopic features that do not differ significantly from normal. C: following 14 days of CH, the vascular lumen has expanded, and chemosensory cell lobules appear to be elongated. D: bosentan treatment during CH appears to enhance vascular dilation and lessen the space occupied by cell lobules. Scale bar = 50 μm.

**Fig. 3.** Mean volumes of tissue components in normal (N) and experimental carotid bodies: normoxic plus bosentan treatment (N+B), CH, and CH plus bosentan treatment (CH+B). Data are expressed as percentages of normal control values. Note that CH expanded all components and that concurrent treatment with bosentan prevents expansion of chemosensory cell lobules and connective tissue (other tissue). Expansion of vascular volume is unimpeded by bosentan.

**Fig. 4.** Immunoperoxidase staining for PCNA, a protein cofactor necessary for activation of DNA polymerase. A: semithin section of normal carotid body shows a highly developed vascular network composed of sinusoidal vessels and capillaries (*). Lobules (L) of type I and type II cells are surrounded by a loose matrix of connective tissue. B: tissue from a normoxic rat treated with bosentan for 14 days shows microscopic features that do not differ significantly from normal. C: following 14 days of CH, the vascular lumen has expanded, and chemosensory cell lobules appear to be elongated. D: bosentan treatment during CH appears to enhance vascular dilation and lessen the space occupied by cell lobules. Scale bar = 50 μm.
previous studies that indicated CH-induced mitotic activity in type I cells (5), and they further indicate that bosentan inhibits the carotid body cell proliferation induced by hypoxia.

To identify cell types involved in cell cycling, we conducted double-label immunofluorescent studies using PCNA antibody combined with an antibody for TH, a signature marker for O2-sensitive type I cells. Only carotid body tissue from animals exposed to hypoxia for 3 days are shown in Fig. 5 since the incidence of PCNA staining is negligible in tissue from normal and normal plus bosentan-treated animals. In untreated hypoxic animals (Fig. 5A), there is a high incidence of TH-positive type I cells (fluorescein, green), which colocalize PCNA (rhodamine, red) in their nucleus. However, many PCNA-positive cells do not express TH, indicating mitotic activity in nonchemosensory cells located in connective tissue outside of cell lobules. Some of these cells appear to be closely associated with vascular lumen (Fig. 5A, arrows). Figure 5B shows a section from a 3-day hypoxic bosentan-treated rat. In this tissue, the incidence of PCNA-positive cells is markedly reduced both in lobular TH-positive type I cells and extralobular nonchemosensory cells. PCNA staining was quantified by determining the percentage of PCNA-positive type I cells and the number of PCNA-stained non-type I cells per unit area of tissue. Data show that following 3 days of hypoxia, 34.2 ± 0.5% (mean ± SE) of TH-positive type I cells also expressed PCNA. However, concurrent treatment with bosentan significantly (P < 0.05) reduced the incidence of PCNA expression to 23.5 ± 0.4%. Likewise, the density of PCNA-positive cells that did not contain TH also decreased significantly in bosentan-treated animals from 5.04 ± 0.48 cells/μm2 in 3-day CH specimens (n = 3) to 3.07 ± 0.49 cells/μm2 (n = 4; P < 0.05) in tissue concurrently treated with bosentan.

Treatment with bosentan also altered hypoxia-evoked responses recorded from the CSN. Data shown in Fig. 6A illustrate typical integrated nerve activity from normal, 9-day CH, and 9-day CH plus bosentan-treated preparations. It is evident that CH induces a dramatic increase in both the basal and the hypoxia-evoked discharge consistent with afferent nerve hyperexcitability. However, in a CH preparation concurrently treated with bosentan, the basal discharge rate is only slightly elevated, and acute hypoxia evokes activity that is marginally above normal. Summary data in Fig. 6B indicate that bosentan potently and significantly mitigates hyperexcitability induced by CH with respect to basal and hypoxia-evoked CSN activity.

**DISCUSSION**

Our data show for the first time that exposure to CH in the presence of a potent ET_{A/B} receptor antagonist markedly and selectively alters CH-induced changes in the morphology of the carotid body in experimental animals. Following 14 days of CH, carotid body mass increased some 2.5-fold in untreated animals, whereas concurrent treatment with bosentan significantly attenuated organ growth. Previous studies (9, 11) have shown that bosentan suppresses CH-induced tissue remodeling in the lung and heart, and our assessment of the relative mass of the right vs. left ventricles confirm that bosentan is able to mitigate CH-induced cardiac hypertrophy. In the carotid body, morphometric data indicate that CH-induced hyperplasia involving lobules of type I and type II cells fails to develop in bosentan-treated animals, and that the volume occupied by other cells within the connective tissue matrix likewise remained unchanged. However, bosentan did not prevent expansion of sinusoidal elements in the microvascular network. ET is...
a powerful vasoconstrictor, and therefore it was not unexpected that vascular expansion occurred during treatment with the ET<sub>AB</sub> antagonist in CH.

In a previous study of CH, we (8) showed that ET and ET<sub>A</sub> receptor expression is upregulated in type I cells and in some cells associated with the vascular lumen, which is consistent with a role for endogenous ET in carotid body blood flow. However, bosentan did not alter the relative volume of carotid body vasculature in normal animals, suggesting that ET is not a major contributor to local vascular regulation in normoxic conditions. In an earlier study, Pequignot et al. (20) showed that concurrent treatment with DL-propranolol inhibited CH-induced vascular expansion in rat carotid body, indicating that vasodilation involves a β-adrenergic mechanism.

Morphometric data for the carotid body from normal and hypoxic animals have been published using a variety of histological techniques. The methods used in our study are similar to those of Pequignot and Hellstrom (19), which employed semithin plastic sections. However, we find substantially less tissue space occupied by lobules of type I/type II cells in normal tissue (~30% vs. ~46%) and relatively more connective tissue and other components (~54% vs. ~37%). The reasons for these differences are not apparent, but they may be related to the use of rats of diverse genetic backgrounds or to differing criteria for the identification of chemosensory cell lobules. However, we did not observe any substantial difference in the volume occupied by vascular lumen in animals not exposed to CH, and mitotic activity was low in our normal animals.

Analysis of PCNA expression indicates that CH induces a high incidence of mitotic activity in both chemoendocrine cell lobules and vascular and extralobular connective tissue elements. Although PCNA staining was very low in normoxic tissue, ~35% of TH-positive type I cells were stained following 3 days of hypoxia. This is consistent with previous reports (5, 26) of type I cell hyperplasia and CH-induced mitotic activity. In bosentan-treated animals, mitotic activity in the O<sub>2</sub>-sensitive cells was significantly attenuated. These findings are consistent with induction of type I cell mitosis during increased receptor occupation by elevated levels of ET. Pacigo and colleagues (18) previously showed that ET elicits mitosis in type I cells cultured in vitro.

We observed that mitotic activity was also suppressed by bosentan in unidentified extralobular cells. In an earlier study, Bee et al. (5) observed mitosis in type I cells and in cells associated with the carotid body vasculature following 1–7 days of 10% O<sub>2</sub> breathing. ET is a potent mitogenic agent that has been shown to act in concert with specific growth factors to promote cell cycling in fibroblasts and vascular smooth muscle cells (4, 21). Given the extensive carotid body vascular network and connective tissue matrix, it is likely that the extralobular PCNA-positive cells in the present study consist of smooth muscle cells and fibroblasts.

In addition to suppressing mitotic activity and tissue hyperplasia in hypoxic animals, concurrent treatment with bosentan markedly suppressed the development of chemoendocrine hyperexcitability, which is of overriding functional significance in the adaptation to CH. In a previous study of a specific ET<sub>A</sub> receptor antagonist, BQ-123, we (8) showed that a receptor-saturating dose had only a marginal effect on CSN activity evoked by an acute hypoxic challenge in normal preparations. However, following CH, BQ-123 blocked the excessively high nerve discharge, indicating the participation of endogenous ET in the hyperexcitable response. Thus one possible explanation of the present findings is that bosentan retained in the tissue following chronic treatment continues to block ET receptors during the in vitro experiments. This possibility seems unlikely because bosentan and other ET receptor antagonists are water soluble and readily washed out of superfused tissue (8, 14). It is more likely that chronic treatment with bosentan suppresses a distinct ET<sub>AB</sub> receptor mediated process that develops during CH. In this regard, it is interesting that bosentan acting via ET receptors is a potent anti-inflammatory drug (2, 12, 16). Indeed, multiple studies in other tissues have shown that ET peptides are multifunctional proinflammatory agents capable of initiating and promoting an immune response. In particular, ET has been implicated in the adhesion of monocytes and neutro-
phils to vascular endothelial cells, the first step in extravasation of circulating immune cells (2). Furthermore, blocking of ETA and ETB receptors with bosentan has been shown to mitigate experimentally induced inflammatory bowel disease and eosinophilic airway inflammation (2, 12). In addition, bosentan inhibits NF-κB and activator protein-1 expression in heart and kidney in genetically modified hypertensive rats, a result consistent with ET activation of inflammatory signaling pathways (16).

Numerous studies within the last decade have established that primary sensory neuron hyperexcitability associated with multiple chronic pain conditions involves tissue invasion by activated immune cells and the release of inflammatory cytokines (27). Thus the current findings are consistent with the hypothesis that increased responsiveness involves, in addition to changes in type I cells, an ET-mediated inflammatory response in carotid body and that inflammation is blocked by bosentan and/or other ET receptor antagonists. Furthermore, it is unknown whether CH induces phenotypic changes in chemoafferent neurons consistent with the development of hyperexcitability.

GRANTS

This work was supported by National Institute of Neurological Disorders and Stroke Grants NS-12636 and NS-07938.

REFERENCES